The effects of rumen nitrogen balance on nutrient digestion, protein metabolism, and performance of dairy cows as influenced by diet composition

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Faculty of Agricultural Sciences
University of Hohenheim

Prof. Dr. Uta Dickhoefer
Institute of Agricultural Sciences in the Tropics (Hans-Ruthenberg Institute)
Animal Nutrition and Rangeland Management in the Tropics and Subtropics

submitted by
Deepashree Dilip Kand

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Examination committee

Supervisor and reviewer:	Prof. Dr. Uta Dickhöfer
Co-reviewer and examiner:	Prof. Dr. Andreas Susenbeth
Additional examiner:	Jun.-Prof. Dr. Amélia Camarinha Silva
Head of the examination committee:	Prof. Dr. Jörn Bennewitz

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Dedication

To My Beloved Grandmother

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Summary

Feeding excess dietary crude protein (CP) beyond the requirements of dairy cattle and microbes in the rumen increases production costs for farmers, excretion of nitrogen (N) to the environment, and has negative effects on the cows' health and reproductive performance. However, studies have shown an improved conversion of feed protein into milk protein (*i.e.*, milk N use efficiency) by reducing the dietary CP intake, while benefiting from the cows' capacity to re-use a share of the urea circulating in their blood. Yet, N requirements of rumen microbes need to be satisfied to not inhibit nutrient fermentation and microbial CP synthesis in the rumen, feed intake, and diet digestibility, and thus energy and utilizable CP supply to the animals.

German feeding recommendation system uses the rumen nitrogen balance (RNB = (CP intake – (rumen undegraded CP + microbial CP)/6.25; e.g. all in g/d) as an indicator for the N supply to rumen microbes. To avoid N shortage or surplus to the rumen microbes, one aims at feeding RNB = 0 g/d to high performing dairy cows, however, RNB = 0 g/d disregards the capacity of cattle to recycle dietary N via the rumino-hepatic pathway. Therefore, researchers have been interested in exploring the effects that diets with negative RNB may have on the dairy cattle and their rumen function. Results so far have been inconsistent, with some studies showing no and other even negative effects of negative RNB on feed intake, nutrient digestibility, and milk production. Inconsistent results may be due to the performance level of the animal with high-yielding dairy cows being more sensitive than low performing ones. Moreover, it may be supposed that variable responses to negative RNB in different studies may at least partly be related to varying ingredient composition and the type of main carbohydrate or N sources in the animals' diets. These differences among others in

degradation kinetics and thus the extent and rate of energy and N availability for the rumen microbes may promote the growth of diverse microbial groups that vary in their energy and N requirements. The overall objective of the thesis was to generate a comprehensive understanding on the effects of interactions between the RNB levels and carbohydrate and N sources in cattle diets on rumen fermentation, the efficiency of microbial CP synthesis, and on N use efficiency *in vitro* and *in vivo*.

In vitro study was conducted to evaluate the effects of different levels of RNB (0, −5, −9 g/kg dry matter) on carbohydrate fermentation, protein degradation, and microbial CP synthesis, and whether these effects differ depending on the dietary carbohydrate or N sources. Three carbohydrate sources (sucrose, corn starch, and cellulose) were incubated with urea as the main source of N in experiment 1, whereas experiment 2 tested three N sources (wheat gluten, soy protein, and casein) with corn starch as the main source of carbohydrate. During the three runs, 1 g each (as-fed basis) of grass hay and a mixture with different proportions of N and carbohydrate sources were incubated in duplicate in buffered rumen fluid for 24 h in an *in vitro* ANKOM-RF system. The results showed that decreasing RNB levels increased *in vitro* gas production in all the dietary treatments, likely due to high proportions of carbohydrate in the diets. However, with declining RNB level the feed CP degradation and microbial CP synthesis decreased, particularly in readily fermentable carbohydrate (*i.e.*, sucrose) and slowly degradable N sources (*i.e.*, soy protein and wheat gluten). Overall, the negative effects of declining RNB levels on carbohydrate fermentation, protein degradation, and microbial CP synthesis were more pronounced amongst the diets varying in N (*i.e.*, in experiment 2) than carbohydrate (*i.e.*, in experiment 1) sources.

Based on the results of the *in vitro* work, an *in vivo* study was conducted to understand the effects of two RNB levels (RNB0: 0 g/kg dry matter; RNB-: −3.2 g/kg dry matter and ~

−65 g/d) and two dietary CP sources (Faba bean grain and SoyPass) in high-yielding lactating dairy cows. The faba bean grain and SoyPass were chosen as main protein sources due to their differences in the rate and extent of degradation in the rumen, with faba bean grain having a greater rate and extent of degradation compared to SoyPass source. It was hypothesized that slowly degradable protein source (*i.e.*, SoyPass) will show pronounced differences between the RNB levels, in line with the results from the *in vitro* study. Twenty-four lactating Holstein cows were included in a replicated 4 × 4 Latin square experimental design, of four 20 d periods with each 12 d of adaptation to the experimental diets and 8 d of sample collection.

In contrast to the expectations, negative RNB diet reduced feed intake, apparent total tract digestibility of neutral detergent fiber and CP, the efficiency of microbial CP synthesis, milk yield, and milk protein yield. Besides this, urinary N excretion decreased, and milk N use efficiency increased in RNB- diets. All the negative effects of reduced RNB were more pronounced in diets containing rapidly degradable protein source (*i.e.*, diets with faba bean grain). Furthermore, the total unsaturated fatty acid proportions in milk were lower in RNB- than in RNB0 for both protein sources and in faba bean grain than in SoyPass diets for both RNB levels. In contrast, chews per dry matter intake were greater for RNB- compared to RNB0 for a diet with only faba bean grain. A greater utilizable CP supply beyond the animals' requirements in diets containing SoyPass than faba bean grain most likely increased endogenous N supply to rumen microbes and thus partly compensated for the negative RNB- effects in SoyPass diets on rumen fermentation and efficiency of microbial CP synthesis. Therefore, dietary treatment effects in the *in vivo* study might not be exclusively attributed to differences in dietary RNB.

Contrasting *in vitro* and *in vivo* findings emphasize the need for validated and

standardized laboratory procedures to estimate the utilizable CP supply at the duodenum and to simulate *in vivo* conditions of low N supply to the rumen microbes in a closed system. Nevertheless, the results of the present thesis indicate that the effects of negative RNB levels may vary with dietary composition in dairy cows. Therefore, outlining a single minimum RNB balance threshold for dairy cattle diets may not be appropriate when optimizing N utilization in dairy cows, because several animal and dietary factors modify the requirements of rumen microbes. Eventually, thesis and literature support that reducing RNB in diets of high-yielding dairy cows and greatly improve N use efficiency and reduce the risk of N emissions into the environment, as even at a low RNB level (~ −65 g/d) negative effects were not seen with each of the dietary treatments.

Zusammenfassung

Die Fütterung von Rohprotein (XP) über den Bedarf von Milchrindern und deren Pansenmikroben hinaus führt zu steigenden Produktionskosten und Stickstoff (N)-Ausscheidungen und belasten somit die Gesundheit und Leistung der Milchkühe sowie die Umwelt. Eine reduzierte XP-Aufnahme verbessert die Effizienz der Verwertung von Futterprotein zu Milchprotein (d.h. Milch-N-Nutzungseffizienz), wobei das Tier einen Teils des im Blut zirkulierenden Harnstoffs nutzt. Der N-Bedarf der Pansenmikroben muss jedoch gedeckt sein, um die Fermentation und die mikrobielle Proteinsynthese im Pansen sowie die Futteraufnahme und -verdaulichkeit nicht zu beeinträchtigen, und so ausreichend Energie und nutzbares XP für das Tier bereitzustellen.

Die deutschen Bedarfsempfehlungen für Milchrinder und Aufzuchtrinder verwenden die ruminale Stickstoffbilanz (RNB = (XP Aufnahme – (unabgebautes XP am Darm + mikrobielles XP) als Indikator für die N-Versorgung der Pansenmikroben und empfehlen eine ausgeglichene RNB (z.B. 0 g/Tag). Allerdings schöpft dies die Fähigkeit von Rindern nicht aus, N aus dem rumino-hepatischen Kreislauf wiederzuverwerten. Zahlreiche Studien untersuchten bereits die Effekte negativer RNB in Rationen von Milchkühen. Deren Ergebnisse waren jedoch widersprüchlich, mit keinen oder negativen Effekte bei negativen RNB auf die Futteraufnahme, Nährstoffverdaulichkeit und Milchproduktion. Neben Unterschieden in der absoluten RNB und dem Leistungsniveau der Tiere, könnte ein Grund für die uneindeutigen Ergebnisse die verschiedenen Kohlenhydrat- und N-Quellen in den Rationen der Tiere sein. Diese unterscheiden sich u.a. hinsichtlich ihrer Abbaukinetik und somit dem Umfang und Zeitpunkt der Energie- und N-Verfügbarkeit für die Pansenmikroben, und fördern das Wachstum unterschiedlicher mikrobieller Gruppen und somit ihren Energie-

und N-Bedarf. Das Ziel dieser Arbeit war es daher, die Effekte der Interaktionen zwischen unterschiedlichen RNB und Kohlenhydrat- oder N-Quellen in den Rationen von Milchrindern auf die Pansenfermentation, die Effizienz der mikrobiellen Proteinsynthese und die N-Nutzungseffizienz *in vitro* and *in vivo* zu erlangen.

Eine *in-vitro*-Studie wurde durchgeführt, um den Einfluss unterschiedlicher RNB-Stufen (0, −5, −9 g/kg Trockensubstanz) auf die Kohlenhydratfermentation, den Proteinabbau und die mikrobielle Proteinsynthese zu untersuchen. Zudem wurde untersucht ob diese Auswirkungen von der Kohlenhydrat- oder N-Quelle im Futtermittel abhängig sind. Drei Kohlenhydrat-Quellen (Saccharose, Maisstärke, Zellulose) wurden mit Harnstoff als wesentlicher N-Quelle im ersten Versuch inkubiert, während im zweiten Versuch drei N-Quellen (Weizengluten, Sojaprotein und Kasein) mit Maisstärke als wesentlicher Kohlenhydrat-Quelle untersucht wurden. In je drei Inkubationen wurden in Doppelbestimmung jeweils 1 g Frischsubstanz Heu und 1 g Frischsubstanz einer Mischung mit unterschiedlichen Anteilen an Kohlenhydrat- und N-Quellen in gepuffertem Pansensaft in einem *in vitro* ANKOM-RF System für 24 Stunden inkubiert. In allen Futtermischungen stieg die *in vitro* Gasproduktion mit sinkender RNB, wahrscheinlich auf Grund der zunehmenden Kohlenhydratanteile in den Rationen. Des Weiteren sank der Futterproteinabbau sowie die mikrobielle Proteinsynthese, insbesondere in den Rationen mit leicht fermentierbaren Kohlenhydraten (d.h. Saccharose) und langsam abbaubaren N-Quellen (d.h. Sojaprotein und Weizengluten). Insgesamt waren die negativen Effekte sinkender RNB auf die Kohlenhydratfermentation, den Proteinabbau und die mikrobielle Proteinsynthese ausgeprägter bei Rationen mit variierenden N-Quellen (d.h. in Versuch 2) als mit variierenden Kohlenhydrat-Quellen (d.h. in Versuch 1).

Basierend auf den Ergebnissen der *in-vitro*-Studie wurde ein *in-vivo*-Versuch

durchgeführt, um die Effekte von zwei unterschiedlichen RNB (RNB0: 0 g/kg Trockensubstanz; RNB-: −3.2 g/kg Trockensubstanz und ~ −65 g/Tag) und zwei verschiedenen Proteinquellen (Ackerbohne und SoyPass) bei hochleistenden Milchkühen zu untersuchen. Die Ackerbohne und SoyPass wurden aufgrund ihrer unterschiedlichen Abbaukinetik ihres XP im Pansen als wesentliche Proteinquellen gewählt. Dabei hat die Ackerbohne im Vergleich zu SoyPass eine höhere Abbaugeschwindigkeit. Es wurde angenommen, dass sich wie im vorangegangenen *in-vitro*-Versuch bei der langsam abbaubaren N-Quelle (d.h. SoyPass) stärkere Unterschiede zwischen den RNB-Stufen zeigen wird. Der Versuch mit 24 laktierenden Holstein Kühen wurde in einem 4 × 4 Lateinisches Quadrat angelegt mit vier Perioden von je 12 Tagen Anpassung an die Fütterung und 8 Tagen Daten- und Probensammlung.

Anders als erwartet, reduzierten die Rationen mit negativen RNB die Futteraufnahme, die scheinbare Gesamttraktverdaulichkeiten der Neutral-Detergenzien-Faser und des XP, die Effizienz der mikrobiellen Proteinsynthese, die Milchleistung, der Milchproteinertrag und die N-Urinausscheidung. Außerdem erhöhte sich die Milch-N-Nutzungseffizienz der Kühe mit negativen RNB Ration. Alle diese Effekte waren ausgeprägter in den Rationen mit der schnell abbaubaren Proteinquelle (d.h. Ackerbohne). Die Gesamtanteile an ungesättigten Fettsäuren in der Milch waren bei RNB- niedriger als bei RNB0 und bei Ackerbohne niedriger als bei der SoyPass-Ration. Im Gegensatz dazu war die Kauintensität (d.h. die Anzahl von Bissen pro kg Trockenmasseaufnahme) nur für die Ackerbohne-Ration bei negativer RNB größer als bei RNB0. Eine höhere Zufuhr an nutzbarem XP über den Bedarf der Tiere hinaus in der Ration mit SoyPass als in der Ration mit Ackerbohne erhöhte wahrscheinlich die Verfügbarkeit von endogene N für die Pansenmikroben und kompensierte somit zumindest teilweise die Auswirkungen einer negativen RNB in der SoyPass-Ration auf die

Pansenfermentation und Effizienz der mikrobiellen Proteinsynthese.

Die unterschiedlichen Ergebnisse der *in-vitro*- und *in-vivo*-Versuche verdeutlichen die Notwendigkeit für eine valide und standardisierte Labormethode, um den XP-Zufluss am Duodenum zu schätzen und um *in vivo* Bedingungen mit niedriger N-Versorgung für Pansenmikroben in einem geschlossenen System simulieren zu können. Die Ergebnisse dieser Doktorarbeit zeigen, dass die Effekte einer negativen RNB auf Milchkühe mit der Zusammensetzung der Ration variieren können. Der Bedarf der Pansenmikroben wird durch unterschiedliche Tier- und FutterungsFaktoren beeinflusst. Daher ist die Definition eines einzigen Schwellenwert für die RNB in den Rationen von Milchrindern ungeeignet, insbesondere wenn die N-Nutzung der Tiere erhöht werden sollte. Zudem bekräftigt diese Doktorarbeit die vorhandene Literatur, dass das Potenzial besteht die RNB-Stufe in Rationen hochleistender Milchkühe zu reduzieren, um damit erheblich die N-Nutzungseffizienz zu verbessern und N-Emissionen in die Umwelt zu minimieren, denn sogar bei einer niedrigen RNB-Stufe (~ −65 g/Tag) konnte für alle Proteinquellen kein negative Auswirkungen beobachtet werden.

Chapter 1

1. General introduction

1.1 Environmental impact of nitrogen originating from livestock production

Agricultural policies continue to focus on increasing agricultural productivity through the use of external inputs, both in the developing and the developed world (van Grinsven et al., 2015). Nitrogen (N) is an important nutrient contributing to food security. Global intensification of agriculture over the past decades has led to an increase in N recovery but also N surplus, for instance, from livestock. Inefficient production of food and feed protein from livestock has resulted in N losses both as an unreactive N_2 and as reactive N compounds into the environment (Bouwman et al., 2013). Reactive N compounds such as ammonia (NH_3), nitrous oxide, and nitrate contribute to soil acidification, eutrophication of surface water, pollution of groundwater due to nitrate leaching, and climate change (Sutton et al., 2013) impacts human health.

Awareness and concerns over the detrimental effects of N emissions, on human health and environment have led to the adoption of environmental policies in the European countries (Oenema et al., 2011). Some of these policies particularly aimed at decreasing the emissions of NH_3 into the atmosphere (e.g. the European Union National Emission Ceiling Directive (European Commission, 2001); UNECE Convention on Long-range Transboundary Air Pollution (UNECE, 1999)), and leaching of nitrate to groundwater and surface waters (e.g. European Union Water Framework Directive (European Commission, 2000); European Union Nitrates Directive (European Commission, 1991)). Likewise, a policy such as the Convention on Long-range Transboundary Air Pollution requires European nations to submit an inventory of NH_3 emission annually to understand the achieved reductions to reach the targets of the environmental policies.

Agriculture is the largest contributor of NH_3 emissions in Europe (EEA, 2019), where

livestock production emits two-thirds of the total NH_3 emissions from agriculture in European nations (Eurostat, 2015). Nitrogen emissions such as NH_3 emissions from livestock are influenced by factors like species, breed, diet, and production of the animal. For instance, the total amount of N-consumption and excretion (g/d) by the animal is greater in dairy and beef cattle compared to pig and poultry. Besides, ruminants compared to monogastrics are less efficient in converting dietary N to meat and milk products (g N in product/100 g N intake) (Dewhurst et al., 2000). Huhtanen and Hristov (2009) in a meta-analysis with dairy cows estimated the milk N use efficiency of an average 28 g/100 g N intake (standard deviation $\pm$ 12 g/100 g N intake), with remainder 72 g/100 g N intake being excreted mainly as urine and fecal N. Alongside, N emissions largely results from housing, manure storage, and urine and dung deposition in grazed pastures, or due to manure spreading on the agricultural land. For instance, NH_3 emissions from grazing cattle are relatively lower compared to housed animals, as urinary N mainly in an organic form (*i.e.*, urea) percolates into the soil before the hydrolysis to total NH_3-N can occur (Groenestein et al., 2019). Besides, seasonal variations across Europe causes limitation in grazing of cattle all-round the year, which may lead to more housed cattle and hence more NH_3 emissions.

For housed systems, total mixed ration (TMR) fed to the cattle can support high levels of output per livestock head than pasture-based systems. Formulation of a TMR at high energy and protein concentrations can increase nutrient intakes and therefore milk productivity in high-producing dairy cattle. Nevertheless, a concern to avoid any negative impact on productivity may result in an excessive feeding of the dietary crude protein (CP) to the dairy cattle which in addition to causing environmental impact may also increase production cost and cause negative implications on animal health and their reproductive performance (Lebzien et al., 2006). Therefore, researchers have been focusing for a few decades on the

diet of dairy cattle particularly by investigating the effect of low dietary CP concentration on urine and fecal N excretions, milk N use efficiency, and milk production (Huhtanen and Hristov, 2009).

1.2 Feeding reduced amount of dietary protein as an approach for decreasing nitrogen excretion and increasing milk nitrogen use efficiency in dairy cattle

Dietary CP provides amino acids (AA) for growth, maintenance, and milk production. However, an excess supply of N relative to the needs of the rumen microbes and host animal increases the concentrations of NH_3-N in the rumen that are absorbed into the blood, converted to urea in the liver, and then excreted in the urine (Colmenero and Broderick, 2006). As a result, researchers in past years have evaluated the effect of low dietary CP concentration relative to the requirements of the animal and in some cases even below the requirements of the animal. Results of several previous studies (Lee et al., 2011; Giallongo et al., 2015; Schiavon et al., 2015; Mutsvangwa et al., 2016) have demonstrated that reducing the dietary CP concentration is an effective strategy to decrease urinary N excretions. In line with a decrease in urinary N excretion, the same studies have also shown an improved milk N use efficiency, suggesting an efficient utilization of available dietary CP by rumen microbes (Huhtanen and Hristov, 2009).

Despite the consistent outcome of reducing dietary CP concentration on urinary N excretion and milk N use efficiency from the dairy cattle, the effect of low dietary CP concentrations on dry matter (DM) intake (kg/d), apparent total tract digestibility (ATTD; g/100 g), and milk production (kg/d) in extensive studies with dairy cattle have not been conclusive. For example, Schiavon et al. (2015) reduced the dietary CP concentration from

150 g to 120 g/kg DM and reported a tendency for a decrease in DM intake and ATTD of organic matter (OM), and a decrease in milk yield (MY). Similarly, Lee et al. (2011) reduced the dietary CP concentration from 167 g to 148 g/kg DM and observed a tendency for a decrease in ATTD of OM and a decrease in DM intake and MY. Moreover, Giallongo et al. (2015) who studied similar dietary CP concentrations as Lee et al. (2011), observed no changes in DM intake and MY but a decrease in ATTD of OM at a dietary CP concentration of 148 g/kg DM. Likewise, Mutsvangwa et al. (2016) observed no change in DM intake, ATTD of OM, and MY when the dietary CP concentrations were reduced from 175 g to 149 g/kg DM. It should be emphasized that the above-mentioned four studies, (i) considered high-producing lactating Holstein cows; (ii) were fed diets as TMR for *ad libitum* intake; (iii) had forage proportions in the diets between 50 to 55 g/100 g feed (DM basis); and (iv) observed similar (mean ± standard deviation) lactation number (2.0 ± 0.7 lactations), days in milk (115 ± 36 d), and body weight (664 ± 39 kg). Therefore, animal or intake associated factors may not have majorly caused such contrasting results. However, plausible reasons for such differences may be that the CP concentration of a dietary feed reflects only total N content in the diet, but reveals nothing about the (i) types of compounds (for example true protein, non-protein N), (ii) rate and extent of the dietary CP degradation in the rumen (*i.e.*, rumen degraded protein (RDP)) and their availability to the rumen microbes, and (iii) dietary feed protein that escapes the rumen undegraded (*i.e.*, rumen undegraded feed protein (RUP)). These details on concentrations of RDP and RUP are required (and also provided in recent studies), as not all supplied dietary CP is utilized by rumen microbes for microbial growth or host animal for milk production. As a result, the upcoming sections highlight the importance of understanding RDP and RUP concentrations in the animal's diet.

1.3 Rumen degraded and rumen undegraded protein concentrations in the diet

The RDP and RUP are two components of the dietary feed protein that have separate functions. The RDP is a crucial component influencing rumen fermentation that provides peptides, free AA, and NH_3-N for microbial growth and synthesis of microbial crude protein (MCP) (NRC, 2001). The MCP is the main source of protein synthesized in the rumen and accounts for an average of 59% (range between 34% to 89%) of the non-NH_3-N that passes to the small intestine of dairy cattle (Clark et al., 1992). The MCP is characterized by a high duodenal digestibility and superior AA composition as compared to dietary protein, making it the main source of essential AA for protein synthesis by the host animal (NRC, 2001). Besides the source, extent, and rate of RDP, the synthesis of MCP in the rumen is also influenced by the source and degradation of carbohydrate (CHO) and its availability to the rumen microbes (see section 1.4). Along the line, the above-mentioned factors may have possibly contributed to the contradictory results of the studies discussed in section 1.2.

Another key source of absorbable AA for the host animal besides AA from MCP synthesis is RUP, a dietary CP that escapes the degradation in the rumen (NRC, 2001). Increase in concentration of RUP in the diets of dairy cattle with high protein requirements does improve the AA supply to the animal and may improve the MY provided that an increase in dietary RUP concentration is not at the expense of RDP concentration, as the low RDP concentration in the rumen may impede synthesis of MCP (Santos et al., 1998) and an overall protein supply to the animal at the duodenum. The source of RUP in the diet influences the rate and extent of protein degradation in the rumen (see sub-section 1.4.1). Besides, the concentrations of RUP in the feed can be increased by heat or formaldehyde treatment, a product that may be purchased from the feed industry. For instance, the

compared studies (Lee et al., 2011; Giallongo et al., 2015; Schiavon et al., 2015; Mutsvangwa et al., 2016) in section 1.2 had almost similar (mean ± standard deviation) RDP concentrations (64 ± 4.3 g/kg CP), among which studies used heat-treated protein sources containing high concentrations of RUP, which then combined with different CHO sources, may have influenced the observed results seen in section 1.2. Therefore, along with the CP concentration, supplementary information on RDP and RUP concentration in the feed CP is certainly necessary, as the extent of feed CP degradation in the rumen is fundamental for supplying adequate amounts of RDP and RUP to the rumen microbes and host animal.

1.4 Factors influencing microbial protein synthesis

Previous section highlighted that extent of feed CP degradation in the rumen is central for supplying adequate amounts of RDP to the rumen microbes. However, there are some key factors that influence rumen degradation of CP, availability of CP to the microbes and thereby MCP synthesis such as diet composition (*i.e.*, amount, source and degradation rate of both CHO and protein), synchronization of CHO and protein concentrations including their degradation rate, and digesta passage rate (Bach et al., 2005).

1.4.1 Amount and source of nitrogen in the diet

The MCP synthesis is a function of N availability in the rumen when providing the required amount of CHO to the rumen microbes. Although the concentration of RDP is most crucial for the synthesis of MCP (NRC, 2001), the N for rumen MCP synthesis may partly be derived from non-protein N, such as urea-N recycling via blood and saliva, and lysis of bacteria and protozoa (Lapierre and Lobley, 2001). For instance, Cyriac (2009) found a tendency for decreased MCP synthesis (g/d), as a result of reduced microbial growth and CP intake when

RDP concentration in the diets of lactating dairy cows decreased from 635 g to 507 g/kg CP. Contrastingly, Mutsvangwa et al. (2016) studied two RDP (630 g and 690 g/kg CP) and two CP (149 g and 175 g/kg DM) concentrations in the diets of lactating dairy cows and showed no effect on DM intake, MCP synthesis (g/d), and MY. Accordingly, the authors suggested that urea-N recycling may have helped to gain an additional N to maintain N supply to the rumen microbes (for a short-term), and therefore milk production. Furthermore, it may also be supposed that the deficit N to the rumen microbes was compensated partly by lysis of bacteria and protozoa. Nevertheless, the concentration of RDP according to the requirement of rumen microbes is necessary for rumen microbial growth. Alongside this, the source of RDP is equally important to understand the rate and extent of degradation of protein in the rumen. For example, N sources such as blood meal or corn gluten meal, that had a low rate and extent of degradation in the rumen (or high RUP concentration), tended to have lower efficiency for MCP synthesis compared to sunflower meal in a study of Erasmus et al. (1994). Yet, the degradable N sources only when combined with an appropriate CHO source may contribute to increased MCP synthesis. For example, Wang et al. (1997) in the *in vitro* study, reported a greater synthesis of MCP when high RDP source (casein) was combined with a rapidly fermentable CHO (starch) compared to slowly degradable CHO (cellulose). Weisbjerg et al. (1994) supplemented barley as an energy source in combination with urea or soybean meal or a mixture of urea and soybean meal to the cows. The study showed an improved efficiency for MCP synthesis when diets contained barley and a mixture of urea and soybean meal. Accordingly, Weisbjerg et al. (1994) suggested energy from barley was better utilized in the presence of urea and soybean meal, possibly because AA, peptides, and NH_3-N concentrations were available to the rumen microbes for CHO fermentation. Consequently, dietary protein sources, their amount, and their rate of degradation are critical

to breakdown AA and peptides to NH_3-N that may influence CHO degradation, and growth of different microbial consortium (McAllan and Smith, 1974), and thus MCP synthesis. Yet, for an improved MCP synthesis, the source and amount of CHO (as energy) in the rumen are equally important.

1.4.2 Amount and source of carbohydrate in the diet

The MCP synthesis is a function of energy availability. Carbohydrate fermentation provides energy in the form of adenosine triphosphate (ATP). After transporting an extracellular AA (from N source) inside the microbial cells, the fate of the absorbed AA depends on the availability of ATP within the microbial cells. When available, ATP will be utilized for the maintenance and growth of rumen microbes (Obara et al., 1991). If ATP is insufficient to drive protein synthesis, AA will be deaminated to NH_3 and ultimately excreted from the cytoplasm of the cell (Tamminga, 1979). Therefore, inadequate energy from the CHO fermentation in the rumen is the first limiting factor for MCP synthesis when N is supplied to the rumen microbes in the required amounts.

The fermentation of CHO is dependent on the amount, extent, and rate of degradation of CHO sources in the rumen that may then influence the synthesis of MCP in the rumen. For instance, increasing dietary non-structural CHO (NSC; e.g. starch, sugar, pectin) concentrations in presence of required N supply to the rumen microbes promotes microbial growth (Schwab et al., 2005). Accordingly, Stokes et al. (1991) simultaneously increased NSC concentrations (380 g, 310 g, 240 g/kg DM) and decreased RDP proportions (730 g, 640 g, 500 g/kg CP) in diets of lactating dairy cows. Thereafter, the authors observed an improved estimated efficiency of MCP synthesis (g N/kg OM truly digested) at the NSC and RDP concentrations of 310 g/kg DM and 640 g/kg CP, respectively suggesting that those

conditions were optimal for efficient MCP synthesis. Albeit, Stokes et al. (1991) could not establish a clear effect of MCP synthesis on increased NSC concentrations due to simultaneous reduction in RDP concentrations. Moreover, Fanchone et al. (2013) studied the effects of CP concentrations (110 g and 142 g/kg DM) and CHO sources (starch concentration at 151 g and 307 g/kg DM, or neutral detergent fiber concentration at 360 g and 490 g/kg DM) at a similar RDP proportion of 660 g/kg CP (DM basis) in the diets of lactating dairy cows. Accordingly, Fanchone et al. (2013) showed a tendency for improved efficiency of MCP synthesis (g N/kg fermented OM) and ATTD of DM in starch-rich diets irrespective of dietary CP concentrations. Lykos et al. (1997) demonstrated that an increased rate of NSC degradation in the rumen from 6.0% to 7.9%/h in lactating dairy cows increased MY and milk protein yield by 2.5 kg/d and 130 g/d, respectively, and tended to increase the efficiency of rumen MCP synthesis (g N/kg fermented OM). Consequently, increased proportions of NSC can have positive effects on rumen MCP synthesis, however, high concentrations of NSC in the diet may change the rumen fermentation pattern, may reduce rumen pH, cause acidosis, and may alter microbial growth (Andrade-Montemayor et al., 2009).

Microbes that degrade NSC have high maintenance requirements, grow rapidly, and use NH_3, peptides, and AA as N sources, in contrast, microbes that degrade structural CHO (e.g. cellulose, hemicellulose, and lignin) have low maintenance requirements relative to their needs, grow slowly and use NH_3-N as their main N source (Russell et al., 1992). Structural CHO (*i.e.*, both forage amount and forage particle size) are not directly responsible for the enhancement of MCP synthesis but are essential in diets of ruminants to promote eating and ruminating activity, salivation, rumen buffering capacity to maintain rumen pH (Zebeli et al., 2012), and milk fat concentration (Beauchemin et al., 1994). The presence of high

structural CHO and low NSC concentrations in the rumen slows the microbial growth, causes a large quantity of energy and N to be utilized for maintenance, increases the lysis of microbial cells, and reduces the passage of microbes from the rumen (Clark et al., 1992). Nevertheless, an increase in structural CHO concentrations in high NSC diets may allow rumen microbes to utilize the energy for growth more efficiently due to the uniform release of energy throughout the day (Clark et al., 1992). Consequently, depending on the type and amount of CHO, the energy required for microbial growth can be modified, and thus the synthesis of MCP may be influenced.

1.4.3 Synchronizing the availability of nitrogen and carbohydrate

Besides providing adequate concentrations of RDP and CHO to the rumen microbes, synchronizing the supply of both RDP and rumen fermentable CHO to the rumen microbes may also be necessary to improve the efficiency of MCP synthesis. Synchronization of nutrients can be performed by (i) modifying rate and supply of CHO and N sources, by combining different fast and slow RDP and rumen degradable CHO sources, to match the release rate of CHO and RDP obtained from the individual feeds in the rumen (Herrera-Saldana et al., 1990; Shabi et al., 1998), and (ii) by changing the feeding frequency and pattern (Kolver et al., 1998; Richardson et al., 2003).

Several studies on the effects of synchronous and asynchronous diets have shown an improved efficiency of rumen MCP synthesis in synchronous diets (Aldrich et al., 1993; Chumpawadee et al., 2006; Seo et al., 2010). In contrast, some studies (Richardson et al., 2003; Kaswari et al., 2007) that showed no difference in the studied variables between a synchronous and asynchronous diet have challenged the concept of synchronization. Along this line, studies with no negative effect on rumen MCP synthesis with asynchronous diet

suggested that rumen microbes are able to cope effectively to nutrient deficiency (i) due to the continuous availability of feed as a TMR; (ii) by the synthesis of intracellular storage polysaccharides in presence of abundant energy, or (iii) by using cellular storage of polysaccharides and recycled urea-N when the amount of CHO and N, respectively are limited (Valkeners et al., 2004; Ichinohe and Fujihara, 2008). Moreover, the extent to which NH_3-N is available and utilized by the microbes in the rumen for MCP synthesis is influenced by various other factors such as type of diet, ruminal fermentation characteristics, and intake of the animal (NRC, 2001). As a result, those above-mentioned studies who evaluated the effect of synchronization of CHO and protein degradation in the rumen may not have observed an improved efficiency of MCP synthesis. Consequently, a comprehensive understanding of the source of energy and protein and their rate of degradation as well as their synchronization effect in the rumen would be advantageous.

1.4.4 Passage rate

Passage rate is related to the level of feed intake, where high feed intake levels increase rumen passage rates, which are associated with improved efficiency of microbial protein (g microbial N/kg digestible OM intake) (Clark et al., 1992). Furthermore, a high DM intake would reduce the mean retention time in the rumen, increase the fractional outflow rate, and may reduce the extent of CP and CHO degradation in the rumen. Thus, a modest increase in the flow of RUP to the small intestine may be observed with an increased digesta passage rate (Bach et al., 2005). Besides, a faster passage rate may also cause some amount of NSC (e.g. starch) in the diets to be digested postruminally, where such postruminally digested starch may be used more effectively for the synthesis of milk than starch digested in the rumen (Nocek and Tamminga, 1991). In contrast to NSC-rich diets, the diets rich in

structural CHO (high forage diets) may decrease the passage rate due to greater particle size and increase its rumen mean retention time and consequently, increase chewing and rumination time (Zebeli et al., 2012). Regardless, it is important to understand that an increase in passage rate may have negative effects on diet digestibility, intake, and in extreme conditions on animal health.

Overall, various factors such as amount and source of CHO and RDP in the diet, their rate of degradation in the rumen, and DM intake influences MCP synthesis and milk production and composition. Therefore, accurate estimation of MCP synthesis and extent and rate of degradation of protein source to understand RUP concentration is essential to improve the accuracy of protein feeding to animals (relative to their requirements) and to reduce environmental burdens from animal production (Dijkstra et al., 2013).

1.5 Estimation of the nitrogen requirements of rumen microbes and their host animals in current protein evaluation systems

A detailed understanding of N supply to the rumen microbes for MCP synthesis is essential to avoid impeding the AA supply to the host animal, and thereby, for instance, decrease MY in dairy cattle (Stern et al., 1994; Dijkstra et al., 1998). For the aforementioned purpose, protein evaluation systems have been developed by several countries to estimate the N supply to the rumen microbes based on various assumptions (Aschemann, 2012) as follows.

The German protein recommendation system for dairy cows and heifers estimates utilizable crude protein (uCP) at the duodenum where the uCP is defined as total CP at the duodenum minus endogenous protein (Lebzien and Voigt, 1999). This recommendation

system uses rumen nitrogen balance (RNB) as an indicator for the N supply to the rumen microbes and is calculated as the difference between dietary CP intake and uCP (RNB = (CP intake – (RUP + MCP))/6.25; e.g. all in g/d) (GfE, 2001). To avoid a shortage of N for rumen microbes and to maximize MCP synthesis, the dietary RNB should be close to zero both in g/kg DM and g/d (GfE, 2001). A positive RNB indicates an excess of N in the rumen that inevitably leads to an increased renal N excretion particularly urinary excretion of urea synthesized from NH_3-N (Reynolds and Kristensen, 2008). In contrast, a negative RNB may indicate a deficiency of N supply to rumen microbes that may reduce the efficiency of MCP synthesis or in some circumstances maintain the efficiency of MCP synthesis through increased recycling of urea-N via the rumino-hepatic cycle (GfE, 2001). Along the line, GfE (2001) proposes that 20% of the synthesized MCP (10.1 g CP/MJ metabolizable energy; ME) may be derived from the N available through urea-N recycling. Lebzien et al. (2006) showed that a maximum negative RNB of −0.36 g/MJ ME (*i.e.*, RNB of −4.2 g/kg DM (-63 g/d), with ME of 11.75 MJ/kg DM) could be accepted in diets of dairy cattle. Nevertheless, the maximum negative RNB may be debatable considering the variations arising due to available CHO and N sources in the diet (see section 1.4).

The French protein recommendation system for dairy cattle (INRA, 2018), calculates the metabolizable protein value as the sum of truly digestible proteins in the intestine coming from the feed and the MCP synthesized in the rumen. In the updated French system, rumen protein balance calculated as the difference between CP intake and non-NH_3 CP flowing out the duodenum (*i.e.*, RUP, MCP, and endogenous CP), is similar to the German protein system. Moreover, the system considers urea-N recycling of 0 to 9% for MCP synthesis in the rumen.

The Dutch protein recommendation system for dairy cows, calculates for each feed, a

true protein digested in the intestine (DVE = DarmVerteerbaar Eiwit), and the rumen degradable protein balance (OEB = Onbestendig Eiwit Balans*, i.e.*, rumen-degradable protein balance) as elaborated by van Duinkerken et al. (2011). The DVE characterizes the amount of true protein from various sources digested in the small intestine and denotes the requirements of dairy cows as DVE units. The OEB is calculated as a difference in potential MCP synthesis based on available RDP and rumen fermented OM (*i.e.,* energy) available for microbial fermentation in the rumen. Similar to the German protein system, the optimum OEB value recommended in a diet is zero or slightly positive.

In the United Kingdom protein recommendation system (AFRC, 1993), the metabolizable protein consists of digestible RUP and digestible microbial true protein. The digestible microbial true protein for each diet is calculated as protein synthesized from fermentable energy sources in the feed and rumen degraded N (AA or non-protein N). The proportion of true protein in MCP is assumed 0.75, while the digestibility of the microbial true protein is considered 0.85 in the intestine.

The NorFor protein recommendation system for dairy cattle (Volden, 2011) is used in Nordic countries. In this, the supply of metabolizable protein to the animal is based on the amount of AA absorbed in the small intestine and the protein balance in the rumen. The rumen microbial AA supply is estimated based on the degradation of starch, neutral detergent fiber, crude lipid, and CP of each feed in the rumen. The urea-N recycling in this system is estimated to be 4.6% of the dietary CP concentration.

In summary, different terminology is used when estimating rumen MCP synthesis in various protein evaluation systems, yet all the protein evaluation systems are dependent on a similar principle that the amounts of available energy and protein to microbes are the main factors influencing the MCP synthesis in the rumen. Nevertheless, the important difference

between these evaluation systems is that most protein evaluation systems acknowledge the importance of urea-N recycling, but not all of them account for it. This omission of urea-N recycling may lead to an overestimation of RDP requirements of the animals as proposed by Huhtanen and Hristov (2009).

However, within the scope of the present thesis among others, the German protein evaluation system (GfE, 2001) was applied. This is because the estimation of uCP supply in the duodenum in both *in vitro* and *in vivo* system is straightforward, and without any substantial requirement for dietary parameters.

1.6 Studies on utilizable crude protein at the duodenum and rumen nitrogen balance

The German protein system aims at feeding RNB = 0 g/d (see section 1.5) to high performing dairy cows (GfE, 2001); however, RNB = 0 g/d does not consider the capacity of cattle to recycle dietary N via the rumino-hepatic pathway. Therefore, researchers have been interested in examining the effects of negative RNB diets on dairy cows and their rumen function. In this regard, several *in vivo* studies (see Table 1.1 for the studies) estimated the uCP supply at the duodenum in negative RNB diets, with an intention to increase the N use efficiency in milk by reducing the dietary CP intake, and while profiting from the cattle's capacity to re-use a share of the urea circulating in their blood (Leiber, 2014). Nevertheless, studies in Table 1.1 (Holthausen et al., 2000; König et al., 2005; van de Sand et al., 2008) with low dietary RNB in dairy cows (−1.6 to −3 g/kg DM; −37 to −94 g/d) observed no negative effects, for instance, on feed intake and MY (~30 kg/d). In contrast, few studies (Riemeier, 2004; Steinwidder et al., 2009; Aschemann, 2012) observed reduction in MY (~30 kg/d) and milk urea (mg/kg milk) with reduced dietary RNB of −0.5 to

−7.0 g/kg DM (−11 to −106 g/d) in dairy cows. Moreover, Bulang et al. (2006) showed no explicit increment patterns for DM intake, ATTD of OM, MY, and milk urea when the most positive RNB diet was compared to less positive RNB diets in the study. Such, variable response in the above-mentioned studies with various RNB levels (as see Table 1.1) may be due to the performance level of the animal with high-yielding dairy cows being more sensitive than low yielding ones. Consequently, from Table 1.1 it may be supposed that besides cattle breed, MY, and sample size (number of animals in the study) differences, variations may also be related to changes in the ingredient composition, forage to concentrate ratio, and the type of main CHO or N sources in the animals' diets.

Along the lines, dietary factors like source, extent and degradation rate of RDP and CHO influence the growth of diverse bacterial groups and MCP synthesis while the extent and rate of RUP influence absorbable dietary AA supply to the host animal (see section 1.3). As the uCP supply at the duodenum is estimated from MCP synthesis in the rumen and RUP concentrations (see section 1.5), it may be postulated that at a similar RNB level, dietary N and CHO sources and their degradation rate may influence the amount and composition of uCP supply at the duodenum. For instance, RUP supply may represent a greater proportion of uCP at the duodenum in diets containing high RUP, wherein diets with high RDP proportion may increase MCP synthesis, and therefore MCP synthesis may represent a substantial proportion of uCP at the duodenum, thus influencing the AA profile. Besides, the simultaneous availability of dietary N and CHO in the rumen and in proportions required by rumen microbes may enhance the efficiency of MCP synthesis (Jacobs, 2014). In this line, diets enhancing efficiencies of rumen microbial growth may increase requirements for RDP and result in a reduction of minimum RNB thresholds.

In vivo technique would be the 'gold standard' method to infer the effects of various CHO

and N sources at different RNB levels. Nevertheless, constraints on feeding low RNB diets and the need for rumen- or even rumen- and duodenum-fistulated animals limits carrying out such *in vivo* trials. As a result, uCP estimation and RNB calculation have increasingly been performed *in vitro* using the modified Hohenheim gas test (Steingass and Südekum, 2013). This modified Hohenheim gas test follows the standard procedure of the Hohenheim gas test (Menke and Steingass, 1988) with slight modification. The measured NH_3-N concentration in the incubation medium is the basis for estimating uCP in g/kg DM (Steingass and Südekum 2013), thereafter dietary RNB (g/kg DM) according to GfE (2001) is calculated. The modified Hohenheim gas test procedure shows potential for calculating effective uCP (g/kg DM) at selected rumen passage rates (*i.e.*, 2%, 5%, 8%/h) to provide a more suitable uCP supply for ruminants fed at various feeding levels (Westreicher-Kristen et al., 2015). In addition, *in vitro* estimation of the uCP (g/kg DM) based on dietary CP, RUP, and ME concentrations are feasible for a large number of individual feeds. Hence, screening of various N and CHO sources differing in their degradation at different RNB levels and then studying the selected dietary sources *in vivo* may be the best possible approach.

Table 1.1 Summary of studies reporting rumen nitrogen balance (RNB; g/kg dry matter, DM) levels and utilizable crude protein (uCP; g/kg DM) either estimated using fistulated animals or using the equation from Lebzien et al. (1996).

Study	Lactating animal breed	n[1]	F:C[2]	Major protein source	Major energy source	RNB	CP[3]	uCP	RUP[3] (g/kg CP)	RNB (g/d)	DM intake (kg/d)	ATTD OM[3] (g/100 g)	Milk yield (kg/d)	Milk urea (mg/kg milk)
						g/kg DM								
Holthausen et al. (2000)	Holstein	4	57:43	corn silage	corn silage	0.0	156	155	NA[3]	0	--	NA	--	
						−5.0	127	155	NA	−94	--	NA	--	↓
Kluth et al. (2003)	Holstein	39	71:29	solvent extracted soybean meal	corn silage	2.1	184	170	NA	44	--	--	--	--
		36				1.8	185	175	NA	39	--	--	--	--
		35				0.3	172	172	NA	6	--	--	--	--
Riemeier (2004)	Holstein	7	52:48	corn silage	corn silage	1.2	158	127	31	18	DR[3]			
						−1.2	139	139	35	−18	DR	↓	↓	↓
						−4.6	119	120	29	−70	DR	↓	↓	↓
						−7.0	101	112	23	−106	DR	↓	↓	↓
König et al. (2005)	Holstein	10	65:35	corn silage	corn silage	0.4	143	131	NA	10	--	NA	--	
			64:36			−1.8[4]	116	130	NA	−37[4]	--	NA	--	↓
Bulang et al. (2006)	Holstein	28	66:34	corn silage	corn silage	2.0	163	169	33	46	↑		↑	
		29	69:31	grass silage	grass silage	1.0	169	178	35	21		↑		↑

		29	69:31	lucerne silage	lucerne silage	0.0	163	167	33	0	↑			↑
van de Sand et al. (2008)	Holstein	48	74:26	grass silage	corn silage	−3.0	144	163	NA	−53	--	--	--	--
						0.5	166	163	NA	10	--	--	--	--
Steinwidder et al. (2009)	Holstein, Simmental and Brown Swiss	36	56:44	grass silage	grass silage	3.5	180	158	24	81		--		
						1.6	161	151	23	35	↓	--	↓	↓
						−0.5	142	145	22	−11	↓	--	↓	↓
Aschemann (2012)	Holstein	9	59:41	corn silage	corn silage	2.9	156	137	22	48	DR		--	
						−1.6	122	114	17	−27	DR	↓	--	↓

-- No difference between the treatments in that particular study.
↓ Significant decrease at that RNB compared to the most positive RNB level in that particular study.
↑ Significant increase at that RNB level compared to other RNB levels in that particular study.
[1] Number of cows per treatment unless otherwise specified.
[2]Forage to concentrate ratio of the diets.
[3]CP, crude protein; RUP, rumen undegraded protein; ATTD, apparent total tract digestibility; OM, organic matter; NA, not available; DR, diet restricted.
[4]Average of periods and pastures proportionally calculated.

1.7 Objectives and thesis outline

Researchers have been interested in exploring the effects of negative dietary RNB on the dairy cattle and their rumen function. However, inconsistent studies on negative effects of reduced RNB on feed intake, ATTD of nutrients, and milk production, suggest that effects of negative dietary RNB may at least partly be related to varying ingredient composition and the type of main CHO or N sources in the animals' diets, particularly in high-yielding dairy cows.

The above may be supposed because different CHO sources vary in the type, extent, and rate of CHO rumen degradation, and therefore the supply of energy that may be used by diverse rumen microbial consortium. Likewise, protein feeds that vary in the type of N source and rate of rumen degradation may influence CHO degradation, growth of diverse microbial groups that use various N sources, and therefore microbial turnover rates. Additionally, different N sources and their rate and concentration of RDP or RUP may also influence the amount and composition of uCP at the duodenum and thus animal performance. Therefore, the overall objective of the present doctoral thesis was to generate a comprehensive understanding on the effects of interaction between the RNB levels and CHO and N sources in cattle diets on rumen fermentation, the efficiency of MCP synthesis, and N use efficiency *in vitro* and *in vivo*.

In Chapter 2, two *in vitro* studies were conducted to understand the effects of different levels of RNB on CHO fermentation, CP degradation, and MCP synthesis and illustrate whether these effects differ depending on the dietary CHO or N sources. The outcome of the *in vitro* studies formed the basis for the *in vivo* study with RNB levels and dietary protein sources, within the scope of this doctoral work.

The outcomes of the conducted *in vivo* study were divided into Chapters 3 and 4. In

Chapter 3 interactions between the RNB level and the dietary protein source varying in their rate and extent of rumen degradation on nutrient intake, MCP synthesis, N partitioning, and N use efficiency in lactating dairy cows were explored. Additionally, in Chapter 4 the effects of interaction between the RNB level and the dietary protein source on nutrient intake, diet digestibility, feeding behavior, milk performance, and milk composition in lactating dairy cows were investigated.

In Chapter 5, the main findings of Chapters 2 − 4 and general relevance on the estimation of uCP at the duodenum and RNB as an indicator for adequate N supply to rumen microbes were discussed. Furthermore, in the general discussion, some constraints of the study were highlighted while laying out the way forward for future research. Finally, in Chapter 6 conclusions were drawn based on the findings from Chapters 2 − 4 with their interpretations on a broad-scale level.

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Chapter 2

2. The effects of rumen nitrogen balance on *in vitro* rumen fermentation and microbial protein synthesis vary with dietary carbohydrate and nitrogen sources

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2.1 Abstract

The objectives were to understand the effects of different levels of rumen nitrogen balance (RNB) on carbohydrate (CHO) fermentation, protein degradation, and microbial crude protein synthesis and to determine whether these effects differ depending on the dietary CHO or nitrogen (N) sources. Three RNB levels RNB0 (*i.e.*, RNB ~ 0 g/kg dry matter), RNB-5 (*i.e.*, RNB ~ −5 g/kg dry matter), and RNB-9 (*i.e.*, RNB ~ −9 g/kg dry matter) were tested in two *in vitro* experiments. Three CHO sources (*i.e.*, sucrose, corn starch, and cellulose) were incubated with urea as main source of N in experiment 1, whereas experiment 2 tested three N sources (*i.e.*, wheat gluten, soy protein, and casein) with corn starch as main source of CHO. During three runs, 1 g each (as-fed basis) of grass hay and a mixture with different proportions of the N and CHO sources were incubated in duplicate in buffered rumen fluid for 24 h.

Cumulative gas production was higher for RNB-9 than for RNB0 for all diets ($P < 0.01$; except sucrose), likely due to higher proportions of CHO in the diets. Yet, total short-chain fatty acid concentrations in inoculum only increased with declining RNB for soy protein and casein ($P \leq 0.02$), and there were no differences in proportions of acetate ($P \geq 0.31$), butyrate ($P \geq 0.31$), and propionate ($P \geq 0.33$). Ammonia-N concentrations in inoculum decreased with declining RNB for all diets ($P < 0.01$), with greater differences in wheat gluten and soy protein than in casein diets. Proportions of branched-chain fatty acids increased in experiment 1 ($P \leq 0.03$; except cellulose) and decreased from RNB0 to RNB-9 in experiment 2 (except wheat gluten; $P < 0.01$). Additionally, microbial crude protein synthesis was lower for RNB-9 than RNB0 in experiment 2 ($P < 0.01$; except casein), with no or only minor RNB effects observed in experiment 1. In conclusion, decreasing RNB has only minor effects on *in vitro* CHO fermentation. However, feed protein degradation and microbial crude protein

synthesis decrease with declining RNB, with less pronounced effects in slowly degradable CHO and rapidly degradable N sources.

Keywords: microbial protein, nitrogen use efficiency, protein nutrition, rumen fermentation, rumen nitrogen balance, ruminants

2.2 Introduction

Feeding animals with excess feed protein not only increases production costs and has negative implications for animal health and reproductive performance, but also causes higher nitrogen (N) excretions via urine, which in turn, amplifies the risk of ammonia emissions (Lebzien et al., 2006). There is a great scope for enhancing efficiency of feed protein conversion (*i.e.*, milk or meat protein per unit of protein intake) by reducing dietary crude protein (CP) intake, thus exploiting the ability of ruminants to compensate for a limited N availability in the rumen by re-utilizing a share of their circulating urea in blood (*i.e.*, rumino-hepatic cycle) (Leiber, 2014). However, N requirements of rumen microbes should be satisfied in order to maximize duodenal flow of microbial protein as the main source of essential amino acids for the ruminant host (Schwab et al., 2005).

The German feeding recommendation system uses the rumen nitrogen balance (RNB) as an indicator for the N supply to rumen microbes (GfE, 2001). The RNB is calculated as the difference between dietary CP intake and utilizable crude protein at the duodenum (uCP), where uCP is defined as total CP at the duodenum minus endogenous protein (RNB = (CP intake – (undegraded feed CP + microbial CP))/6.25; e.g. all in g/d) (Lebzien and Voigt, 1999). To avoid N shortage for rumen microbes, the RNB of ruminant diets should be close to zero or even positive (GfE, 2001). Nevertheless, studies have shown that feeding diets with RNB at −3 g/kg dry matter (DM; ~ −53 to −59 g/d) to lactating dairy cows with milk

yields of about 28 kg/d did not reduce animal feed intake and performance (König et al., 2005; van de Sand et al., 2006). Similarly, Holthausen et al. (2000) did not find any effect of RNB as low as −5 g/kg DM (~ −94 g/d) on feed intake and milk yield (~ 30 kg/d) in lactating dairy cows. Instead, Riemeier (2004) found a tendency of a decrease in milk yield (~ 23 kg/d) and milk fat concentration along with a decrease in rumen fermentation and microbial crude protein synthesis in dairy cows fed diets with RNB of −4.2 g/kg DM (~ −63 g/d). Hence, previous findings on the effects of negative RNB are inconsistent despite similar feed intake levels of the animals, which may at least partly be related to differences in the ingredient composition and the type of main carbohydrate (CHO) or N sources in the animals' diets.

Different CHO sources differ in the type of CHO (*i.e.*, mono-, di-, oligo-, or polysaccharides, structural or non-structural polysaccharides), the extent and rate of their rumen degradation, and thus the energy supply to and use by diverse rumen microbial groups. Similarly, protein feeds differ in the type of N source (*i.e.*, non-protein N, amino acid-N) and rate of rumen degradation, which in turn may influence CHO degradation (Lebzien et al., 2006), growth of diverse bacterial groups (McAllan and Smith, 1974) that use various types of N sources (Bryant and Robinson, 1962), and thus microbial turnover rates. Moreover, providing dietary N and energy such that they are available simultaneously and in proportions required by rumen microbes is expected to optimize both, diet digestion and efficiency of microbial protein synthesis (Jacobs, 2014). In this line, diets resulting in greater rates and efficiencies of rumen microbial growth may increase requirements for rumen-degradable N and result in greater minimum RNB thresholds. Nevertheless, to our knowledge, no study has analyzed so far the effects of different RNB depending on the dietary CHO or N sources. Therefore, the present research analyzed in an *in vitro* system the effects of three RNB levels in six diets differing in CHO or N sources.

The aim was (i) to understand the effects of different levels of RNB on rumen CHO and protein degradation, efficiency of microbial crude protein synthesis, and microbial biomass yield; and (ii) to elucide how these effects are influenced by the type of dietary CHO or N sources. It was hypothesized that (i) low dietary N combined with an increase in fermentable CHO supply to rumen microbes will negatively affect CHO fermentation variables; and (ii) different CHO and N sources as well as levels of N and energy supply to rumen microbes will result in a change in rumen microbial population thus influencing microbial crude protein synthesis.

2.3 Material and methods

Two *in vitro* experiments were performed to test the effects of RNB levels on rumen fermentation as affected by different CHO and N sources. The first experiment tested the RNB effects using three CHO sources, whereas in the second experiment, three N sources were used to create the experimental treatments.

2.3.1 Experiment diets and nutrient composition

In experiment 1, three pure CHO sources differing in type of CHO and rate of rumen degradation (*i.e.*, sucrose (4661.1, Roth GmbH, Karlsruhe, Germany), corn starch (9444.1, Roth GmbH, Karlsruhe, Germany), and cellulose (5873.1, Roth GmbH, Karlsruhe, Germany)) were added to unprotected urea (a non-protein N source with rapid rumen degradation) (131754.1211, Applichem Panreac, Darmstadt, Germany) and grass hay to create diets with three calculated RNB levels: RNB0 (*i.e.*, RNB close to 0 g/kg DM), RNB-5 (*i.e.*, RNB close to −5 g/kg DM), and RNB-9 (*i.e.*, RNB close to −9 g/kg DM). In experiment 2, three sources of N differing in rate of rumen degradation (*i.e.*, wheat gluten (4601.1, Roth

GmbH, Karlsruhe, Germany), soy protein (066-974, ProFam® 974 ADM, Decatur, IL, USA), and casein (C5679, Sigma-Aldrich, Steinheim, Germany)) were added to corn starch (having intermediate rate of rumen degradation compared to sucrose and cellulose) and grass hay to create similar RNB levels as in experiment 1 (*i.e.*, 0, −5, −9 g/kg DM).

Chemical composition of the individual ingredients was determined in triplicates to calculate the expected nutrient concentrations of each formulated diet (Table 2.1 and Table 2.2). The concentrations of DM, crude ash, and ether extract were determined according to VDLUFA (1997). The N concentration of the individual feedstuffs was determined using Dumas combustion method (CN analyzer, Vario Max CN Elementar, Hanau, Germany) (VDLUFA, 1997) to calculate the CP concentrations by multiplying the N concentrations by 6.25. Neutral detergent fiber and acid detergent fiber concentrations of grass hay (both inclusive residual ash) were determined following the methods of VDLUFA (1997) using an ANKOM200 Fiber Analyzer (ANKOM Technology, Macedon, NY, USA). For neutral detergent fiber analysis, heat-stable amylase (ANKOM Technology, Macedon, NY, USA) was used. The metabolizable energy (ME) concentration of the grass hay was estimated from *in vitro* gas production (GP) in triplicate in two runs according to Close and Menke (1986) using the following equation:

$$ME = 2.2 + 0.136 \times GP + 0.057 \times CP + 0.00285 \times CL^2 ;$$

where: ME = metabolizable energy in MJ/kg DM; GP = gas production in ml/ 200 mg of substrate dry mass after 24 h; CP = crude protein concentration of grass hay in g/kg DM; CL = crude lipid concentration of grass hay in g/kg DM.

The ME concentrations (MJ/kg DM) of the pure substrates sucrose, corn starch, and cellulose were obtained from Schiemann et al. (1971). As there was some CP present in cellulose (6.9 g/kg DM) and corn starch (9.9 g/kg DM), rumen CP degradability of cellulose

was assumed to be 50%, whereas that of CP in corn starch was assumed to be 80% (Huntington, 1997; Niwińska, 2012). Urea was considered to be completely degraded in the rumen. The ME concentration of wheat gluten was obtained from Schiemann et al. (1971), of casein from Agroscope (2016), and of soy protein from van Eys et al. (2004). The rumen CP degradabilities of casein (90%) and wheat gluten (75%) were taken from Agroscope (2016), whereas those of grass hay (45%) and soy protein (80%) were estimated using equations from Kirchhof (2007). For this, concentrations of different CP fractions described by Sniffen et al. (1992) and Russell et al. (1992) were analyzed following procedures developed by Licitra et al. (1996).

The grass hay contained (per kg DM): 936.9 g organic matter, 55.6 g CP, 634.7 g neutral detergent fiber, 359.4 g acid detergent fiber (both inclusive residual ash), 12.4 g ether extract, and 234.2 g non-fibrous CHO (*i.e.*, 1000 – (crude ash + CP + neutral detergent fiber + ether extract); all in g/kg DM). The ME concentration of grass hay was 7.0 MJ/kg DM. The low-quality grass hay was chosen in order to reduce the contribution of that source to total N and energy concentration in the mixed diets. The formulated diets had a forage to concentrate ratio of 50:50 (on DM basis). The information on chemical composition and ME concentrations of the ingredients was used to design the diets at three RNB levels. A pre-trial was conducted wherein the CHO and N diets were incubated at three RNB levels to verify the theoretically assumed uCP and RNB. For this, the uCP was estimated according to Steingass and Südekum (2013) and the RNB calculated as RNB = (CP - uCP)/6.25 (all in g/kg DM). Additionally, an uCP standard (CP = 254 g/kg DM) was included containing (per kg DM) 450 g rapeseed meal, 300 g field beans, and 250 g sugar beet pulp, resulting in a target uCP of 183 g/kg DM after 24 h incubation.

Table 2.1 Ingredient composition and chemical composition for the experimental carbohydrate diets at different rumen nitrogen balance (RNB) levels.

	Carbohydrates sources								
	Sucrose			Corn starch			Cellulose		
RNB (g/kg DM[1])	0	−5	−9	0	−5	−9	0	−5	−9
Ingredient composition of diets (g/kg as fed basis)									
Grass hay	500.0	500.0	500.0	500.0	500.0	500.0	500.0	500.0	500.0
Sucrose[2]	476.0	485.0	491.0	0.0	0.0	0.0	0.0	0.0	0.0
Corn starch[2]	0.0	0.0	0.0	472.0	483.0	492.0	0.0	0.0	0.0
Cellulose[2]	0.0	0.0	0.0	0.0	0.0	0.0	468.0	478.0	487.0
Urea	24.0	15.0	9.0	28.0	17.0	8.0	32.0	22.0	13.0
Chemical composition of the diets (g/kg DM)									
DM (g/kg FM[1])	958	958	959	908	908	907	926	926	927
Organic matter	969	969	969	968	968	968	967	967	967
Crude protein	104	75	56	121	87	58	132	101	72
aNDF[1,3]	317	317	317	317	317	317	758	767	776
ADF[1,3]	180	180	180	180	180	180	566	574	582
Ether extract	6.7	6.7	6.7	6.7	6.7	6.7	7.3	7.3	7.3
NFC[1,4]	541	569	588	522	557	585	69	91	111
uCP[1,5,6] (g/kg DM)	111 ± 4.1	107 ± 3.2	105 ± 5.3	126 ± 3.8	123 ± 4.7	118 ± 5.7	138 ± 5.6	137 ± 4.6	133 ± 3.1
RNB[6] (g/kg DM)	−0.7 ± 1.2	−5.3 ± 0.8	−7.8 ± 0.8	−0.8 ± 0.6	−5.9 ± 0.7	−9.0 ± 0.9	0.4 ± 1.0	−5.1 ± 0.5	−8.7 ± 0.5

[1]DM, dry matter; FM, fresh matter; aNDF, neutral detergent fiber determined using heat-stable amylase and expressed inclusive of residual ash; ADF, acid detergent fiber expressed inclusive of residual ash; NFC, Non-fibrous carbohydrates; uCP, utilizable crude protein at the duodenum.

[2]Sucrose (crude lipid 0.9 g/kg DM); corn starch (crude protein 9.9 g/kg DM, crude lipid 1.2 g/kg DM, crude ash 1.5 g/kg DM); cellulose (crude protein 6.9 g/kg DM, crude lipid 2.5 g/kg DM, crude ash 3.8 g/kg DM); urea (462 g N/kg DM).

[3]aNDF and ADF concentrations are similar in sucrose and corn starch diets as the proportion of grass hay was the same for all diets.

[4]NFC (g/kg DM) = 1000 − (crude protein + crude ash + aNDF + ether extract); all in g/kg DM.

[5]uCP was verified after incubation as described by Steingass and Südekum (2013) to further calculate RNB as RNB (g/kg DM) = (crude protein − uCP/6.25), all in g/kg DM.

[6]Arithmetic means ± standard deviations, n = 6.

Table 2.2 Ingredient composition and chemical composition for the experimental protein diets at different rumen nitrogen balance (RNB) levels.

	Nitrogen sources								
	Wheat gluten			Soy protein			Casein		
RNB (g/kg DM[1])	0	−5	−9	0	−5	−9	0	−5	−9
Ingredient composition of diets (g/kg as fed basis)									
Grass hay	500.0	500.0	500.0	500.0	500.0	500.0	500.0	500.0	500.0
Wheat gluten[2]	115.0	70.0	30.0	0.0	0.0	0.0	0.0	0.0	0.0
Soy protein[2]	0.0	0.0	0.0	127.5	67.5	27.5	0.0	0.0	0.0
Casein[2]	0.0	0.0	0.0	0.0	0.0	0.0	157.0	104.0	47.0
Corn starch	385.0	430.0	470.0	372.5	432.5	472.5	343.0	396.0	453.0
Chemical composition of the diets (g/kg DM)									
DM (g/kg FM[1])	912	910	908	917	913	909	920	916	911
Organic matter	967	967	968	963	965	967	966	966	967
Crude protein	136	96	60	145	93	57	176	128	75
aNDF[1,3]	317	317	317	317	317	317	317	317	317
ADF[1,3]	180	180	180	180	180	180	180	180	180
Ether extract	7.1	7.1	7.1	7.1	7.1	7.1	7.1	7.1	7.1
NFC[1,4]	507	547	583	493	548	585	466	515	567
uCP[1,5,6] (g/kg DM)	134 ± 3.2	128 ± 0.9	116 ± 0.7	150 ± 0.6	125 ± 0.2	111 ± 2.0	178 ± 2.1	161 ± 1.0	129 ± 0.6

RNB[6] (g/kg DM)	−0.6 ± 0.5	−5.1 ± 0.2	−9.0 ± 0.2	−0.7 ± 0.1	−5.1 ± 0.03	−8.6 ± 0.3	−0.5 ± 0.2	−5.3 ± 0.2	−8.6 ± 0.3

[1]DM, dry matter; FM, fresh matter; aNDF, neutral detergent fiber determined using heat-stable amylase and expressed inclusive of residual ash; ADF, acid detergent fiber expressed inclusive of residual ash; NFC, Non-fibrous carbohydrates; uCP, utilizable crude protein at the duodenum.

[2]Wheat gluten (crude protein 904 g/kg DM, crude lipid 3.7 g/kg DM, crude ash 7.3 g/kg DM); soy protein (crude protein 890 g/kg DM, crude lipid 5.0 g/kg DM, crude ash 41.7 g/kg DM); casein (crude protein 917 g/kg DM, crude lipid 0.53 g/kg DM, crude ash 13.4 g/kg DM); corn starch (crude protein 9.9 g/kg DM, crude lipid 1.2 g/kg DM, crude ash 1.5 g/kg DM).

[3]aNDF and ADF concentrations are similar in sucrose and corn starch diets as the proportion of grass hay was the same for all diets.

[4]NFC (g/kg DM) = 1000 − (crude protein + crude ash + aNDF + ether extract); all in g/kg DM.

[5]uCP was verified after incubation as described by Steingass and Südekum (2013) to further calculate RNB as RNB (g/kg DM) = (crude protein − uCP/6.25), all in g/kg DM.

[6]Arithmetic means ± standard deviations, n = 6.

2.3.2 In vitro experiments

The *in vitro* experiments were performed using the ANKOM RF technique (Cornou et al., 2013), an automated wireless system used to measure the GP. As compared to other *in vitro* batch incubation techniques (Close and Menke, 1986), the method allows for incubation of greater amounts of substrate which is a prerequisite for more comprehensive and advanced analysis in particular of feed residues and microbial mass during and after incubation. Individual ingredients were weighed separately into 500 ml bottles (Duran GmbH, Mainz, Germany) to result in a total amount of 2 g of mixed substrate per flask.

Rumen fluid was obtained from three rumen-fistulated Jersey cows (200 − 260 d of lactation; 580, 520, and 480 kg body weight) before the morning feeding using a tube attached to a vacuum pump. Each cow was fed for ad libitum a total mixed ration containing (per kg DM) corn silage (243 g), grass silage (243 g), grass hay (162 g), barley straw (29.5 g), and a commercial concentrate mixture (200 g: 50 g winter barley, 40 g corn grain, 30 g faba beans, 30 g pea, and 50 g rapeseed cake), rapeseed meal (103 g), and a mineral mixture (20.4 g: 2.1 g calcium, 6.8 g phosphorus, 2.2 g magnesium, 0.1 g sodium, 9.2 g potassium). The total mixed ration had a forage to concentrate ratio of 67:33 (DM basis) and contained 141 g CP/kg DM. The cows had free access to clean drinking water. The rumen fluid (3.8 l/incubation) was transported in insulated flasks to the laboratory and was filtered through a nylon bag with 100-µm-pore size. The N-free McDougall's buffer solution (McDougall, 1948) was used instead of the standard buffer according to Menke and Steingass (1988) to minimize the overall N concentration in the rumen inoculum. The pH of the buffer was adjusted to 6.8 under continuous flushing of carbon dioxide and constant magnetic stirring in a thermostatically controlled water bath (39°C). Then, buffer solution and rumen fluid were mixed in a ratio of 2:1 and 300 ml of the rumen inoculum filled into the

already pre-warmed (39°C) bottles. Thereafter, the headspace was saturated with carbon dioxide, and the bottles sealed and incubated for 24 h. Total N concentration in rumen inoculum before incubation as determined on ten of the incubation days averaged 70 mg/300 ml (standard deviation 6.72).

The three CHO (experiment 1) and N sources (experiment 2) were each incubated at three RNB levels in three independent runs. Each RNB level was incubated in duplicate per sampling time (*i.e.*, 4, 12, and 24 h), resulting in a total of 18 flasks per incubation (*i.e.*, 1 CHO or N source x 3 RNB x 3 sampling times x 2 replicates). The six blank bottles containing only buffered rumen inoculum (blank) were included per incubation: two bottles for 0 h and four bottles for 24 h of which two bottles were side-necked with a possibility to sample the buffered rumen after 4 and 12 hours of incubation. In addition, one CHO or N source at 3 RNB levels along with uCP standard were incubated in duplicate for cross-checking the RNB again (similar to pre-trial) for a period of 24 h. The variables determined were GP, concentrations of short-chain fatty acids (SCFA) and ammonia-nitrogen (NH_3-N), liquid- (LAM) and solid-associated microbial mass (SAM), and undegraded feed. In cellulose treatment, the sampling was done till 24 h; however, GP thereafter was determined in duplicate until 72 h of incubation. The total GP and total SCFA were corrected for GP and SCFA concentrations, respectively, in the blank syringes at respective sampling times.

2.3.3 Extraction and chemical composition of the microbial pellet

At each sampling time, the incubation was terminated and the pH of the rumen inoculum was recorded by using the digital pH meter (WTW Multi 340i, WTW, Weilheim, Germany). The contents of the bottles were then transferred in polyethylene bottles and centrifuged to separate the LAM, SAM, and undegraded feed. For this, the bottles were first centrifuged at

500 *x g* for 10 min at 4°C (Hettich Rotanta, Tuttlingen, Germany). The supernatant was decanted and centrifuged at 20,000 *x g* for 10 min at 4°C (Avanti™ J-25, Becker Coulter™, Indianapolis, IN, USA). After centrifugation, 10 ml of the supernatant were collected and stored in a freezer (−20°C) to determine the SCFA and NH_3-N concentrations. The pellet collected from this second centrifugation step was considered as LAM. The pellet obtained from the first, 500 *x g* centrifugation underwent a detachment procedure to separate the residual feed particulate matter and the adhered microbes. For this, the pellet was first incubated at 38°C (15 min) in 40 ml of a saline methylcellulose solution (0.9% v/w sodium chloride + 0.1% v/w methylcellulose) and then stored at 4°C for 24 h (Dickhoefer et al., 2016; Ranilla and Carro, 2003). Thereafter, the mixtures were centrifuged at 500 *x g* for 10 min. The pellet of this third centrifugation was considered to represent the undegraded feed. The supernatant was decanted and centrifuged at 20,000 *x g* for 10 min to obtain a pellet representing the SAM. The LAM, SAM, and undegraded feed pellets were then lyophilized, weighed, ground using a ball mill (Retsch, MM200, Haan, Germany) for 2 min at a frequency of 30/s, and then stored at room temperature until analysis.

Carbon and N concentrations in LAM, SAM, and undegraded feed pellets were determined using a CN analyzer according to VDLUFA (1997). The microbial N yield was calculated by multiplying the N concentrations with the weight of the LAM and SAM pellets. Moreover, the microbial crude protein synthesis was expressed as sum of microbial N yield of LAM and SAM. Similarly, N in undegraded feed was calculated by multiplying the N concentrations with the weight of undegraded feed.

2.3.4 Short-chain fatty acid analysis

For SCFA analysis (extraction procedure mentioned in section 2.3) 2 ml (in duplicate) of

the 10-ml-aliquot of the supernatant collected during centrifugation was transferred into vials and further centrifuged at 20,000 *x g* for 10 min, 4°C (Avanti™ 30, Beckman Coulter™, Indianapolis, IN, USA). The 720-µl-aliquot of the supernatant of this centrifugation step was pipetted into a 1.5-ml-vial, mixed with 80 µl of an internal standard (1 ml methylvaleric acid dissolved in 99 ml formic acid), and stored overnight at 4°C to precipitate the soluble proteins (Castro-Montoya et al., 2011). The next day, the mixtures were again centrifuged at 20,000 *x g* (10 min, 4°C). Thereafter, 750 µl of the acidified, deproteinized supernatants were transferred into 1.5-ml-glass vials and analyzed for SCFA (µmol/ml) by a gas chromatograph (GC 14-A Shimadzu Corp., Kyoto, Japan) that was equipped with an auto injector (AOC–20i, Shimadzu Corp., Kyoto, Japan). The stainless steel column used for separation was 3 m long with an internal diameter of 3 mm and was packed with 10% SP™–1000, 1% phosphoric acid, 100/120 Chromosorb® WAW (Supelco 221 Inc., Bellefonte, PA, USA).

2.3.5 Ammonia-nitrogen analysis

The NH_3-N (mg/l) concentrations were determined according to Weatherburn (1967) in duplicates. For this, 20 µl of the 10-ml-aliquot of the supernatant collected during centrifugation (see section above) was placed in a 2-ml-vial and 900 µl of reagent A (2.5 g phenol + 12.5 mg sodium-nitroprusside dissolved in 250 ml distilled water) was added. The mixture was then centrifuged for 45 s at 10,000 *x g* for 10 min, 4°C (Biofuge, Heraeus, Hanau, Germany). After 4 min, 900 µl of reagent B ((2.5 g sodium hydroxide + 2.1 ml sodium hypochlorite (containing 12% chlorine)) was added and the mixture then incubated for 20 min at 35°C. After incubation, the solution was transferred to a semi-micro cuvette and the samples were read at 625 nm using a spectrophotometer (Varian Cary 50 Bio, UV-Visible, Palo Alto, CA, USA).

2.3.6 Statistical analysis

Statistical analyses were conducted using the software SAS 9.4 (SAS Institute Inc., Cary, NC, USA). The main effect of RNB for the different CHO or N sources and interactions of RNB and CHO or N source at distinct sampling hours (n = 6; 2 duplicates x 3 incubations) was tested using mixed procedure (PROC MIXED) according to:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + f_k + e_{ijk};$$

where: Y_{ijk} = dependent variable; μ = overall mean; α_i = effect of the i^{th} RNB level; βj = effect of j^{th} CHO or N source; $(\alpha\beta)_{ij}$ = the interaction effect of i^{th} RNB level on j^{th} CHO or N source, f_k = random effect of the incubation run on i^{th} and j^{th} treatment; and e_{ijk} = residual random error of experiment.

Least squares means at different RNB levels within the CHO or the N source were compared at a significance level of $P < 0.05$, whereas the tendencies were declared at $P \geq 0.05$ to < 0.10.

2.4 Results

2.4.1 Rumen nitrogen balance verification

The RNB of the diets verified in the pre-trial were similar to the theoretically calculated RNB of the diets with no large variation between the incubation runs, as seen from the low standard deviations of the verified means (Table 2.1 and 2.2). Among the CHO sources, RNB0 ranged between 1.4 to −1.4 and RNB-9 between −7.6 to −9.3, while for N sources RNB0 ranged between −0.2 to −1.1 and RNB-9 between −8.3 to −9.3.

2.4.2 Experiment 1 – carbohydrate sources

The RNB affected GP after 24 h of incubation for all CHO sources (except sucrose diets)

with higher GP for RNB-9 than for RNB0 ($P < 0.01$; Figure 2.1). Although sucrose diets showed a similar increasing trend like other CHO sources for GP after 24 h of incubation, no significant difference ($P \geq 0.21$) between the RNB levels was observed. No differences in GP after 24 h of incubation were observed between RNB levels for all CHO sources when it was expressed in ml/g CHO source ($P = 0.24$; data not shown). Total SCFA concentrations differed between CHO sources ($P < 0.01$) but not between RNB levels at all the incubation times ($P \geq 0.37$; Table 2.3). However, differences were observed in the proportions of individual branched-chain fatty acids (BCFA) between RNB levels, with BCFA proportions being higher for RNB-9 compared to the other two RNB levels in corn starch diets after 12 h ($P < 0.01$) and in corn starch and sucrose diets after 24 h ($P \leq 0.01$) of incubation. There was an interaction effect between RNB and CHO source for iso-valerate proportions after 12 h ($P < 0.02$) and 24 h ($P < 0.02$) of incubation, where iso-valerate proportions differed between RNB levels in corn starch diets but were below the detection level in all sucrose and cellulose diets. The pH at all incubation times did not differ between RNB levels ($P \geq 0.42$) but varied between CHO sources ($P < 0.01$) after 4 and 24 h of incubation. Mean pH of RNB levels after 4 h of incubation ranged from 6.73 to 6.95 and was highest in cellulose diets ($P < 0.01$), whereas after 24 h of incubation, mean pH of RNB levels ranged from 6.65 to 6.86 and were highest in corn starch diets compared to the other CHO diets ($P < 0.01$; data not shown).

The NH_3-N concentrations were lower at RNB-9 compared to the other two RNB levels ($P < 0.01$; Figure 2.1) across all CHO sources. The NH_3-N concentrations after 4 and 12 h of incubation differed between the CHO sources ($P < 0.01$ for both incubation times; Figure 2.1). Furthermore, there was an effect of the interaction between RNB and CHO source for

NH_3-N concentration after 4 h ($P < 0.01$) and 12 h ($P = 0.03$) of incubation, with larger differences seen between the RNB levels for sucrose and corn starch diets compared to cellulose diets. The N in undegraded feed at all the incubation times differed between RNB levels (after 4 and 12 h; $P < 0.01$ and 24 h; $P = 0.01$) as well as between CHO sources ($P < 0.01$ at all the incubation times; Table 2.4). However, irrespective of the incubation time and CHO source, the microbial N yield in LAM did not differ between RNB levels ($P \geq 0.46$), whereas microbial N yield in SAM after 4 h ($P = 0.04$) and 24 h ($P = 0.03$) of incubation were lower at RNB-9 compared to the other RNB levels in sucrose diets. These effects were however, only minor. No differences in total microbial crude protein synthesis (in mg N/g DM or mg N/µmol SCFA) ($P \geq 0.31$) were found between RNB levels for all CHO sources. However, microbial crude protein synthesis after 4 h ($P < 0.01$) and 12 h ($P < 0.01$) of incubation were higher in sucrose diets compared to the other two CHO sources.

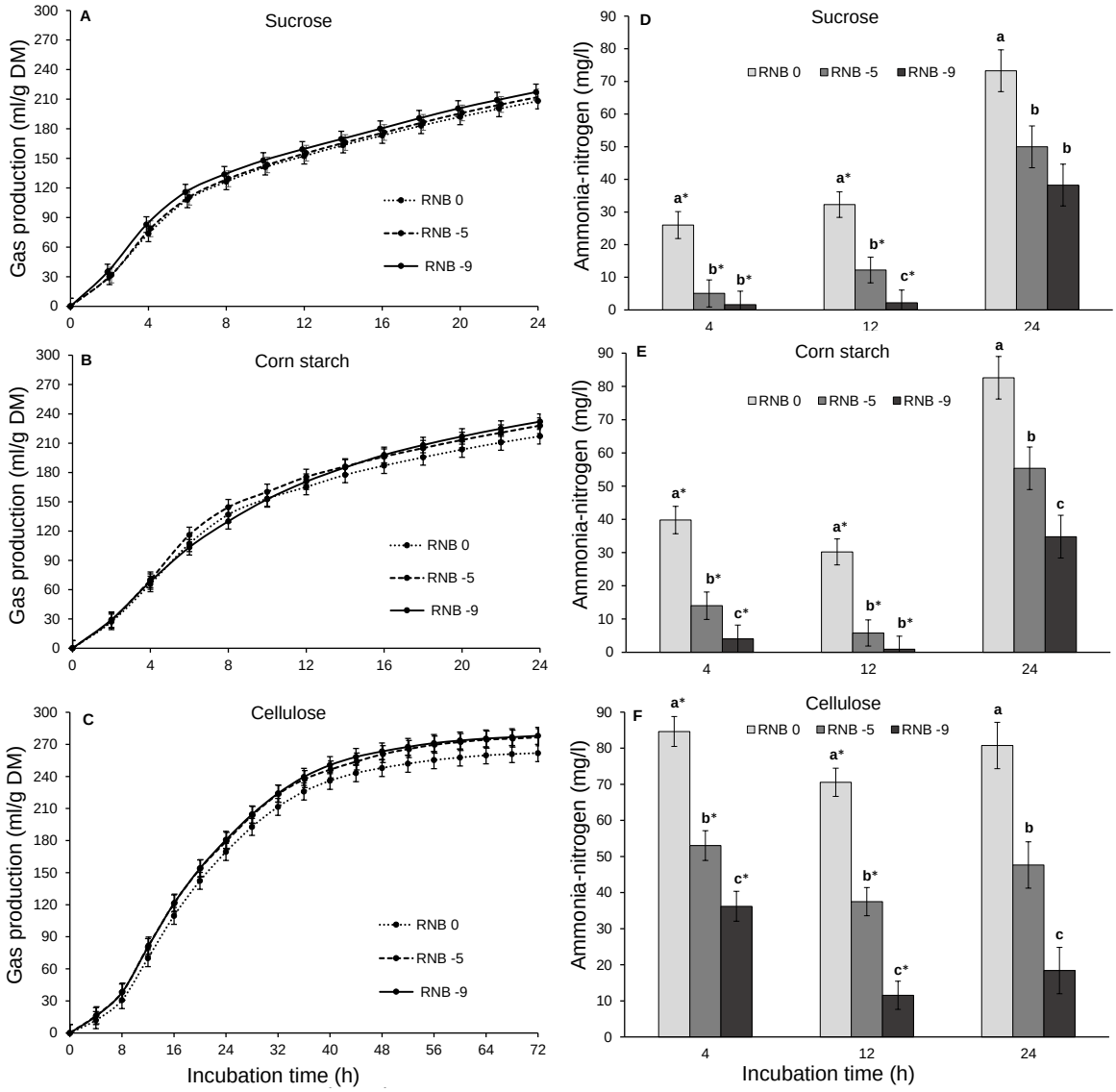

Figure 2.1 Measured gas production (A, B, C) and ammonia-nitrogen concentrations (D, E, F) from experimental diets differing in carbohydrates sources at three rumen nitrogen balance (RNB) during 24 h of incubation for sucrose and corn starch diets and during 72 h of incubation for cellulose diets.

Error bars (at particular incubation time) indicate measure of variation within the RNB levels for each carbohydrate source. Different superscripts (a, b, c) over the bars (at particular incubation time) indicate significant differences ($P < 0.05$) between the RNB levels for each carbohydrate source. Asterisk (*) over the bars (at particular incubation time) indicate significant ($P < 0.05$) RNB and carbohydrate interaction effect. DM, dry matter. RNB0, RNB-5, and RNB-9, RNB in the diet at 0 g, −5 g, and −9 g/kg DM, respectively.

Table 2.3 Effect of rumen nitrogen balance (RNB) and carbohydrate (CHO) source on short-chain fatty acids (SCFA) yield and profile in rumen inoculum at different *in vitro* incubation times (least square means; n = 6).

Variables	Time	CHO sources									SEM[1]	*P*-value		
		Sucrose			Corn starch			Cellulose				RNB	CHO	RNB x CHO
RNB (g/kg DM[1])		0	−5	−9	0	−5	−9	0	−5	−9				
Total SCFA[2] (µmol/ml inoculum)	4	12.1	12.8	12.6	17.3	15.9	16.6	4.1	4.5	4.2	1.08	0.99	< 0.01	0.79
	12	20.3	21.1	20.8	23.8	24.2	23.0	11.6	11.3	11.5	1.26	0.84	< 0.01	0.94
	24	28.6	29.5	30.1	27.0	27.4	26.6	21.7	22.4	23.7	0.63	0.37	< 0.01	0.69
SCFA proportions (µmol/100 µmol total SCFA)														
Acetate	4	52.0	53.0	53.1	64.6	63.7	64.3	66.1	66.2	64.6	1.24	0.98	< 0.01	0.88
	12	52.0	53.5	52.6	64.0	63.8	63.6	57.2	57.3	57.3	0.98	0.92	< 0.01	0.97
	24	56.5	57.2	56.4	65.1	65.3	65.3	56.6	56.5	56.8	0.72	0.91	< 0.01	0.92
Propionate	4	37.3	36.9	36.7	22.9	23.7	23.1	27.7	27.7	28.9	1.19	0.95	< 0.01	0.90
	12	35.6	35.0	35.5	21.4	21.7	22.0	37.6	37.6	37.5	1.46	0.96	< 0.01	0.99
	24	30.1	29.7	29.9	20.9	21.1	21.3	37.4	37.2	36.8	1.18	0.97	< 0.01	0.98
Butyrate	4	10.5	10.0	9.9	11.1	11.1	10.9	5.7	5.6	5.8	0.54	0.85	< 0.01	0.99
	12	11.7	11.0	11.4	13.0^{x}	12.7^{xy}	12.0^{y}	4.8	4.8	4.9	0.75	0.33	< 0.01	0.47
	24	12.5	12.2	12.7	12.0^{x}	11.6^{xy}	11.1^{y}	5.5	5.9	5.9	0.64	0.80	< 0.01	0.31

	4	0.03	0.04	0.12	0.28	0.29	0.38	nd[1]	nd	nd	0.03	0.30	< 0.01	0.85
Iso-butyrate	12	0.04	0.05	0.09	0.31[b]	0.37[b]	0.54[a]	nd	nd	nd	0.04	0.03	< 0.01	0.09
	24	0.13[b]	0.19[b]	0.29[a]	0.34[b]	0.40[b]	0.52[a]	0.05	0.09	0.12	0.03	< 0.01	< 0.01	0.47
	4	0.17	0.13	0.17	0.92	0.95	0.98	0.54	0.60	0.62	0.07	0.88	< 0.01	0.99
Valerate	12	0.64	0.41	0.37	0.94	0.93	0.97	0.39	0.38	0.38	0.06	0.48	< 0.01	0.45
	24	0.76	0.75	0.79	1.09	0.99	0.99	0.37	0.37	0.39	0.06	0.72	< 0.01	0.80
	4	nd	nd	nd	0.19	0.24	0.35	nd	nd	nd	0.04	0.68	< 0.01	0.81
Iso-valerate	12	nd	nd	nd	0.31[b]	0.48[b]	0.83[a]	nd	nd	nd	0.06	0.04	< 0.01	0.02
	24	nd	nd	nd	0.49[b]	0.62[b]	0.85[a]	nd	nd	nd	0.06	0.03	< 0.01	0.02

[1]SEM, standard error of the mean; DM, dry matter; nd, not detected.

[2]Total SCFA corrected for the SCFA concentrations in the inoculum in the blank syringes at respective sampling times.

[a,b]Means with different superscripts in same row within the same CHO source differed significantly at $P < 0.05$.

[x,y]Means with different superscripts in same row within the same CHO source tended to differ at $P \geq 0.05$ to < 0.10.

Table 2.4 Effect of rumen nitrogen balance (RNB) and carbohydrate (CHO) source on the microbial crude protein synthesis at different *in vitro* incubation times (least square means; n = 6).

Variables	Time	CHO sources									SEM[1]	*P*-value		
		Sucrose			Corn starch			Cellulose				RNB	CHO	RNB x CHO
RNB (g/kg DM[1])		0	−5	−9	0	−5	−9	0	−5	−9				
N[1] yield in LAM[1] (mg/g DM of incubated substrate)	4	7.6	7.3	6.6	5.5	5.6	4.6	4.4	4.0	4.1	0.35	0.59	< 0.01	0.97
	12	7.8	7.6	7.1	5.6	5.3	4.5	5.2	5.0	4.9	0.31	0.46	< 0.01	0.98
	24	7.0	6.9	6.5	6.2	5.8	5.7	6.1	6.2	6.5	0.15	0.87	0.21	0.91
N yield in SAM[1] (mg/g DM of incubated substrate)	4	0.96^{a}	0.75^{ab}	0.61^{b}	0.41	0.36	0.35	0.51	0.57	0.51	0.05	0.40	< 0.01	0.40
	12	0.77	0.75	0.71	0.58	0.56	0.39	0.86	0.86	0.85	0.05	0.54	0.03	0.90
	24	0.59^{a}	0.56^{a}	0.44^{b}	0.59	0.55	0.54	0.83^{x}	0.76^{xy}	0.72^{y}	0.03	0.02	< 0.01	0.99
N in undegraded feed (mg/g N in incubated substrate)	4	38.4^{c}	54.1^{b}	68.0^{a}	38.4^{c}	52.6^{b}	74.1^{a}	19.2^{b}	27.5^{ab}	36.5^{a}	3.49	< 0.01	< 0.01	0.14
	12	33.4^{c}	48.0^{b}	63.4^{a}	37.8^{c}	53.8^{b}	77.9^{a}	25.3^{b}	32.2^{b}	44.5^{a}	3.20	< 0.01	< 0.01	0.05
	24	24.8^{c}	34.1^{b}	46.2^{a}	24.0^{c}	29.9^{b}	43.5^{a}	26.1^{b}	38.7^{b}	55.0^{a}	2.13	0.01	< 0.01	0.45
Microbial crude protein synthesis														
mg N/g DM of incubated substrate	4	8.6	8.1	7.2	5.9	6.0	5.0	4.9	4.6	4.6	0.38	0.32	< 0.01	0.90
	12	8.6	8.4	7.8	6.2	5.9	4.9	6.1	5.9	5.8	0.31	0.33	< 0.01	0.96
	24	7.6	7.5	6.9	6.8	6.4	6.2	6.9	7.0	7.2	0.16	0.62	0.12	0.72
mg N/µmol of	4	0.61	0.64	0.58	0.35	0.41	0.31	1.41	1.24	1.50	0.10	0.97	< 0.01	0.85

SCFA	12	0.41	0.39	0.35	0.26	0.25	0.22	0.55	0.52	0.52	0.03	0.31	< 0.01	0.97
	24	0.29	0.27	0.27	0.25	0.23	0.24	0.32	0.31	0.30	0.01	0.80	0.08	1.00

[1]SEM, standard error of the mean; N, nitrogen; DM, dry matter; LAM, liquid-associated microbes; SAM, solid-associated microbes; SCFA, short-chain fatty acids.

[a,b,c]Means with different superscripts in same row within the same CHO source differ significantly at $P < 0.05$.

[x,y]Means with different superscripts in same row within the same CHO source tended to differ at $P \geq 0.05$ to < 0.10.

2.4.3 Experiment 2 – nitrogen sources

The RNB affected GP after 24 h of incubation across all N sources with higher GP for RNB-9 than for RNB0 ($P < 0.01$; Figure 2.2). However, GP after 4, 12, and 24 h of incubation in ml/g of corn starch were lower for RNB-9 compared to the other two RNB levels for all the N sources ($P < 0.01$; data not shown). Total SCFA concentrations after 24 h of incubation were higher for RNB-9 than for RNB0 in soy protein ($P = 0.02$) and casein diets ($P = 0.03$), and a tendency was observed for wheat gluten diets ($P = 0.07$). Moreover, proportions of individual BCFA after 24 h of incubation differed between RNB levels for all N sources ($P \leq 0.01$). There was an effect of the interaction between RNB and N source for valerate proportions after 12 h ($P < 0.04$) and 24 h ($P < 0.01$) of incubation, with greater differences observed between RNB levels for wheat gluten diets compared to soy protein and casein diets (Table 2.5). Mean pH across the N sources after 24 h of incubation was higher for RNB0 (pH = 6.74) than for RNB-9 (pH = 6.69) ($P < 0.01$). Mean pH across the RNB levels after 24 h of incubation ranged from 6.68 to 6.77 and was higher in soy protein and casein diets compared to wheat gluten diets ($P < 0.01$; data not shown).

The NH_3-N concentration differed between RNB levels ($P < 0.01$) as well as between N sources at all the incubation times ($P < 0.01$; Figure 2.2). There was also an effect of the interaction between RNB and N source on NH_3-N concentration after 4 and 12 h of incubation ($P < 0.01$ for both incubation times) with greater differences between RNB levels for soy protein and wheat gluten diets compared to casein diets. The N in undegraded feed (mg/g N in incubated substrate) after 4 h ($P < 0.01$) and 12 h ($P = 0.02$) of incubation differed between RNB levels as well as between N sources ($P < 0.01$ at all the incubation times; Table 2.6). Microbial crude protein synthesis (mg N/g DM) was lower for RNB-9 than for RNB0 in the soy protein (after 12 h: $P = 0.04$; 24 h: $P = 0.01$) and in wheat gluten diets (24

h: P = 0.02). In contrast to experiment 1, microbial crude protein synthesis (mg N/µmol SCFA) after 12 h (P = 0.01) and 24 h (P < 0.01) of incubation was lower for RNB-9 than for RNB0 in all N sources.

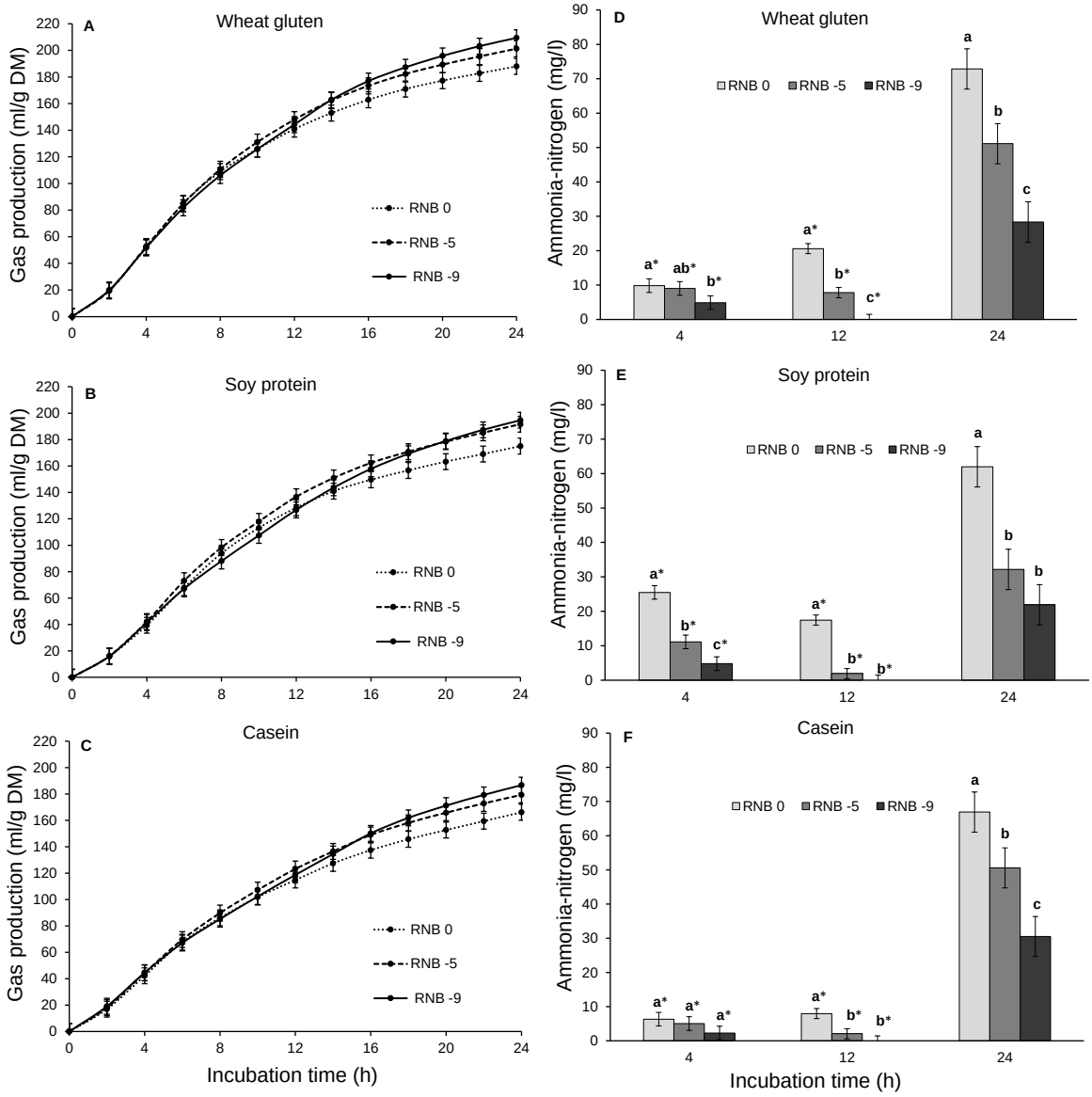

Figure 2.2 Cumulative gas production (A, B, C) and ammonia-nitrogen concentrations (D, E, F) from experimental diets differing in nitrogen source at three rumen nitrogen balances (RNB) during 24 h of *in vitro* incubation.

Error bars (at particular incubation time) indicate measure of variation within the RNB levels for each nitrogen source. Different superscripts (a, b, c) over the bars (at particular incubation time) indicate significant differences ($P < 0.05$) between the RNB levels for each nitrogen source. Asterisk (*) over the bars (at particular incubation time) indicate significant ($P < 0.05$) RNB and nitrogen interaction effect. DM, dry matter. RNB0, RNB-5, and RNB-9, RNB in the diet at 0 g, −5 g, and −9 g/kg DM, respectively.

Table 2.5 Effect of rumen nitrogen balance (RNB) and nitrogen (N) source on the short-chain fatty acids (SCFA) yield and profile in rumen inoculum at different *in vitro* incubation times (least square means; n = 6).

Variables	Time	N source									SEM[1]	*P*-value		
		Wheat gluten			Soy protein			Casein				RNB	N	RNB x N
RNB (g/kg DM[1])		0	−5	−9	0	−5	−9	0	−5	−9				
Total SCFA[2] (µmol/ml inoculum)	4	10.2	10.5	9.2	9.3	8.5	9.1	8.7	8.2	5.1	0.52	0.52	0.05	0.78
	12	21.3	21.3	21.8	17.2	17.1	18.0	16.5	15.6	18.9	0.64	0.61	0.02	0.92
	24	27.6^{y}	28.9^{xy}	30.2^{x}	22.9^{b}	26.4^{a}	26.8^{a}	23.8^{b}	23.7^{b}	27.2^{a}	0.55	< 0.01	< 0.01	0.50
SCFA proportions (µmol/100 µmol total SCFA)														
Acetate	4	59.3	59.1	58.4	64.5	65.8	66.5	59.7	65.2	59.2	1.01	0.54	0.02	0.72
	12	60.2	59.6	59.7	58.7	60.2	60.2	59.5	56.8	57.0	0.58	0.88	0.28	0.74
	24	60.7	60.9	61.8	62.0	61.9	63.5	61.4	61.0	61.9	0.33	0.31	0.27	0.98
Propionate	4	28.0	28.1	29.7	27.7	27.5	26.7	27.7	27.9	25.0	0.78	0.95	0.75	0.94
	12	24.1	25.1	25.5	24.9	26.5	26.5	25.5	28.3	28.0	0.54	0.31	0.22	0.99
	24	22.6	23.0	23.0	22.3	23.3	22.9	22.5	23.3	23.4	0.23	0.49	0.91	1.00
Butyrate	4	11.5	11.4	11.0	7.0	6.2	6.5	11.8	9.5	11.5	0.56	0.44	< 0.01	0.76
	12	13.3	13.5	13.4	14.0	12.0	11.7	12.4	12.8	13.0	0.38	0.80	0.63	0.71
	24	13.2	13.2	13.3	12.8	12.7	12.1	12.2	12.7	12.8	0.28	0.95	0.45	0.93

	4	0.24	0.30	0.19	0.08	0.07	0.06	0.17	0.09	0.00	0.03	0.50	0.03	0.84
Iso-butyrate	12	0.33	0.33	0.33	0.50	0.48	0.57	0.53	0.47	0.50	0.02	0.70	< 0.01	0.87
	24	0.53	0.51	0.47	0.62^{x}	0.57^{xy}	0.47^{y}	0.85^{a}	0.70^{a}	0.52^{b}	0.03	< 0.01	< 0.01	0.22
	4	0.91	0.94	0.60	0.71	0.36	0.21	0.72^{ab}	0.94^{a}	0.20^{b}	0.13	0.04	0.10	0.54
Valerate	12	1.67^{a}	1.27^{b}	0.80^{c}	1.13^{a}	0.57^{b}	0.53^{b}	1.23^{a}	1.03^{ab}	0.86^{b}	0.07	< 0.01	< 0.01	0.04
	24	2.15^{a}	1.57^{b}	1.01^{c}	1.35^{a}	0.99^{b}	0.71^{c}	1.73^{a}	1.33^{b}	0.83^{c}	0.09	< 0.01	< 0.01	< 0.01
	4	0.13	0.11	0.06	nd[1]	nd	nd	0.04	0.09	0.03	0.02	0.72	0.04	0.90
Iso-valerate	12	0.37	0.27	0.23	0.73^{x}	0.40^{y}	0.53^{xy}	0.77	0.67	0.63	0.05	0.23	< 0.01	0.89
	24	0.79	0.63	0.46	0.97^{a}	0.66^{ab}	0.34^{b}	1.35^{a}	0.97^{b}	0.53^{c}	0.07	< 0.01	0.01	0.47

[1]SEM, standard error of the mean; DM, dry matter; nd, not detected.

[2]Total SCFA corrected for the SCFA concentrations in the inoculum in the blank syringes at respective sampling times.

[a,b,c]Means with different superscripts in same row within the same N source differ significantly at $P < 0.05$.

[x,y]Means with different superscripts in same row within the same N source tended to differ at $P \geq 0.05$ to < 0.10.

Table 2.6 Effect of rumen nitrogen balance (RNB) and nitrogen (N) source on the microbial crude protein synthesis at different incubation times (least square means; n = 6).

Variables	Time	N sources									SEM[1]	*P*-value		
		Wheat gluten			Soy protein			Casein				RNB	N	RNB x N
RNB (g/kg DM[1])		0	−5	−9	0	−5	−9	0	−5	−9				
N yield in LAM[1] (mg/g DM of incubated substrate)	4	6.4	6.0	5.9	7.0	6.5	6.3	6.3	6.4	5.9	0.11	0.15	0.16	0.88
	12	8.2^{a}	7.0^{ab}	6.2^{b}	8.1^{a}	7.0^{ab}	5.7^{b}	8.6	7.5	7.2	0.24	< 0.01	0.24	0.93
	24	8.0^{a}	7.6^{ab}	6.8^{b}	7.8^{a}	7.4^{a}	6.4^{b}	7.3	7.2	6.9	0.13	< 0.01	0.32	0.58
N yield in SAM[1] (mg/g DM of incubated substrate)	4	0.34	0.40	0.37	0.82	0.81	0.86	0.56	0.44	0.53	0.05	0.94	< 0.01	0.95
	12	0.93	0.90	1.00	0.67	0.73	0.57	0.67	0.63	0.47	0.07	0.89	0.16	0.96
	24	1.06	0.95	0.89	0.91	0.79	0.60	0.66	0.56	0.67	0.07	0.64	0.18	0.93
N in undegraded feed (mg/g N in incubated substrate)	4	42.4^{c}	52.6^{b}	70.4^{a}	34.1^{c}	43.8^{b}	55.2^{a}	34.9^{b}	37.6^{b}	51.2^{a}	2.27	< 0.01	< 0.01	0.19
	12	37.1^{c}	48.3^{b}	64.2^{a}	33.9^{c}	46.7^{b}	63.2^{a}	32.8^{b}	37.6^{b}	53.0^{a}	2.56	0.02	< 0.01	0.69
	24	25.9^{c}	33.8^{b}	50.6^{a}	25.3^{c}	36.0^{b}	52.0^{a}	25.1^{c}	32.5^{b}	42.4^{a}	2.06	0.05	< 0.01	0.21
Microbial crude protein synthesis														
mg/g DM of incubated substrate	4	6.7	6.4	6.3	7.8	7.3	7.2	6.9	6.8	6.4	0.14	0.34	0.02	0.98
	12	9.1	7.9	7.2	8.8^{a}	7.7^{ab}	6.3^{b}	9.3	8.1	7.7	0.28	0.03	0.42	0.95
	24	9.1^{a}	8.6^{ab}	7.7^{b}	8.7^{a}	8.2^{ab}	7.0^{b}	8.0	7.8	7.6	0.17	0.01	0.14	0.55

mg N/µmol of SCFA	4	0.66	0.64	0.77	0.91	0.92	0.82	0.79^{y}	0.84^{xy}	1.24^{x}	0.04	0.36	0.06	0.44
	12	0.43	0.38	0.33	0.57^{a}	0.49^{ab}	0.37^{b}	0.57^{x}	0.54^{xy}	0.43^{y}	0.02	0.01	0.01	0.90
	24	0.33^{a}	0.30^{ab}	0.25^{b}	0.38^{a}	0.31^{ab}	0.26^{b}	0.34^{ab}	0.36^{a}	0.28^{b}	0.01	< 0.01	0.20	0.46

[1]SEM, standard error of the mean; DM, dry matter; LAM, liquid-associated microbes; SAM, solid-associated microbes; SCFA, short-chain fatty acids.

[a,b,c]Means with different superscripts in same row within the same N source differ significantly at $P < 0.05$.

[x,y]Means with different superscripts in same row within the same N source shows tended to differ at $P \geq 0.05$ to < 0.10.

2.5 Discussion

The effects of RNB levels on rumen fermentation as affected by different CHO and N sources were studied in experiment 1 and 2, respectively. Different CHO sources namely, a disaccharide (sucrose), a non-structural polysaccharide (corn starch), and a structural polysaccharide (cellulose) were studied in experiment 1 in combination with urea as the main source of N. Urea was chosen as N source due to its rapid and complete rumen degradation. Moreover, as urea contains high amount of N, using urea as main N source also helped to adjust RNB without any major changes in ingredient composition of the diets. In contrast, different sources of N, a highly soluble animal protein (casein) and two partially soluble plant proteins (soy protein and wheat gluten), which differ in their rate of degradation (Annison, 1956), were studied with corn starch as CHO source in experiment 2. It was hypothesized that a more negative RNB would reduce *in vitro* GP, concentration of SCFA, NH_3-N concentration, and microbial crude protein synthesis due to a lack of N that would limit the rumen microbial activity. Moreover, it was expected that effects of varying RNB would depend on the dietary CHO and N sources, due to, for instance, differences in the kinetics of their rumen degradation or associated shifts in microbial species composition.

2.5.1 Effect of rumen nitrogen balance on carbohydrate fermentation

In contrast to our expectations, reducing RNB did not hamper microbial activity and CHO fermentation, but increased GP after 24 h of incubation in both experiments (except sucrose diets), which was likely due to a greater amount of the respective CHO source in RNB-9 than in the diets of the other two RNB levels (Table 2.1 and 2.2). The similar GP per unit of CHO across the RNB levels in experiment 1 supports this explanation. Similarly, van Dung et al. (2014) observed an increase in *in vitro* GP with declining dietary CP concentration from

190 g to 100 g/kg DM, which was possibly also due to the greater proportion of cassava meal in diets with low than with high CP concentrations. Accordingly, total SCFA concentrations increased and pH decreased with declining RNB in experiment 2 of the present study. Similar to results of van Dung et al. (2014), no differences in total SCFA concentrations and pH with declining RNB were also observed for all CHO sources in experiment 1 which is likely related to (i) the smaller differences in the amount of CHO between the diets of different RNB levels in experiment 1; and (ii) the greater differences in total SCFA concentrations across the RNB levels between the CHO than between the N sources. Total SCFA concentrations were greater in wheat gluten than in casein diets, likely due to their greater CHO proportions. Contradictory to our expectations, RNB also had no or only minor effects on proportions of major SCFA (*i.e.*, acetate, propionate, and butyrate) in both experiments, suggesting that differences in the amounts of the CHO sources between the RNB levels were too small to result in pronounced shifts in the species composition of the microbial consortium or to alter fermentation pathways. Similar to findings of Beuvink and Spoelstra (1992) and Belanche et al. (2012), acetate proportions were lower and propionate proportions greater in cellulose than in corn starch diets in experiment 1, which may be due to a lower extent and slower rate of degradation of grass hay in cellulose diets. Overall, results indicate that N availability did not limit nor alter CHO fermentation in any of the CHO or N sources.

2.5.2 Effect of the interaction between rumen nitrogen balance and carbohydrate or nitrogen source on carbohydrate fermentation

Despite pronounced differences in the fermentation characteristics between CHO or N sources, no significant effects of the interactions between RNB level and CHO or N source

were detected for GP, total SCFA concentrations, and proportions of acetate, propionate, and butyrate in experiment 1. Similarly, Fanchone et al. (2013) and Klusmeyer et al. (1990) testing two dietary N levels and two energy (*i.e.*, starch, fiber) or N sources (*i.e.*, corn gluten, soy bean meal), respectively, found similar effects of N level on SCFA concentration and proportions in the rumen of lactating dairy cows irrespective of the CHO or N source in their diets. Hence, results do not confirm our hypothesis that the effects of RNB on CHO fermentation differ depending on the CHO or N source. The lack of differences in the effects of declining RNB between CHO or N sources is most likely related to the fact that N availability was not limiting microbial activity and thus CHO fermentation even at RNB-9. An increased use of N from microbial origin might have at least partly compensated for the lower feed N supply in RNB-9 diets. Moreover, decreasing dietary N supply might have reduced microbial growth rates, resulting in lower N requirements of rumen microbes per unit of CHO fermented, which is partly confirmed by the declining microbial crude protein synthesis in experiment 2 (see below). Additionally, the lack of effects of the interaction between RNB level and CHO source for SCFA concentrations and proportions of acetate, propionate, and butyrate in experiment 1 was likely also related to the absence of an overall RNB effect on these variables (see above).

Overall, results suggest that RNB effects on CHO fermentation *in vitro* do not differ depending on the CHO or N source in ruminant diets. Yet, differences in the responses between experiments 1 and 2 for SCFA concentration and GP (in ml/g CHO source) indicate that RNB effects might differ between diets that vary greatly in their ingredient and chemical composition.

2.5.3 Effect of rumen nitrogen balance on protein degradation and microbial crude protein synthesis

As expected, NH_3-N concentration of rumen inoculum decreased with declining RNB for all diets, which is at least partly due to the lower proportions of N sources and thus lower CP concentrations in the RNB-9 and RNB-5 diets as compared to the RNB0 diets. For all RNB-9 diets, NH_3-N concentrations after 24 h of incubation were below 50 mg/l, which is considered optimum for maximal growth of mixed microbial populations *in vitro* (Satter and Slyter, 1974). Although such low NH_3-N concentrations can be sufficient for continued hydrolytic activity of microbes (Wallace, 1979), they may hamper microbial growth in particular of SAM, which have higher NH_3-N requirements than LAM (McAllan and Smith, 1983). Accordingly, declining dietary RNB decreased microbial crude protein synthesis as well as concentrations and proportions of BCFA (*i.e.*, from deamination of amino acids; El-Shazly, 1952) in experiment 2, suggesting that lack of N inhibited growth of rumen microbes in these diets. A similar trend of a reduced microbial crude protein synthesis with negative RNB was also observed for sucrose and corn starch diets in experiment 1, although differences between RNB levels were much smaller compared to experiment 2 and partly not significant according to ANOVA. The more pronounced effect of RNB on variables related to microbial growth and feed protein degradation in experiment 2 than experiment 1 might be explained by the greater differences in the ingredient composition between the RNB diets (e.g. in terms of CHO concentration) in experiment 2 than experiment 1.

The N in undegraded feed (mg/g N in incubated substrate) increased with declining RNB levels in experiment 1 and 2 diets, suggesting decline in dietary CP degradation and/or increase in protozoal and fungal mass in the undegraded feed fraction. Moreover, proportions of valerate, that is also formed from amino acids (Chen and Russell, 1988),

decreased with declining RNB, because of lower dietary N concentrations in diets tested in experiment 2. In contrast, an increase in BCFA proportions with declining RNB was found in experiment 1 for corn starch and sucrose diets (not detected in cellulose diets), and valerate proportions did not differ between RNB levels in these diets. As urea used in experiment 1 is a non-protein N source, a considerable proportion of the BCFA in sucrose and corn starch diets must have originated from the breakdown of microbial protein. Hence, rumen BCFA proportions might have increased with declining RNB due to predation and digestion of bacteria by protozoa or autolysis of rumen microbes in response to a lack of N (Dawes, 2013; Lapierre and Lobley, 2001; Morrison and Mackie, 1996) in RNB-9 diets in experiment 1. Predation and autolysis of microbes most likely also occurred in RNB-9 diets of experiment 2 as indicated by a decrease in microbial crude protein synthesis (in mg N/g DM or mg N/µmol SCFA) with declining RNB. Yet, the decline in BCFA production from dietary true-protein sources probably exceeded the increase in BCFA formation from microbial protein at low RNB, resulting in an overall decrease in BCFA proportions.

2.5.4 Effect of the interaction between rumen nitrogen balance and carbohydrate or nitrogen source on protein degradation and microbial crude protein synthesis

Similar to our expectations, the effects of RNB level differed depending on the CHO or N source for several variables related to ruminal protein degradation and microbial crude protein synthesis. The NH_3-N concentrations in incubation medium decreased with declining RNB level after 4 and 12 h of incubation for all diets tested in experiment 1. Yet, the differences were more pronounced for sucrose and corn starch diets as compared to the cellulose diets, respectively. Accordingly, microbial N yield in SAM decreased with declining RNB in sucrose, but not in corn starch and cellulose diets.

The limited RNB effects on protein-related variables in cellulose diets are most likely associated to its lower degradability and slower rate of degradation and suggest that, in contrast to the other two CHO sources, N supply from incubation medium, urea, and grass hay was sufficient to cover the N requirements of rumen microbes. In the same line, Fanchone et al. (2013) suggested greater N requirements for microbial growth in starch-rich than in fiber-rich diets of lactating cows based on observed differences in rumen NH_3-N concentrations. Additionally, dietary CP concentrations in the present study were higher for cellulose than for corn starch and sucrose diets (Table 2.1). As a result, the increases in predation and autolysis of bacteria and rate of NH_3-N use for microbial crude protein synthesis from RNB0 to RNB-9 diets were likely less pronounced for cellulose than for the other two CHO sources. The interaction effects observed for BCFA proportions in experiment 1 were probably related to the fact that their concentrations were below the detection level for cellulose and sucrose diets, which hampered statistical analysis of actual interactions.

In experiment 2, differences between RNB levels in NH_3-N concentrations of the incubation medium after 4 and 12 h of incubation were more pronounced for soy protein and wheat gluten as compared to casein diets. Moreover, synthesis of microbial crude protein declined with decreasing RNB level for wheat gluten and soy protein, but not for casein diets, corresponding to the smaller and partly non-significant differences between RNB levels in BCFA proportions for wheat gluten and soy protein (in case of iso-butyrate), likely related to a greater BCFA release from microbial protein breakdown. The greater differences in BCFA proportions and less pronounced effects of declining RNB on NH_3-N concentrations in casein than in wheat gluten and soy protein diets could be related to the faster degradation rate of casein as compared to the other protein sources which allowed for a sufficient supply

of N compounds from dietary sources by the time starch was degraded. Moreover, CP concentration was higher for casein than for wheat gluten and soy protein diets. From experiment 2, it can thus be suggested that differences exist in RNB effects on protein degradation and rumen microbial crude protein synthesis depending on the dietary N source possibly due to shifts in microbial species composition and/or the synchrony in N and energy supply to microbes. These changes did not affect SCFA concentration and profiles, because N requirements are lower for hydrolytic activity than for microbial growth (Wallace, 1979), so that N availability at low RNB did not limit CHO fermentation. In the same line, also Belanche et al. (2012) found varying responses to declining dietary N levels in the abundance of selected bacterial species in the rumen of lactating dairy cows depending on the ingredient composition of the diets (*i.e.*, varying CHO sources), without any effects on fermentation profile.

Despite limited differences in RNB effects between N sources for SCFA concentration and profiles in experiment 2, valerate proportions in incubation medium decreased with declining RNB level for all diets, showing an interaction effect between RNB levels and N source. The observed interactions for valerate were possibly due to pronounced differences between wheat gluten diets and other N sources. As proline, which is a precursor for valerate (McDonald, 2011), is a major amino acid in wheat gluten, differences in the proportion of this N source greatly affected valerate production.

Use of the *in vitro* batch system allowed for testing a wide range of RNB in multiple pure CHO and N sources without any confounding animal or dietary effects. However, situations *in vitro* clearly differ from those *in vivo* which may hamper transferability of our findings. On the one hand, there is a continuous removal of N from the rumen due to NH_3-N absorption and outflow of microbial and feed crude protein, which could aggravate the N deficit at rumen

level as compared to *in vitro* situations. On the other hand, diets are composed of diverse types of CHO and N sources with varying rumen degradation and passage rates and more frequent feed intake results in continuous substrate supply. Moreover, there is a constant inflow of N via rumen wall and saliva (*i.e.*, urea-N recycling) *in vivo*, which accounts for approximately 20% of the N supply for microbial protein synthesis (GfE, 2001) and increases with declining RNB (Lebzien et al., 2006). This recycling of N may compensate for low rumen-degradable N intake. In this line, a recent study by Fanchone et al. (2013) did not find any significant interactions between level of dietary N and type of energy source (*i.e.*, fiber *versus* starch) on duodenal microbial biomass flow. However, the study comprised only four cows, and actual rumen protein levels (*i.e.*, difference between N intake and duodenal nonammonia N flow multiplied by 6.25) differed for starch- and fiber-rich diets, hampering detection of any possible interaction effects. Hence, further *in vivo* studies are necessary to confirm our observed differences in RNB effects between different CHO and N sources.

2.6 Conclusions

Decreasing RNB does not reduce *in vitro* CHO fermentation in diets only varying in the type of one CHO or N source, due to additional N supply from breakdown of microbial protein. Yet, differences may occur in RNB effects between diets with more contrasting ingredient and chemical composition. Highly negative RNB reduce microbial crude protein synthesis, particularly in diets with readily fermentable CHO and slowly degradable N sources. Hence, minimum RNB in diets of ruminants for optimum rumen fermentation and microbial crude protein synthesis may vary with diet composition. However, further research is needed to clarify reasons for such differences in the RNB effects between feed N and CHO sources and to resolve whether they might be less pronounced *in vivo* with more

continuous supply and removal of energy and N from dietary and endogenous origin.

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Chapter 3

3. The effects of rumen nitrogen balance on nutrient intake, nitrogen partitioning, and microbial protein synthesis in lactating dairy cows offered different dietary protein sources

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3.1 Abstract

The aim was to study the effects of rumen nitrogen balance (RNB), dietary protein source, and their interaction on feed intake, nitrogen (N) partitioning, and rumen microbial crude protein (MCP) synthesis in lactating dairy cows. Twenty-four lactating Holstein cows were included in a replicated 4 × 4 Latin square experimental design com- prising four 20-d periods, each with 12 d of adaptation to the experimental diets and 8 d of sampling. The dietary treatments followed a 2 × 2 factorial arrangement (*i.e.*, 4 treatments) with 2 main protein sources [faba bean grain (FB) and SoyPass (SP; Beweka Kraftfutterwerk GmbH, Heilbronn, Germany)] offered at 2 dietary RNB levels each [0 g/kg of dry matter, DM (RNB0) and −3.2 g/kg of DM (RNB−)]. The RNB was calculated as the difference between dietary crude protein (CP) intake and the rumen outflow of undegraded feed CP and MCP and divided by 6.25. Composition of concentrate mixtures was adjusted to create diets with desired RNB levels. Each of these protein sources supplied ≥35% of total dietary CP. Both diets for each protein source were isoenergetic but differed in CP concentrations. The main effects of RNB, protein source, and their interactions were tested using PROC MIXED in SAS 9.4 (SAS Institute Inc., Cary, NC). The DM intake (kg/d) was lower for RNB− than for RNB0 in diets containing FB, whereas no differences were seen between the RNB levels for SP diets. The RNB− decreased N intake and urinary N excretion but increased milk N use efficiency in both FB and SP diets, with greater differences be- tween the RNB levels for FB diets than for SP diets. Similarly, duodenal MCP synthesis (g/kg of digestible organic matter intake) estimated from purine derivatives in the urine was lower for RNB− than for RNB0 in FB diets but similar between the RNB levels in diets containing SP. Low RNB of approximately −65 g/d (approximately −3.2 g/kg of DM) in diets reduced feed intake, N balance, and performance

in high-yielding dairy cows with possibly more pronounced effects in diets containing rapidly degradable protein sources.

Keywords: dairy cow, microbial protein synthesis, rumen nitrogen balance, protein nutrition, nitrogen use efficiency

3.2 Introduction

Feeding excess dietary crude protein (CP) beyond the requirements of dairy cattle and microbes in their rumen increases production costs, excretion of nitrogen (N) in particular via urine, and metabolic load of the animals for eliminating surplus dietary N. There may be an opportunity to improve feed protein conversion (*i.e.*, milk protein per unit of protein intake) by matching dietary CP intake to actual requirements of rumen microbes and their host and by exploiting the animal's capacity to reuse a share of the urea circulating in their blood (Leiber, 2014). Although this approach can be beneficial from economic, animal health, and ecological point of view, a shortage of N supply to rumen microbes may also hamper nutrient fermentation, decrease microbial crude protein (MCP) synthesis and thus feed intake and utilizable crude protein supply (uCP) in the animals.

The German feeding recommendation system uses the rumen nitrogen balance (RNB) as an indicator for the adequacy of N supply to rumen microbes (GfE, 2001). The RNB is calculated as the difference between dietary CP intake and the rumen outflow of rumen undegraded protein (RUP) and MCP: (RNB = (CP intake – (RUP + MCP))/6.25) (all in g/d). Reducing the dietary RNB has been studied previously as a measure to efficiently utilize the available N in the rumen. Results of several studies in the literature on low RNB in diets of dairy cows (*i.e.*, −1.8 to −5 g/kg dry matter (DM); −37 to −94 g/d) did not observe any negative effect on, for instance, feed intake, MCP synthesis, and milk yield (MY; ~ 30 kg/d)

(Holthausen et al., 2000; König et al., 2005; van de Sand et al., 2008). In contrast, other studies with dairy cows have already observed negative effects on rumen MCP synthesis and MY at dietary RNB of only −0.5 to −1.6 g/kg DM (−11 to −27 g/d) (Steinwidder et al., 2009; Aschemann et al., 2012a; Aschemann et al., 2012b). Variable responses to negative RNB in different studies may at least partly be related to varying carbohydrate and protein sources included in the diets (Kand et al., 2018). Along this line, Kand et al. (2018) studied the effects of three RNB levels (*i.e.*, 0, −5, and −9 g/kg DM) on rumen fermentation in diets containing different carbohydrate (*i.e.*, sucrose, corn starch, and cellulose) and N (*i.e.*, casein, wheat gluten, and soy protein) sources *in vitro.* The authors found that low RNB negatively affects MCP synthesis with more pronounced effects in diets with slowly degradable N than in those with rapidly degradable N sources, likely due to evident time lapse in availability between rapidly available carbohydrate and slowly degradable N.

Dietary protein sources differ in the extent and rate of breakdown to ammonium and amino acids, which in turn affects carbohydrate degradation (Lebzien et al., 2006), growth of diverse microbial populations (McAllan and Smith, 1974) that use different N sources, and thus microbial turnover rates and MCP synthesis. Additionally, different protein sources and their rate and amount of RUP or rumen degraded protein (RDP) may also influence the amount and composition of uCP at the duodenum and thus animal MY.

Therefore, the aim of the study was to understand the effects of the interaction between the level of RNB and the dietary protein source on intake, partitioning of N excretion, N use efficiency, and rumen MCP synthesis. It was hypothesized that at low RNB, N use efficiency for rumen MCP and milk protein synthesis would increase and that urinary N excretion would decrease because of reduced N supply to rumen microbes. Differences would be more pronounced in the diets with a slowly degradable protein source, as seen in our previous *in*

vitro study (Kand et al., 2018). Additionally, differences in the extent and rate of rumen degradation between the protein sources may influence the amount and composition of uCP supply at the duodenum.

3.3 Materials and methods

The animal trial reported herein was conducted at the Meiereihof experimental farm of the University of Hohenheim, Stuttgart, Germany (48°42'50.6"N and 9°13'03.0"E) and was performed in accordance with ethical and animal welfare legislation.

3.3.1 Experimental design, feeding, and animals

The experimental design followed a 4 × 4 Latin square and comprised of four 20-d periods, each with 12 d of adaptation to the experimental diets and 8 d of sample collection (*i.e.*, sampling period). Dietary treatments followed a 2 × 2 factorial arrangement with two main protein sources offered at two dietary RNB levels each (*i.e.,* four treatments). Faba bean grain (FB) and SoyPass (SP) (Beweka Kraftfutterwerk GmbH, Heilbronn, Germany) were selected as main protein sources due to differences in the extent of degradation in the rumen. For diet formulation, the extent of CP degradation in rumen was considered to be 850 g/kg CP for FB (DLG, 1997) and 270 g/kg CP for SP (Harstad and Prestløkken, 2000). The two RNB levels were 0 g/kg DM (RNB0) and −3.2 g/kg DM (RNB-). The latter RNB level was selected to achieve a RNB of − 70 to – 80 g/d at an estimated mean DM intake of the animals of ∼24 kg/d, where according to literature (Jilg et al., 1999; Riemeier, 2004) a tendency for negative effects on feed intake and animal performance may be visible. The FB supplied 37% and 35% and the SP 35% and 36% of total dietary CP for the two RNB0 and RNB-, respectively.

The diets were fed as a total mixed ration (TMR) with either RaPass (Beweka Kraftfutterwerk GmbH, Heilbronn, Germany) and FB or rapeseed cake and SP (Table 3.1). Dietary RNB was adjusted by varying the ingredient composition of the concentrate mixture, whereas forage to concentrate ratio of 55:45 (on DM basis), forage ingredients, and their proportions in the diet were constant in all diets. All the dietary treatments met the uCP requirements of dairy cows according to the recommendations of GfE (2001), assuming a body weight (BW) of 650 kg, a MY of 30 kg/d, and a DM intake of 24 kg/d.

For diet formulation, CP concentrations (per kg DM) were analyzed for grass hay (73 g), second-cut grass hay (76 g), barley straw (43 g), barley grain (113 g), sugar beet pulp molasses (127 g), rapeseed cake (362 g), RaPass (392 g), FB (297 g), and SP (521 g). The CP concentrations of corn silage (63 g) and grass silage (155 g) of the previous-year's harvest were considered. In order to avoid opening silage bales before the start of the experiment, previous-year's CP concentrations of the silages were considered. The metabolizable energy (ME) concentrations of forages, barley grain, and rapeseed cake were obtained from Spiekers et al. (2009), whereas RUP proportions for the same were obtained from DLG (1997). The extent of degradation of RaPass was obtained from the feed producer (Beweka Kraftfutterwerk GmbH, Heilbronn, Germany). The uCP supply from the individual feeds were then estimated following the regression equation 9 of Lebzien et al. (1996) to calculate dietary RNB (GfE, 2001). Samples of all diet ingredients and the TMR were taken and analyzed to confirm the nutrient and ME concentrations and rumen CP degradabilities assumed in diet formulation (see "Sample collection and Processing", and Table 3.2).

All forage ingredients and the four concentrate mixtures were weighed and mixed thoroughly each morning using a mixer wagon (Power Champ L, MARMIX GmbH & Co. KG, Unterwachingen, Germany). Diets were fed *ad libitum* once daily at approximately 08:45 h,

ensuring a daily feed refusal of approximately 10% of offered feed (on DM basis). Additionally, the external fecal marker titanium dioxide (TiO_2) (60797, Kronos® 1171, Kronos Worldwide Inc. Dallas, TX, USA) was mixed with the concentrate mixtures of each of the four diets. From d 8 till d 20 of the experimental periods, the concentrate mixtures containing TiO_2 were mixed each morning to achieve a dietary concentration of ~ 1.4 g TiO_2 /kg of DM and an intake of 34 g/d of TiO_2 (at a DM intake of ~ 24 kg/d of each cow). Each diet was offered in three separate feeding troughs; the same troughs assigned for each group of cows across all experimental periods to avoid any perturbation. The troughs were equipped with electronic scales, computer-regulated access gates, and a transponder sensor (Waagen Döhrn GmbH & Co.KG, Wesel, Germany) to automatically record feed intake (on fresh matter (FM) basis) of individual cows.

Table 3.1 Ingredients of the four offered experimental diets varying in rumen nitrogen balance (RNB) levels and protein source fed as a total mixed ration to the lactating dairy cows.

	Protein source			
	Faba bean grain		SoyPass	
Ingredient (g/kg dry matter)	RNB0[1]	RNB-[1]	RNB0	RNB-
Corn silage	245	245	245	245
Grass silage	105	105	105	105
Grass hay	141	141	141	141
Second-cut grass hay	23	23	23	23
Barley straw	36	36	36	36
Faba bean grain	196	145	0	0
RaPass[2]	98	27	0	0
SoyPass[2]	0	0	118	102
Rapeseed cake	0	0	135	66
Barley grain	77	116	77	168
Sugar beet pulp molasses[2]	52	84	95	59
Feed sugar[3]	0	50	0	30
Mineral mixture[3,4]	27	28	24	25

[1]RNB0, RNB in the diet 0 g/kg dry matter; RNB-, RNB in the diet is −3.2 g/kg dry matter.
[2]Beweka Kraftfutterwerk GmbH, Heilbronn, Germany.
[3]Bergophor Futtermittelfabrik Dr. Berger GmbH & Co. KG, Kulmbach, Germany
[4]Contained the following (g/100 g, as-fed basis), faba bean grain (RNB0): 8.5 g beta-carotene, 7.6 g feed salt, 31.9 g calcium carbonate, 13.4 g monosodium phosphate, 33.6 g mineral feed GM134, and 5.0 g magnesium oxide; faba bean grain (RNB-): 7.6 g beta- carotene, 4.6 g feed salt, 35.2 g calcium carbonate, 16.8 g monosodium phosphate, 30.5 g mineral feed GM134, and 5.3 g magnesium oxide; SoyPass (RNB0): 10.9 g beta-carotene, 10.8 g feed salt, 28.3 g calcium carbonate, 6.5 g monosodium phosphate, and 43.5 g mineral feed GM134; SoyPass (RNB-): 9.2 g beta- carotene, 7.3 g feed salt, 31.2 g calcium carbonate, 12.8 g monosodium phosphate, 36.7 g mineral feed GM134, and 2.8 g magnesium oxide. Mineral feed composition (as fed): 200 g calcium, 40 g magnesium, 50 g sodium, 7 g zinc, 5 g manganese, 1.1 g copper, 35 mg selenium, 60 mg iodine, 20 mg cobalt, 250,000 IU Vitamin A, 65,000 IU Vitamin D, 5000 mg Vitamin E, and 120 mg Vitamin B7.

Twenty-four lactating Holstein cows (10 primiparous, 14 multiparous) were used and divided into four groups of six cows each. All the four groups of cows had a similar (mean ± standard deviation) MY (35.2 ± 5.9 kg/d), days in milk (120 ± 83 d), lactation number (2.8 ± 1.7 lactations), and BW (681 ± 44 kg) before the start of the experiment. Arrangement of the animal groups remained unchanged throughout the experiment. The four groups of cows were randomly assigned to one of the four experimental diets before the start of the experiment. The cows were housed in a free-stall barn with free access to clean drinking water.

Cows were milked twice daily (05:00 and 16:00 h) in a 2 × 6 tandem milking parlor (Westfalia, GEA Farm Technologies, Bönen, Germany), where MY was automatically recorded for each cow by an electronic milk meter (Metatron P21; GEA Farm Technologies, Bönen, Germany). Additionally, BW of the cows was recorded at the exit of the milking parlor after each morning milking using an automated walk-over weighing system (GEA Farm Technologies, Bönen, Germany).

3.3.2 Sample collection and processing

Offered and refused feed. During every sampling period, about 0.5 kg FM each of all offered and refused feed were collected in the morning, weighed, and stored at −20°C. At the end of every sampling period, these samples were lyophilized for 72 h (LYO GT2 Basis, SRK Systemtechnik GmbH, Riedstadt, Germany) and weighed to determine their DM concentrations. In addition, samples of individual diet ingredients were collected at the end of the *in vivo* experiment and lyophilized for 72 h. The samples of the offered and refused feed as well as their ingredients were then ground to pass a 1-mm sieve (SM 1, Retsch

GmbH, Haan, Germany). Thereafter, samples of each offered and refused feed were pooled by period according to the weighted daily DM intake of each treatment group of animals (*i.e.*, six cows).

Feces and urine. For feces and urine spot sampling, the animals were randomly divided into two sampling groups of 12 animals each (*i.e.*, three cows per dietary treatment) at the beginning of the experiment to avoid long sample collection at each sampling time. The group division remained constant throughout the experiment. Sampling of each group was alternated between morning (8:00 h) and afternoon (16:00 h) for eight days to compensate for day-to-day and diurnal variations in the concentrations TiO_2 in feces (Glindemann et al., 2009) and of purine derivative (PD), creatinine, and N in urine (Dickhoefer et al., 2015). Fecal samples (~ 500 g FM) were collected directly from the rectum of each cow and stored at -20°C. At the end of each sampling period, the samples were thawed at room temperature overnight, thoroughly homogenized, and pooled by cow and period by taking the same quantity of feces (400 g FM) from each daily sample. The pooled samples were stored at -20°C until the end of the experiment and then lyophilized for 72 h (LYO GT2 Basis, SRK Systemtechnik GmbH, Riedstadt, Germany), weighed, and ground to pass a 2-mm sieve (SM 1, Retsch GmbH, Haan, Germany).

Simultaneous to fecal sampling, urine spot samples of at least 400 ml were collected by initiating urination through gently massaging the skin under the vulva of the animal. Urine samples were homogenized and filtered through a funnel containing a gauze of pore size 0.5 mm to remove foreign materials (e.g. hair, feces, feed, dust). Of the filtered urine, 200 ml were acidified with sulfuric acid (20%, v/v) to reduce its pH below 3, and the amount of sulfuric acid was recorded. About 100 ml of this acidified urine were filtered through filter

paper (DP 400 185, Ø 185 mm, average pore size 7–12 µm, Hahnemühle FineArt GmbH, Dassel, Germany*)* and 20 ml of the filtrate were diluted with distilled water (1:5). Three aliquots of 12 ml each of this diluted urine were taken and stored at −20°C for PD and creatinine analysis. The remaining 100 ml of the acidified, undiluted urine was transferred into plastic tubes and immediately frozen at −20°C. At the end of each sampling period, these urine samples were thawed overnight and pooled by cow and period by taking the same amount of urine (50 ml) from each individual sample (corrected for the amount of sulfuric acid added). Of the pooled samples, two aliquots of 50 ml each were taken and stored at −20°C until urinary N analysis.

Milk. Samples of milk (~ 100 ml per cow) were collected during milking at 16:00 h on d 14, 16, 18, and 20 of the experimental period, and at 5:00 h on the respective following mornings on d 15, 17, and 19 and d 1 of the subsequent adaptation period. The afternoon milk samples were refrigerated until the next morning and were then pooled together per cow per day to have one milk sample. For pooling, 20 ml of afternoon milk was taken, whereas the amount of morning milk equaled the ratio between morning and afternoon MY (kg/cow) multiplied by 20 ml. The pooled milk samples (~ 45 ml) were conserved with 150 µl Bronysolv BL (90.051.001, ANA.LI.TIK. Austria, Vienna, Austria) and refrigerated at 4°C until milk protein analysis.

3.3.3 Laboratory analysis

Samples of offered and refused feed, individual diet ingredients, and feces were analyzed in duplicate according to official analytical methods in Germany (VDLUFA, 2007; all methods listed in this paragraph are from this source). Analysis were repeated, when the coefficient

of variations of duplicate determinations of the same feed sample were > 5%. The samples were analyzed for DM (method 3.1) and subsequently for crude ash (method 8.1), to calculate organic matter (OM) concentrations. The N concentrations of the offered and refused feed, individual feed, feces, and acidified urine were determined using Kjeldahl procedure (method 4.1.1) and CP concentrations were calculated (CP = N × 6.25). Additionally, starch was analyzed in samples of corn silage, FB, barley grain, and offered diets (method 7.2.5) using an enzymatic kit (Test-Combination Nr. 10 207 748 035, R-Biopharm AG, Darmstadt, Germany). Samples of the offered diets were analyzed for crude lipid by ether extraction procedure (method 5.1.1). The concentrations of neutral detergent fiber (NDF; method 6.5.1), acid detergent fiber (method 6.5.2), and acid detergent lignin (method 6.5.3) (all with residual ash) were determined in samples of offered diets using an Ankom200 Fiber Analyzer (ANKOM Technology, Macedon, NY, USA), whereas only NDF was analyzed in samples of refused feed. Sodium sulfite and heat-stable α-amylase (ANKOM Technology, Macedon, NY, USA) were used for all NDF analyses.

Concentrations of non-protein N, neutral-detergent-insoluble N, and acid-detergent-insoluble N were determined in duplicate for samples of offered diets according to Licitra et al. (1996). Moreover, samples of FB, SP, and RaPass were incubated *in situ* to determine degradability and rate of degradation of DM, OM, and CP in the rumen following the procedure of Titze et al. (2019). For this, three dry, rumen-fistulated Jersey cows with BW (mean ± standard deviation) of 529 ± 76.6 kg were used. Cows were fed a TMR containing (per kg DM) corn silage (325 g), grass silage (325 g), grass hay (226 g), barley straw (108 g), a commercial concentrate mixture (10 g), and a mineral-amino-acid-vitamin mixture (6 g). About 7 g of each protein source was weighed in duplicate into a polyester bag (pore size of 50 ± 15 µm, R1020, ANKOM Technology, Macedon, NY, USA) and incubated for in

each cow for 0, 2, 4, 8, 16, 24, 48, and 72 h. Residues left in the bag after incubation, washing, and drying were pooled per feed sample, cow, and incubation time for DM (method 3.1), crude ash (method 8.1) to calculate OM, and N (method 4.1.2) analysis according to VDLUFA (2007). The CP concentrations of the residues were calculated by multiplying the N concentrations by 6.25.

Samples of offered diets were also incubated in triplicate *in vitro* for 24 h on two days each following procedures by Menke and Steingass (1988) to estimate ME concentrations from *in vitro* gas production and proximate nutrient concentrations. Additionally, samples of offered diets were incubated in triplicate *in vitro* for 8 h and 24 h on two days each according to Steingass and Südekum (2013) to estimate uCP from ammonium concentrations in inoculum (modified Hohenheim gas test). Moreover, an uCP standard (CP = 254 g/kg DM) was included with a target uCP of 232 g, 183 g, and 97 g/kg DM after 8 h, 24 h, and 48 h of incubation, respectively.

During *in vitro* tests for ME and uCP estimation, rumen fluid was collected from two lactating, rumen-fistulated Jersey cows (490 and 560 kg BW) using an electric vacuum pump. The cows were fed with a TMR that comprised of (per kg DM) corn silage (243 g), grass silage (243 g), grass hay (162 g), barley straw (29.5 g), rapeseed meal (103 g), a commercial concentrate mixture (200 g), and a mineral-amino-acid-vitamin mixture (20.5 g).

Table 3.2 Chemical composition of the four offered experimental diets varying in rumen nitrogen balance (RNB) and protein source fed as a total mixed ration to the lactating dairy cows (arithmetic mean ± one standard deviation; n = 4).

Variable	Protein source			
	Faba bean grain		SoyPass	
	RNB0[1]	RNB-[1]	RNB0	RNB-
Chemical composition (g/kg DM[2] unless noted)				
DM (g/kg fresh matter)	408 ± 2.9	414 ± 5.1	412 ± 3.3	415 ± 4.0
Crude ash	74 ± 1.4	71 ± 0.6	75 ± 2.8	72 ± 3.8
CP[2]	159 ± 3.4	128 ± 2.2	174 ± 1.7	150 ± 3.1
Crude lipid	21 ± 1.8	19 ± 0.8	36 ± 1.9	28 ± 0.7
Starch	123 ± 4.5	121 ± 5.5	83 ± 3.2	112 ± 9.3
Neutral detergent fiber	405 ± 5.6	388 ± 9.5	420 ± 5.8	407 ± 7.1
Acid detergent fiber	216 ± 7.0	203 ± 3.7	220 ± 3.9	208 ± 7.4
Acid detergent lignin	27 ± 2.0	20 ± 1.4	35 ± 1.1	28 ± 1.5
Non-protein nitrogen (g/kg CP)	394 ± 22	400 ± 13	325 ± 19	301 ± 15
NDIN[2] (g/kg CP)	212 ± 10	178 ± 8.4	275 ± 7.6	300 ± 13
ADIN[2] (g/kg CP)	62 ± 1.3	47 ± 0.8	72 ± 1.3	89 ± 1.6
ME[2] (MJ/kg DM)	10.5 ± 0.07	10.5 ± 0.10	10.7 ± 0.11	10.7 ± 0.07
RUP[2] (g/kg CP) (k = 5% and 8%/h) + *in situ*[3]	375	319	402	414
Theoretical uCP[2,4]	157	143	177	169
Theoretical RNB[4]	−0.03	−3.20	−0.03	−3.18
uCP – Total mixed ration[5]	160 ± 3.0	146 ± 2.2	174 ± 1.7	167 ± 2.6
RNB – Total mixed ration[5]	−0.3 ± 0.24	−2.9 ± 0.11	−0.2 ± 0.16	−2.7 ± 0.16
uCP – (RUP; k = 5% and 8%/h) + *in situ*[3,6]	165	149	178	170
RNB – (RUP; k = 5% and 8%/h) + *in situ*[3,6]	−0.7	−3.4	−0.3	−2.9

[1]RNB0, RNB in the diet at 0 g/kg DM; RNB-, RNB in the diet at −3.2 g/kg DM.
[2]DM, dry matter; CP, crude protein; NDIN, neutral detergent insoluble nitrogen; ADIN, acid detergent insoluble nitrogen; ME, metabolizable energy; RUP, rumen undegraded protein; uCP, utilizable crude

protein.
[3]The RUP concentrations for individual feed ingredients (except FB, SP, and RaPass) were predicted from their concentrations of CP fractions using the equation of Kirchhof (2007) for forages and of Shannak et al. (2000) for concentrates, each at passage rate of 0.05/h and 0.08/h, respectively. The RUP concentration for FB, SP, and RaPass were determined *in situ* at the passage rate of 8%/h.
[4]Theoretical uCP (g/kg DM) and RNB (g/kg DM) calculated during ration formulation.
[5]Total mixed rations were incubated (n = 24; 4 periods x 3 triplicates x 2 incubation runs). The uCP (g/kg DM) was determined after incubation at a passage rate of k = 8 %/h, as described by Steingass and Südekum (2013) to further calculate RNB (g/kg DM) according to GfE (2001).
[6]The microbial CP of the individual feed was determined by multiplying ME concentration by 10.1 g CP/MJ ME (GfE, 2001), where ME concentration was estimated from proximate nutrient concentrations and gas production during *in vitro* incubation. The uCP (g/kg DM) was calculated as the sum of RUP and microbial CP (Lebzien and Voigt, 1999) and the RNB (g/kg DM) was calculated according to GfE (2001).

In samples of offered diets and feces, TiO_2 was analyzed by spectrophotometry as described by Boguhn et al. (2009) with slight modifications. The PD and creatinine analyses in urine were performed following the procedures described by Balcells et al. (1992) and George et al. (2006) with minor modifications. For this, diluted urine samples from two consecutive sampling days were thawed, thoroughly mixed, and pooled by animal by taking the same amount of urine (10 ml) from each individual sample. The four pooled urine samples per animal and period were then further diluted with 20 mM of ammonium dihydrogen phosphate in the ratio of 1:4 and centrifuged at 22,000 *x g* at 4°C for 10 min (Avanti™ 30, Beckman Coulter™, Indianapolis, IN, USA). Of this centrifuged sample, 20 µl of the supernatant were inserted into 1.5-ml-glass vials that were then loaded into a high-performance liquid chromatograph (Varian 920-LC, Palo Alto, CA, USA). For PD, only allantoin and uric acid concentrations were determined in urine, because xanthine and hypoxanthine have been reported to be almost absent in urine of cattle (Verbic et al., 1990; González-Ronquillo et al., 2004).

Milk samples were analyzed for protein and urea concentrations in duplicate by Milchprüfring Baden-Württemberg e. V. (Kirchheim unter Teck, Germany) according to ASU

L 01.00-78, 2002-05 (DIN ISO 6922) and 05022100.QMD, 2011-03, respectively, using Fourier-transform infrared spectroscopy (Bentley FTS, Bentley Instruments, Chaska, MN, USA).

3.3.4 Calculations

The RNB of individual feed ingredients were estimated as the difference between their CP concentration and the potential rumen outflow of RUP and MCP divided by 6.25 (GfE, 2001). The ruminal RUP concentrations for individual feed ingredients (except FB, SP, and RaPass) were predicted from their concentrations of CP fractions using the equation of Kirchhof (2007) for forages and of Shannak et al. (2000) for concentrates, each at passage rate of 0.05/h and 0.08/h, respectively. The effective CP degradability (g/kg CP) of FB, SP, and RaPass was calculated using the equation of McDonald (1981) with the modification of Wulf and Südekum (2005). A passage rate of 0.08/h was assumed to represent high feed intake levels (Wulf and Südekum, 2005). The *in situ* RUP proportion (g/kg CP) was then calculated as 1000 – effective degradability (g/kg CP). The MCP flow was calculated from the ME concentrations assuming an efficiency of MCP synthesis of 10.1 g CP/MJ ME (GfE, 2001). The ME concentrations were estimated from Menke and Steingass (1988), using their equation 16e for forages, and 14b for concentrates. For estimating ME concentrations of individual feeds, crude lipid concentrations of FB, barley grain, and rapeseed cake were obtained from Agroscope (2016), and those of grass and corn silages from Spiekers et al. (2009). The previous year's crude lipid concentrations (per kg of DM) were taken for grass hay, second-cut grass hay, and barley straw. The crude lipid concentrations of SP, RaPass, and sugar beet molasses were obtained from the supplier (Beweka Kraftfutterwerk GmbH, Heilbronn, Germany). The ME concentrations of the offered diets were estimated using

equation 14f of Menke and Steingass (1988).

For estimating the daily DM intake of individual cows, the DM concentration of the actually ingested feed was calculated as the difference in the amount of feed offered to and refused by each group of 6 cows (on DM basis) divided by the total FM intake of the entire group of 6 cows multiplied by 1000. By multiplying these DM concentrations of the ingested feed with the FM intake of each individual cow recorded by the trough on the respective day, the daily DM intake was estimated and the mean value across the eight days was calculated. Similarly, the concentrations of OM, N, CP, and NDF in ingested feed were calculated as the difference in the respective concentrations in offered and refused feed (on a DM basis) for each group of cows, and then divided by their total DM intake. By multiplying these nutrient concentrations in ingested feed with the DM intakes of individual cows, the mean OM, N, CP, and NDF intakes across the sampling periods were estimated.

Fecal DM excretion was calculated from the average daily intake of TiO_2 (g/animal) across the sampling period divided by the TiO_2 concentration (g/kg DM) in the feces of the respective cow, assuming a fecal recovery of TiO_2 of 100% (Glindemann et al., 2009). The digestible organic matter (DOM) intake was calculated from the cows' daily OM intake and their fecal OM excretion.

Urinary N excretion (g/animal and d) was calculated as the difference between the N intake and the N deposition in BW change (positive or negative) and the N losses via feces, milk, and skin (all in g/animal). The change in BW across each period was calculated as difference between the average BW of first 5 d of two consecutive adaptation periods. The N deposition (g/animal and d) with BW change was calculated as the change in BW (g/animal and d) multiplied by 22.4 g N/kg BW change (Kirchgeßner, 1997). Milk protein yields were calculated from the content of protein in milk (g/kg milk) multiplied by the daily

MY (kg/d) of each animal. The milk N yield was calculated by dividing the milk protein yield by 6.38. The N losses via the skin and hair (g/d) were calculated as 0.018 (g N/kg$^{0.75}$ BW) times metabolic BW of the animals (kg$^{0.75}$) (GfE, 2001). The daily urine volume (l/animal) was calculated by dividing the daily estimated urinary N excretion (g/animal) by the N concentration (g/l) in the urine of the respective animal.

Daily urinary PD excretion (mmol/animal) was determined by multiplying the estimated urine volume (l/animal and d) with the PD concentration (mmol/l) in the urine of the respective animal. Duodenal absorption of microbial PD (mmol/animal and d) was calculated according to Verbic et al. (1990). From this, duodenal microbial N flow (g/animal and d) was estimated from absorbed PD following equation 5 of Chen and Gomes (1992). The ratios between the urinary concentrations of PD and creatinine and between PD and N, as well as the PD to creatinine index were calculated as indicators for rumen N turnover (Chen et al., 1995).

3.3.5 Statistical analysis

For statistical analysis, arithmetic means per cow and period were calculated for all the variables, with the dataset comprising of total number of 96 observations (*i.e.*, 4 periods x 4 treatments x 6 animals). Statistical analyses were conducted using the software SAS 9.4 (SAS Institute Inc. Cary, NC, USA). The main effect of RNB level, protein source, and their interactions (n = 24 cows) were tested for all the variables using mixed model (PROC MIXED) according to:

$$Y_{ijklm} = \mu + G_i + R_j + P_k + T_l + (RP)_{jk} + (RT)_{jl} + (PT)_{kl} + (RPT)_{jkl} + A_{mi} + e_{ijklm}$$

where: Y_{ijkl} = dependent variable; μ = overall mean; G_i = effect of the animal group during feeding (i = 1–4); R_j = effect of the RNB level (j = 1–2); P_k = effect of the protein source (k =

1–2); T_l = effect of the period (l = 1–4); $(RP)_{jk}$ = the interaction effect of j^{th} RNB level and the k^{th} protein source; $(RT)_{jl}$ = the interaction effect of j^{th} RNB level and l^{th} period; $(PT)_{kl}$ = the interaction effect of k^{th} protein source and l^{th} period; $(RPT)_{jkl}$ = the interaction effect of j^{th} RNB level, k^{th} protein source, and l^{th} period; A_{mi} = random effect of the m^{th} animal in i^{th} group; and e_{ijkl} = residual random error of experiment.

Least square means were compared with Tukey test at a significance level of $P < 0.05$, whereas the tendencies were declared at $P \geq 0.05$ to < 0.10. As the interactions of RNB x period, protein x period, and RNB x protein x period were not significant, the model was re-run without these effects. The fixed effect of group was not significant and is thus not reported in the results section.

3.4 Results

3.4.1 Nutrient intake

The CP concentrations of the diets ranged from 128 g to 174 g/kg DM (Table 3.2), and were similar to our target values. The RDP and rate of *in situ* CP degradation of feeds were 710 g, 350 g, and 370 g/kg CP and 17%, 6.1%, and 6.7%/h, for FB, SP, and RaPass, respectively, at a passage rate of 8%/h (data not shown). The analyzed starch concentrations and greater proportions of sugar beet molasses, and feed sugar suggest greater non-fibrous carbohydrate concentrations in RNB- compared to RNB0 diets. Dietary NDF and ME concentrations were very similar across all diets and ranged between 388 to 420 g/kg DM and 10.5 to 10.7 MJ/kg DM, respectively. The uCP supply estimated using the modified Hohenheim gas test and the RNB of the diets derived thereof were also similar to our target values. The RNB (g/d) with FB source was −7.3 g/d and −68.2 g/d for RNB0 and

RNB- diets, respectively. Similarly, RNB (g/d) with SP source was −4.9 g/d and −65.3 g/d RNB0 and RNB- diets, respectively.

The DM and OM intakes were lower for RNB- than for RNB0 in diets containing FB ($P \leq 0.01$, for both variables; Table 3.3) with no differences seen between RNB levels in diets containing SP source. There was also an interaction between RNB level and protein source for CP intake ($P < 0.01$), as CP intake decreased in RNB- diet with greater reduction seen in the FB compared to the SP diet, and greater differences between protein sources in RNB- compared to RNB0 diets.

Table 3.3 Effects of rumen nitrogen balance (RNB) and protein source on intake, fecal excretion, and milk yield in lactating dairy cows (least square means, n = 24).

Variable	Protein source				SEM[1]	*P*-value			
	Faba bean grain		SoyPass			RNB	Protein	RNB x Protein	Period
	RNB0[2]	RNB-[2]	RNB0	RNB-					
Intake (kg/d)									
Dry matter	24.4[a]	23.5[b]	24.3[a]	24.2[a]	0.51	< 0.01	0.16	0.03	< 0.01
Dry matter (g/kg body weight$^{0.75}$ and day)[3]	183.0[a]	176.4[b]	182.2[a]	180.7[a]	3.9	< 0.01	0.20	0.07	0.04
Organic matter	22.5[a]	21.6[b]	22.3[a]	22.3[a]	0.48	0.01	0.17	0.03	< 0.01
Crude protein	3.90[b]	3.01[d]	4.23[a]	3.65[c]	0.081	< 0.01	< 0.01	< 0.01	< 0.01
Digestible organic matter	15.1[a]	14.6[b]	15.2[a]	15.1[a]	0.32	0.06	0.09	0.32	< 0.01
Milk yield (kg/d)	30.4[c]	28.2[d]	32.7[a]	31.5[b]	1.55	< 0.01	< 0.01	0.17	< 0.01

[a,b,c,d]Means with different superscripts in the same row differed significantly at $P < 0.05$.
[x,y]Means with different superscripts in the same row tended to differ at $P \geq 0.05$ to < 0.10.
[1]SEM, standard error of the mean.
[2]RNB0, RNB in the diet at 0 g/kg dry matter; RNB-, RNB in the diet at −3.2 g/kg dry matter.
[3]Mean body weight of each animal across each sampling period.

3.4.2 Nitrogen balance and turnover

An effect of the interaction between RNB level and protein source was found for urinary N excretion ($P < 0.01$; Table 3.4). Urinary N excretions (g/d) were lower for RNB- compared to RNB0 for both protein sources, with greater absolute differences between the RNB levels in FB than in SP diets ($P < 0.01$). Moreover, urinary N excretions were greater for SP than FB for both RNB levels ($P < 0.01$), with greater differences between protein sources in RNB- compared to RNB0 diets. Fecal N excretions (g/d) differed between RNB levels only for FB ($P = 0.02$). In addition, fecal N excretion was greater with SP compared to FB for RNB- level ($P < 0.01$). The excretion of N in milk (g/d) was lower for RNB- compared to RNB0 in cows fed FB diets ($P < 0.01$). There were interactions between RNB level and protein source for the proportion of N intake found in urine, feces, and milk ($P \leq 0.02$). According to the pairwise comparisons, N excretion expressed as proportion of N intake was lower in urine and greater in feces and in milk for RNB- than RNB0 for both protein sources ($P < 0.01$, for all variables); however, absolute differences between the RNB levels were greater in FB than in SP diets. Moreover, urine and fecal N excretions (g/100 g N intake) were greater or lower, respectively, for SP than FB diets at RNB- but not with RNB0. Similarly, excretion of N in milk (g/100 g N intake) was greater for SP than for FB for both RNB levels ($P \leq 0.03$), with greater differences between protein sources in RNB- compared to RNB0 diets.

The estimated PD excretion (mmol/d) was lower for RNB- compared to RNB0 for FB ($P < 0.01$; Table 3.5) but similar between RNB levels in diets containing SP. Accordingly, rumen MCP synthesis (g/kg DM intake and g/kg DOM intake) was lower for RNB- than RNB0 in FB ($P < 0.01$, for both variables) with no differences between RNB levels containing SP. Pairwise comparison suggested that MCP synthesis (g/kg DOM intake) was lower for FB compared to SP for RNB- level ($P < 0.01$), whereas a tendency for a lower MCP (g/kg DOM

intake) was observed for SP than FB for RNB0 level (P = 0.06). The MCP synthesis expressed in g/kg CP intake differed between RNB levels only for SP (P < 0.01), with greater efficiency of N use for MCP synthesis for RNB- compared to RNB0 diet. The MCP (g/kg CP) was lower for SP compared to FB at RNB0 level (P < 0.01).

3.4.3 Period effects

Dietary CP concentrations (g/kg DM) ranged from 125 g to 171 g in period 1, 129 g to 174 g in period 2, 126 g to 175 g in period 3, and 130 g to 174 in period 4. The CP concentration in the diets were slightly lower in period 1 compared to rest of the periods (P < 0.01; data not shown). Consequently, the DM and N intakes, urinary N and PD excretions, and rumen MCP synthesis (g/kg DM intake) were lowest in period 1, followed by period 2, and then periods 3 and 4 ($P \leq 0.03$, for all variables). Milk N excretions were greater in periods 1, 2, and 3 than in period 4 ($P \leq 0.04$). However, as all diets were tested in every period, these period effects do not hamper the analysis of the main effects of RNB level, protein source, and their interaction analyzed in the present study.

Table 3.4. Effects of rumen nitrogen balance (RNB) and protein source on nitrogen (N) intake, excretion, and partitioning via urine, feces, and milk in lactating dairy cows (least square means, n = 24).

Variable	Protein source				SEM[1]	*P*-value			
	Faba bean grain		SoyPass			RNB	Protein	RNB x Protein	Period
	RNB0[2]	RNB-[2]	RNB0	RNB-					
N balance (g/d)									
N intake	623[b]	482[d]	677[a]	584[c]	12.8	< 0.01	< 0.01	< 0.01	< 0.01
Urine N	215[b]	104[d]	246[a]	169[c]	6.1	< 0.01	< 0.01	< 0.01	< 0.01
Fecal N	230[ay]	217[b]	240[ax]	234[a]	6.6	0.01	< 0.01	0.40	0.21
Milk N	167[b]	155[c]	174[a]	170[ab]	6.5	< 0.01	< 0.01	0.06	0.01
N retained with BW[1] change	8.97[b]	3.71[cy]	14.67[a]	8.60[bcx]	1.974	< 0.01	< 0.01	0.81	0.09
Skin and hair N	2.41	2.40[y]	2.40	2.41[x]	0.007	0.83	0.43	0.06	< 0.01
Milk urea (mg/kg milk)	238[b]	119[d]	282[a]	184[c]	7.0	< 0.01	< 0.01	< 0.01	0.33
Urinary N to creatinine ratio (g/g)	13.01[b]	7.18[d]	14.74[a]	10.26[c]	0.345	< 0.01	< 0.01	< 0.01	< 0.01
Fecal N to urinary N ratio (g/g)	1.09[c]	2.35[a]	1.00[c]	1.45[b]	0.11	< 0.01	< 0.01	< 0.01	< 0.01
Milk N to urinary N ratio (g/g)	0.78[c]	1.69[a]	0.71[c]	1.05[b]	0.09	< 0.01	< 0.01	< 0.01	< 0.01
N partitioning, g/100 g N intake									
Urine	34.5[ay]	21.8[c]	36.3[ax]	28.9[b]	0.95	< 0.01	< 0.01	< 0.01	< 0.01
Feces	36.8[c]	45.0[a]	35.6[c]	40.0[b]	0.67	< 0.01	< 0.01	< 0.01	0.02
Milk	26.8[c]	32.0[a]	25.5[d]	29.1[b]	0.74	< 0.01	< 0.01	0.02	< 0.01

[a,b,c,d]Means with different superscripts in the same row differed significantly at $P < 0.05$.

[x,y]Means with different superscripts in the same row tended to differ at $P \geq 0.05$ to < 0.10.

[1]SEM, standard error of the mean; BW, body weight.

[2]RNB0, RNB in the diet at 0 g/kg dry matter; RNB-, RNB in the diet at −3.2 g/kg dry matter.

Table 3.5 Effects of rumen nitrogen balance (RNB) and protein source on purine derivatives (PD), and microbial crude protein synthesis (MCP) in lactating dairy cows (least square means, n = 24).

Variable	Protein source				SEM[1]	*P*-value			
	Faba bean grain		SoyPass			RNB	Protein	RNB x Protein	Period
	RNB0[2]	RNB-[2]	RNB0	RNB-					
PD excretion (mmol/d)									
Allantoin	553[a]	449[b]	519[a]	523[a]	20.5	< 0.01	0.25	< 0.01	< 0.01
Uric acid	72.1[a]	55.8[b]	70.5[a]	68.0[a]	3.24	< 0.01	0.02	< 0.01	< 0.01
Total	625[a]	505[b]	590[a]	591[a]	23.2	< 0.01	0.19	< 0.01	< 0.01
PD to creatinine ratio (mmol/mmol)	3.91[a]	3.61[c]	3.67[bc]	3.73[b]	0.130	< 0.01	0.11	< 0.01	< 0.01
PD to urinary N ratio (mmol/g)	2.94[c]	4.95[a]	2.40[d]	3.53[b]	0.098	< 0.01	< 0.01	< 0.01	< 0.01
PDC[3] index	521[a]	478[c]	489[bc]	497[b]	15.5	< 0.01	0.23	< 0.01	< 0.01
MCP synthesis									
MCP (g/kg dry matter intake)	125[a]	104[b]	118[a]	119[a]	4.3	0.01	0.37	< 0.01	< 0.01
MCP (g/kg DOM[2] intake)	203[ax]	166[b]	188[ay]	190[a]	6.7	< 0.01	0.43	< 0.01	< 0.01
MCP (g/kg crude protein intake)	788[a]	809[a]	675[b]	783[a]	29.4	0.02	0.01	0.11	0.05

[a,b,c,d]Means with different superscripts in the same row differed significantly at $P < 0.05$.
[x,y]Means with different superscripts in the same row tended to differ at $P \geq 0.05$ to < 0.10.
[1]SEM, standard error of the mean; N, nitrogen
[2]RNB0, RNB in the diet at 0 g/kg dry matter; RNB-, RNB in the diet at −3.2 g/kg dry matter.
[3]PDC, purine derivative to creatinine index; DOM, digestible organic matter.

3.5 Discussion

3.5.1 Diet composition and experimental approach

In the present study, differences in dietary nutrient composition were exclusively due to variable composition of the concentrate mixtures, because the forage sources and their proportions were constant in all dietary treatments. To avoid large differences in diet composition amongst animals of the same treatment group, diets were offered as TMR. Tested protein sources provided at least 35% of the total dietary CP to increase likeliness of a dietary treatment effect and diets for each protein source were iso-energetic, with a difference of only 0.2 MJ/kg DM between the diets containing the two protein sources.

The estimated uCP supply and RNB level of the diets were very similar to targeted values during ration formulation. The uCP supply from all diets exceeded the uCP requirements of the cows for maintenance and lactation of 3,040 g/d according to GfE (2001), but was greater with SP compared to FB diet for RNB0 level, which likely increased urea-N recycling and thereby N supply to rumen microbes. Hence, for RNB0, uCP supply to cows receiving the SP diet (4,228 g/d) exceeded that of animals fed the FB diet (3,904 g/d) by 324 g/d. According to the mean absorbability of uCP at the duodenum of 0.85 (GfE, 2001), this would equal to an extra N supply to cows of 44.1 g N/d (= 324/6.25 × 0.85) with SP compared to FB at RNB0. The milk urea-N and milk protein secretion as well as N retention in BW change were also slightly greater for SP compared to FB at RNB0 level (0.8 g, 7.8 g, and 5.7 g N/d, respectively). Nevertheless, the additional N supply to cows most likely increased plasma urea-N concentrations and urea-N recycling to the gastrointestinal tract, as seen by increases in milk urea-N concentrations and yields as well as the greater urine N excretion in SP compared to FB diets (see Table 3.4). As estimated by Lapierre and Lobley (2001), 33% of hepatic urea-N flux is eliminated in urine, whereas 67% of urea synthesized in the

liver re-enters the gastrointestinal tract, of which 50% is available for anabolic purposes to rumen microbes. Accordingly, an extra N supply of 10.0 g/d (= (44.1 − 0.8 − 7.8 − 5.7) × 0.67 × 0.50) might have been available to rumen microbes with SP compared to FB at RNB0. However, partitioning of urea-N recycled to the gastrointestinal tract, as proportion of total dietary N intake or total endogenous urea-N production, is not constant but greater in low-N compared to N-rich diets (Dinh, 2007). Hence, compared to SP diets, a greater proportion of dietary N was likely recycled in form of urea-N in FB diets that had lowest dietary N concentrations, which might have slightly reduced above-mentioned difference in urea-N recycling. Moreover, not all of the recycled N may be utilized when N supply to microbes is adequate (*i.e.*, RNB0). Nevertheless, the greater uCP supply in SP diets most likely increased endogenous N supply to rumen microbes and thus at least partly compensated for the negative RNB- effects in SP diets on rumen fermentation and MCP synthesis. Therefore, dietary treatment effects in the present study might not be exclusively attributed to differences in RNB (g/kg DM or g/d). Such differences in uCP supply (relative to the animals' uCP requirements) should be considered when evaluating RNB effects in the present and other studies, and when deriving recommendations for optimum RNB levels in diets of dairy cows.

Several of the variables were estimated and not measured. For instance, estimation of rumen MCP synthesis from urinary PD may be subject to inaccuracies (Dickhoefer et al., 2015; Dickhoefer et al., 2016), mainly due to differences in the endogenous excretion of PD and the shift in the purine concentrations in microbial mass. Nevertheless, experimental animals were of the same breed and originated from the same herd and dietary treatments did not result in marked changes in the animals' BW. Hence, considerable treatment differences in the endogenous excretion of PD are not expected. Along the line, similar feed

ingredients, identical forages, and their proportions across all the four diets, may suggest that considerable differences in purine N to total N ratio in rumen microbes may be precluded. Moreover, as described in the method section, urinary N excretion (g/animal and d) was calculated from their N balance equations, that is by either measuring or estimating N concentration in feed, milk, and feces, and BW of the animals with acceptable methods and levels of accuracy. As a result, it may be suggested that calculated urine volume (from urine N concentration and excretion), and thereof urinary PD may not be subjected to large inaccuracies.

3.5.2 Effects of the interaction between rumen nitrogen balance and protein source on intake and protein metabolism

The effects of RNB level on several variables related to protein metabolism differed depending on the protein source. In contrast to our hypothesis, differences between RNB levels were more pronounced in FB compared to SP diets. For instance, DM and DOM intakes, fecal N excretion, efficiency of MCP synthesis (in g/kg DM or DOM), and the supply of MCP, RUP, and thus uCP as well as milk N yields were lower with RNB- than RNB0 in FB diets. In contrast, no differences were observed in these variables between RNB levels for SP diets. Moreover, N intake and urinary N excretion were lower and the proportions of ingested N excreted via feces (*i.e.*, lower apparent total tract CP digestibility; Kand and Dickhoefer (2019)) or milk (*i.e.*, milk N use efficiency) and fecal N to urinary N ratio greater at RNB- than at RNB0 for both protein sources. However, the differences between RNB levels were greater in FB compared to SP diets. A significant difference between DM intake and MY and greater difference in N intakes between RNB levels for FB compared to SP diets resulted in an interaction effect between RNB and protein source for milk N use

efficiency.

The marked differences in studied variables between RNB levels for the FB diets are most likely related to the low dietary N and RDP supply to rumen microbes in RNB-, which reduced rumen microbial growth and nutrient fermentation. Although not analyzed in the present study, shifts in the rumen microbial species composition with different RNB levels and protein sources might be anticipated, which could explain differences in nutrient fermentation and efficiency of MCP synthesis. Additionally, greater differences in uCP supply between the RNB levels in FB compared to SP diets likely contributed to these more pronounced RNB effects on milk N yield, and indirectly, on DM intake and rumen MCP synthesis (*i.e.*, due to less urea-N recycling). In contrast, the overall greater uCP supply in SP compared to FB at both RNB levels might have at least partly compensated for the negative effects of low RNB on efficiency of MCP synthesis and milk N yield (see section above). Moreover, as expected, differences in the rate of rumen CP degradation and RUP supply between the protein sources might be also responsible for observed effects. For instance, slow rate of rumen degradation of SP might have resulted in a continuous and slow release of CP degradation products in the rumen and thus their more efficient incorporation in MCP. Instead, the proportion of CP degradation products from FB absorbed in the rumen might have been greater, due to the rapid rumen degradation rate of this protein source, which may have influenced microbial turnover rates and negatively affected MCP synthesis. Moreover, apparent total tract digestibility of CP (Kand and Dickhoefer, 2019) and NDF (Kand et al., 2021) as well as daily MY were also reduced with RNB- for SP, suggesting that there were negative although minor effects on rumen carbohydrate and protein metabolism. However, the lack of effect of RNB on DM and DOM intakes for SP likely offset any pronounced differences in variables related to protein metabolism and animal

performance.

Pronounced differences between the RNB levels were expected for the slowly degradable protein source in line with the results from our previous *in vitro* study (Kand et al., 2018). In the *in vivo* study, surplus RDP (*i.e.*, in FB diets), that is not incorporated into MCP, will be withdrawn from the rumen through absorption or passage, will be partly lost via feces, urine, and milk of the animals, and hence not be available to rumen microbes. Instead, the CP degradation products as well as the synthesized microbial biomass of rapidly degradable protein source remain within the closed *in vitro* system and can be used at a later stage of fermentation or even be recycled by the rumen microbes. Such differences in N metabolism are of particular relevance in diets containing rapidly degradable protein sources such as FB and may explain the contrasting results observed in the present *in vivo* and our previous *in vitro* studies.

Various studies testing the effects of a reduced N supply to rumen microbes, in which the uCP requirements of the animals calculated according to GfE (2001) were satisfied, have shown contrasting outcomes. Previously, Riemeier (2004) at a measured RNB of ≤ −63 g/d and at an uCP supply of ≥ 2,185 g/d (~ 2,080 g/d of cows' uCP requirement) with corn silage as major protein source (≥ ~ 26% of dietary CP) found negative effects on rumen MCP synthesis and a tendency for a reduced MY in dairy cows. In contrast, Mutsvangwa et al. (2016) at a calculated RNB of ≤ −62 g/d and at an uCP supply of ≥ 4,312 g/d (~ 3,787 g/d of cows' uCP requirement) with canola meal as a major protein source (~ 28% of dietary CP) and Afzalzadeh et al. (2010) at a calculated RNB of −67 g/d and at an uCP supply of ~3,540 g/d (~ 2,554 g/d of cows' uCP requirement) with cottonseed meal as major protein source (≥ ~ 50% of dietary CP) did not observe any negative effects on animal production. Along the same line, Giallongo et al. (2015) at a calculated RNB of −62 g/d and at an uCP

supply of ~ 4,768 g/d (~ 4,439 g/d of cows' uCP requirement) with corn silage as a major protein sources (~ 24% of dietary CP) did not observe any negative effects on MCP synthesis and MY. Finally, at similar RNB level and uCP supply than in above-mentioned studies, rumen MCP synthesis, MY, and milk N yield clearly declined at RNB- for FB in the present study, whereas no negative RNB effects were observed for SP. Different approaches to estimate rumen MCP synthesis have partly been used in the present and above-mentioned studies, which should be considered when comparing RNB estimates. Yet, irrespective of an oversupply of uCP and despite the very similar RNB (in g/d) in all of the above-mentioned studies and our experiment, very contrasting animal responses have been observed. Hence, factors such as diet composition (including carbohydrate and protein sources), extent and rate of rumen CP degradation, passage rate, and intake and performance level of the animals indeed also appear to affect the minimum RNB level needed for efficient rumen fermentation. Further *in vivo* studies are thus needed to understand the effects of negative RNB in diets with different protein or carbohydrate sources but similar uCP supply.

3.5.3 Effects of rumen nitrogen balance

The observed greater milk N use efficiency at RNB- compared to RNB0 for both protein sources was possibly due to a more efficient utilization of available dietary CP by rumen microbes and an increased urea-N recycling in RNB- diets. Present results confirm previous findings of greater N use efficiencies in response to lower N and greater fermentable carbohydrate intakes, and therefore, lower RNB in diets of lactating dairy cows (Broderick, 2003; Dijkstra et al., 2013; Riemeier, 2004). In a meta-analysis of the effects of animal and dietary characteristics on the efficiency of N utilization for milk protein production, and on

fecal N, urinary N, and total manure N output, Huhtanen et al. (2008) showed that dietary CP concentrations and RNB were strongly and negatively related to milk N use efficiency. Fecal N to urinary N ratio was greater for RNB- compared to RNB0 for both protein sources, which has been also observed previously by Castillo et al. (2001), Fanchone et al. (2013), and Mutsvangwa et al. (2016). As urinary N is more rapidly degraded and released in form of urea compared to fecal N (Castillo et al., 2001), such reduction in urinary N excretion has positive environmental effects.

However, to match the supply of RDP and uCP to requirements of rumen microbes and their host, respectively, accurate methods to assess rumen degradation rate, MCP synthesis and duodenal uCP flow are required. Different laboratory methods are currently used to estimate uCP supply and thereby RNB of the diet. The two methods use,d in the present study were (i) modified Hohenheim gas test (Steingass and Südekum, 2013), and (ii) separate estimation of MCP from ME concentrations (GfE, 2001) and of RUP from prediction equations of Kirchhof (2007) for forages and of Shannak et al. (2000) for concentrates) and from *in situ* degradation kinetics (for FB, SP, and RaPass). Although estimates of RNB (in g/kg DM) only slightly differed between both the methods (~ 0.5 g/kg DM; see Table 3.2), these small differences become relevant when expressed in g/d, in particular in high-yielding dairy cows with high feed intake level. Hence, validated and standardized lab procedures to estimate dietary uCP and RNB are still needed in order to optimize RNB in dairy cattle nutrition.

Finally, long-term studies are required to understand the effects of negative dietary RNB, for instance, on adaptive changes in urea- N recycling as well as on health and reproduction of dairy cows, to be able to derive reliable estimates of the minimum RNB and to fully capture the economic and ecological implications at herd and farm system levels.

3.6 Conclusions

Negative RNB of ~ 65 g/d (−3.2 g/kg DM) reduce feed intake, N balance, and performance of high-yielding dairy cows. The results suggest that these effects may be more pronounced in diets containing rapidly degradable protein sources. Surplus uCP supply relative to the cows' requirements may at least partly enable to compensate for low RDP availability to rumen microbes due to increased N recycling. Hence, results from our experiment and from the literature support that defining a single minimum RNB threshold for dairy cattle diets is not feasible when optimizing N utilization in dairy cows, because several animal and dietary factors modify the requirements of rumen microorganisms for feed RDP as well as N recycling in cows. Further *in vivo* studies are thus needed to better understand the effects of negative dietary RNB with different protein or carbohydrate sources at similar or even gradients of uCP supply.

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Chapter 4

4. The effects of rumen nitrogen balance on intake, nutrient digestibility, chewing activity, and milk yield and composition in dairy cows vary with dietary protein sources

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4.1 Abstract

The objective was to study the interaction effects of rumen nitrogen balance (RNB) and dietary protein source on feed intake, apparent total tract digestibility (ATTD), eating and ruminating activity, milk yield (MY), and milk composition in lactating dairy cows. Twenty-four lactating Holstein cows were divided in four groups which were randomly assigned to the dietary treatments included in a replicated 4 × 4 Latin square experimental design that comprised four 20-d-periods with each 12 d of adaptation to the experimental diets and 8 d of sampling. The dietary treatments followed a 2 × 2 factorial arrangement with two main protein sources, faba bean grain (FB) and SoyPass (SP), offered at two dietary RNB levels: RNB0 (RNB 0 g/kg dry matter; DM) and RNB- (RNB –3.2 g/kg DM) (*i.e.*, four treatments). The composition of concentrate mixtures was adjusted to create diets with the desired RNB levels. Each of the protein sources supplied ≥ 35% of the total dietary crude protein (CP). Both diets within a protein source had similar forage sources and forage to concentrate ratios, and were iso-energetic, but differed in CP concentrations. The main effects of RNB, protein source, and their interactions were tested by PROC MIXED in SAS 9.4. Interaction effects were observed for daily DM intake and energy-corrected MY, which were lower for RNB- than RNB0 in diets containing FB (23.5 vs 24.4 kg DM/d; 28.6 vs 30.6 kg milk/d), but similar in diets containing SP (24.2 vs 24.3 kg DM/d; 31.3 vs 31.7 kg milk/d). The ATTD of neutral detergent fiber (NDF) was lower for RNB- compared to RNB0 in the FB (44.9 vs 49.1 g/100 g) and SP (48.5 vs 51.9 g/100 g) diets, and greater for the SP than for FB diets. There were interaction effects for ATTD of CP and concentrations of milk urea nitrogen, which were lower for RNB- compared to RNB0 in both, FB (55 vs 63.1 g/100 g CP; 5.65 vs 11.3 mg/dl milk) and SP diets (60 vs 64.4 g/100 g CP; 8.74 vs 13.4 mg/dl milk). However, differences between RNB levels were greater for FB than for SP diets. Furthermore, proportions of milk

fatty acids followed the same pattern as that of dietary fatty acids, but bio-hydrogenation appeared to be greater for RNB- than RNB0 for both protein sources and in FB than in SP diets for both RNB levels. There was an interaction effect on total number of chews per unit of NDF intake, which was greater for RNB- compared to RNB0 for both protein sources. However, the differences between RNB levels were greater in FB than in SP diets. Overall, differences in the animal responses to negative RNB between FB and SP diets suggest a need to better understand the effect of negative RNB levels with different dietary ingredients at similar utilizable CP supply.

Keywords: dairy cow, diet digestibility, protein nutrition rumen nitrogen balance

4.2 Introduction

Feeding dietary crude protein (CP) to dairy cattle beyond their requirements increases excretion of nitrogen (N) to the environment, production costs, and has negative effects on the health and reproductive performance of cows (Lebzien et al., 2006). Nevertheless, efficiency of converting feed protein into milk protein can be increased by reducing dietary CP intake, while profiting from the animal's capacity to re-use a share of the urea circulating in their blood (Leiber, 2014). Yet, in doing so, the N requirements of rumen microbes need to be satisfied to avoid negative impacts on microbial growth and activity, ruminal fermentation, microbial crude protein (MCP) synthesis, and ultimately, on nutrient digestibility, feed intake, and milk yield (MY) and its quality (*i.e.*, nutrient concentrations and fatty acid (FA) profile).

The German feeding recommendation system evaluates the protein supply and requirements of cattle based on the utilizable crude protein at the duodenum (uCP) (GfE, 2001). The uCP (g/d) is defined as total CP at the duodenum minus the endogenous protein

(Lebzien and Voigt, 1999), which can be estimated from changes in the ammonium concentration in rumen inoculum during *in vitro* fermentation (Steingass and Südekum, 2013). Moreover, the rumen nitrogen balance (RNB) is an indicator for N supply to rumen microbes and is calculated by subtracting the uCP supply at the duodenum from the CP intake and dividing that difference by 6.25 (RNB = (CP intake – (rumen undegraded protein, RUP + MCP))/6.25; all in g/d) (GfE, 2001). Positive RNB indicates a surplus in N supply to rumen microbes, whereas negative RNB indicates a deficiency. To avoid N shortage or surplus for rumen microbes, one aims at feeding RNB = 0 g/d to high-performing dairy cows; however, RNB = 0 g/d disregards the capacity of cattle to recycle dietary N via the rumino-hepatic pathway. As a result, researchers have been interested in exploring the effects that diets with negative RNB may have on dairy cows and their rumen function. Responses so far have been variable, with some studies showing no effects of RNB (Holthausen et al., 2000; König et al., 2005; van de Sand et al., 2008) on e.g. feed intake, nutrient digestibility, and milk performance, whereas other studies detected even negative effects (Steinwidder et al., 2009; Aschemann et al., 2012a; Aschemann et al., 2012b). The contradicting results are difficult to explain, but a recent *in vitro* study indicated that the effects of negative RNB on the substrate fermentation may differ with dietary carbohydrate and protein sources (Kand et al., 2018), which highlights the need to account for differences in the ruminal degradation characteristics of the ingredients of dairy cattle diets when studying the effect of negative RNB *in vivo*.

Dietary protein sources differ in their extent and speed of ruminal breakdown to amino acids, peptides, and ammonium, which in turn may influence the composition of rumen microbial consortium, their growth, and, therefore, rumen MCP synthesis (Hoover and Stokes, 1991). Besides, different protein sources with high RUP concentration may also

influence the amount and amino acids profile of the uCP reaching the duodenum (Kung and Rode, 1996). Moreover, a low extent and rate of protein degradation in the rumen coupled with a negative RNB may at least temporarily compromise N supply to rumen microbes, and therefore ruminal fermentation (Klusmeyer et al., 1990), with consequences on carbohydrate degradation, amount and composition of uCP supply, and ultimately MY and milk composition. Yet, it may be hypothesized that low rumen fermentative activity may result in longer ruminal retention times (Martínez et al., 2009). Furthermore, long ruminal retention times may intensify chewing activity (*i.e.*, eating and ruminating expressed in minutes or number of chews per unit of nutrient intake or per day), which in turn could stimulate saliva secretion (Mertens, 1997). One may assume that such an increase in salivation could contribute to enhance the recycling of N circulating from the blood and thus to mitigate possible adverse effects of negative RNB in diets of dairy cows.

Consequently, it was hypothesized, that low dietary N supply to rumen microbes (*i.e.*, negative RNB) may have negative effects on ruminal fermentation, diet digestibility, MY, and its composition and FA profile, while increasing the eating and ruminating activity relative to the animals' nutrient intakes. Similar to results of our previous *in vitro* study (Kand et al., 2018) differences will be more pronounced in the diets containing a slowly degradable protein (*i.e.*, in soy protein or wheat gluten *versus* casein), possibly due to at least temporary differences in the supply of energy and N to the rumen microbes. Therefore, while previous research has focused on either analyzing the effects of different protein sources (Brito et al., 2007) or of reduced rumen degraded protein (RDP) intakes (Mutsvangwa et al., 2016), the objective of the study was to explore the interactions between dietary RNB levels and the protein sources included in the diets with varying rate and extent of ruminal degradation.

4.3 Material and methods

The animal experiment was performed at the experimental station Meiereihof of the University of Hohenheim, Stuttgart, Germany, between October and December 2017. Mean daily ambient air temperatures were between 0.3°C and 10.6°C during the experiment. Procedures followed the regulations of the current ethical and animal welfare legislation. While a companion paper (Kand and Dickhoefer, 2021) studied the effects of the interaction between RNB level and protein source on rumen MCP synthesis and N partitioning in lactating dairy cows, the present study analyzed the response of feed intake, nutrient digestibility, animal behavior, and MY and composition.

4.3.1 Experimental design, cows, and feeding

The experiment followed a 4 × 4 Latin square design, with a 2 × 2 factorial arrangement of dietary treatments. The total experiment consisted of four periods of 20 d each. Each period comprised 12 d of adaptation to the experimental diets and eight days of sample collection.

Twenty-four lactating Holstein-Friesian cows (10 primiparous, 14 multiparous) were included in the study. Four groups of six cows each were formed (*i.e.*, animals were blocked) with mean MY, days in milk, lactation number, and body weight (BW) (mean ± standard deviation) 35.2 ± 6.1 kg/d, 120 ± 85 d, 2.8 ± 1.7 lactations, and 681 ± 45 kg BW, respectively, before the start of the experiment. The assignment of animals to individual groups was not changed throughout the experiment. The cows were housed in a free-stall barn with free access to clean drinking water. Cows were milked twice daily at 05:00 and 16:00 h in a 2 × 6 tandem milking parlor (Westfalia, GEA Farm Technologies, Bönen, Germany), and MY was automatically recorded for each cow at every milking by an electronic milk meter

(Metatron P21; GEA Farm Technologies, Bönen, Germany). In addition, BW of the cows were recorded daily at the exit of the milking parlor after morning milking using an automated walk-over-weighing system (GEA Farm Technologies, Bönen, Germany).

The four groups of cows were randomly assigned to one of the four experimental diets before the start of the experiment (Table 4.1). The dietary treatments consisted of two main protein sources in combination with two dietary RNB levels. Faba bean grains (FB) and SoyPass (SP) (Beweka Kraftfutterwerk GmbH, Heilbronn, Germany) were chosen as main protein sources due to their differences in the rate and extent of degradation in the rumen, as described in (Kand and Dickhoefer, 2021). The four experimental diets were fed as total mixed ration with RaPass (Beweka Kraftfutterwerk GmbH, Heilbronn, Germany) and FB in the FB diets, and rapeseed cake and SP in the SP diets (Table 4.2). RaPass is a commercial rumen-protected protein supplement from rapeseed meal that is treated with xylose to increase the concentration of RUP (Beweka Kraftfutterwerk GmbH, Heilbronn, Germany). The RDP proportion and rate of *in situ* CP degradation were 710 g and 350 g/kg CP and 17% and 6.1%/h, for FB and SP feeds, respectively, at a ruminal passage rate of 8%/h (Kand and Dickhoefer, 2021). The RDP and rate of *in situ* CP degradation were 370 g/kg CP and 6.7%/h, for RaPass, at a ruminal passage rate of 8%/h (Kand and Dickhoefer, 2021). For the two FB diets, FB supplied 37% and 35% of the total dietary CP, whereas SP contributed 35% and 36% of the total dietary CP in both SP diets. The RNB levels of 0 g/kg dry matter; DM (RNB0) and -3.2 g/kg DM (RNB-) were selected for each protein source. The RNB- level was selected to achieve a RNB of ~ − 70 to – 80 g/d at an estimated DM intake of the cows of ~24 kg/d, where according to literature (Jilg et al., 1999; Riemeier, 2004) tendencies for negative effects on animal performance may be visible, without any negative impacts on feed intake.

The forage to concentrate ratio of 55:45 (on DM basis), forage ingredients, and their proportion in the diet was constant across the four diets (Table 4.2). Samples of all diet ingredients and the offered diets were taken and analyzed to confirm the nutrient concentrations assumed in diet formulation (see “sample and data collection, preparation and laboratory analyses” and Table 4.3 and 4.4).

Each of the dietary treatments met the uCP and energy requirements of dairy cows according to the recommendations of GfE (2001), assuming a BW of 650 kg, a MY of 30 kg/d, and a DM intake of 24 kg/d. All experimental diets were iso-energetic.

Each group of animals was randomly assigned to one of the four experimental diets. The diets were fed for *ad libitum* intake, once daily at approximately 08:45 h, ensuring a daily feed refusal of approximately 10% of the offered diet (on DM basis). Each diet was offered in three separate feeding troughs to all six animals per group, which were equipped with computer-regulated access gates, and a transponder sensor as well as electronic scales (Waagen Döhrn GmbH & Co.KG, Wesel, Germany) to automatically record feed intake of individual cows. The transponder sensors also recorded the number of trough visits (n/d) and feeding time at the trough (min/d). As each group of animals was feeding from the same trough, samples of the diet refusals were not taken for individual cows but group-wise. However, the fact that three troughs were accessible to six cows and diets were offered *ad libitum* should have allowed for free intake and similar quality of the diets consumed even by the less dominant animals.

The external fecal marker titanium dioxide (TiO_2) (60797, Kronos® 1171, Kronos Worldwide Inc. Dallas, TX, USA) was used to estimate fecal DM excretion. The marker was mixed with the concentrate mixtures of each of the four diets. From d 8 until d 20 of the experimental periods, the concentrate mixtures containing TiO_2 were mixed with the

corresponding total mixed ration using a mixer wagon (Power Champ L, MARMIX GmbH & Co. KG, Unterwachingen, Germany) to achieve a concentration of ~1.4 g TiO_2/kg DM and an intake of ~34 g/d of TiO_2 by each cow (at a DM intake of ~ 24 kg/d of each cow).

Table 4.1 Sequential design of the experimental diets fed to each animal group during the experiment.

	Period 1	Period 2	Period 3	Period 4
Group 1	Faba bean grain (RNB0[1])	SoyPass (RNB-[1])	SoyPass (RNB0)	Faba bean grain (RNB-)
Group 2	Faba bean grain (RNB-)	SoyPass (RNB0)	SoyPass (RNB-)	Faba bean grain (RNB0)
Group 3	SoyPass (RNB-)	Faba bean grain (RNB-)	Faba bean grain (RNB0)	SoyPass (RNB0)
Group 4	SoyPass (RNB0)	Faba bean grain (RNB0)	Faba bean grain (RNB-)	SoyPass (RNB-)

[1]RNB0, RNB in the diet 0 g/kg DM; RNB-, RNB in the diet is -3.2 g/kg DM.

Table 4.2 Ingredients of the four offered experimental diets varying in rumen nitrogen balance (RNB) levels and protein source fed as a total mixed ration to the lactating dairy cows.

	Protein source			
	Faba bean grain		SoyPass	
Dietary ingredient (g/kg dry matter)	RNB0[1]	RNB-[1]	RNB0	RNB-
Corn silage	245	245	245	245
Grass silage	105	105	105	105
Grass hay	141	141	141	141
Second-cut grass hay	23	23	23	23
Barley straw	36	36	36	36
Faba bean grain	196	145	0	0
RaPass	98	27	0	0
SoyPass	0	0	118	102
Rapeseed cake	0	0	135	66
Barley grain	77	116	77	168
Sugar beet pulp molasses chips[2]	52	84	95	59
Feed sugar[2]	0	50	0	30
Mineral-vitamin mixture[3]	27	28	24	25

[1]RNB0, RNB in the diet 0 g/kg dry matter; RNB-, RNB in the diet is -3.2 g/kg dry matter.

[2]Sugar beet molasses was obtained from Beweka Kraftfutterwerk GmbH, Heilbronn, Germany, and feed sugar was obtained from Bergophor Futtermittelfabrik Dr. Berger GmbH & Co. KG, Kulmbach, Germany.

[3]Mineral mixture (Bergophor Futtermittelfabrik Dr. Berger GmbH & Co. KG, Kulmbach, Germany per g/100 g (as-fed basis), faba bean grain (RNB0): 8.5 g beta-carotene, 7.6 g feed salt, 31.9 g calcium carbonate, 13.4 g monosodium phosphate, 33.6 g mineral feed, and 5.0 g magnesium oxide; faba bean grain (RNB-): 7.6 g beta- carotene, 4.6 g feed salt, 35.2 g calcium carbonate, 16.8 g monosodium phosphate, 30.5 g mineral feed GM134, and 5.3 g magnesium oxide; SoyPass (RNB0): 10.9 g beta-carotene, 10.8 g feed salt, 28.3 g calcium carbonate, 6.5 g monosodium phosphate, and 43.5 g mineral feed; SoyPass (RNB-): 9.2 g beta- carotene, 7.3 g feed salt, 31.2 g calcium carbonate, 12.8 g monosodium phosphate, 36.7 g mineral feed, and 2.8 g magnesium oxide. Mineral feed composition as fed: 200 g calcium, 40 g magnesium, 50 g sodium, 7 g zinc, 5 g manganese, 1.1 g copper, 35 mg selenium, 60 mg iodine, 20 mg cobalt, 250,000 IU Vitamin A, 65,000 IU Vitamin D, 5000 mg Vitamin E, and 120 mg Vitamin B7.

4.3.2 Sample and data collection, preparation and laboratory analyses

During every sampling period, approximately 0.5 kg (as-fed basis) of each offered and refused diet were collected in the morning, weighed, and stored at -20°C. At the end of every sampling period, these samples were lyophilized for 72 h (LYO GT2 Basis, SRK Systemtechnik GmbH, Riedstadt, Germany), weighed to determine their DM concentrations. In addition, samples of individual diet ingredients were collected at the beginning (except of grass and corn silages) and at the end of the experiment and lyophilized for 72 h. The samples of the offered and refused diet as well as their ingredients were then ground to pass a 1-mm sieve (SM 1, Retsch GmbH, Haan, Germany). Thereafter, samples of each offered and refused diets were pooled by period according to the weighted daily DM intake of each treatment group of animals (*i.e.*, six cows).

Fecal samples (~ 500 g FM) were collected daily from the rectum of each cow and stored at -20°C. Sampling of each animal began on d 12 of each period and was alternated between morning (8:00 h) and afternoon (16:00 h) for eight days to compensate for day-to-day variations in the concentrations of nutrients and TiO_2 in feces. At the end of each sampling period, the samples were thawed at room temperature, homogenized, and pooled by cow and period by taking the same quantity of feces (400 g FM) from each daily sample. The pooled samples were then lyophilized for 72 h (LYO GT2 Basis, SRK Systemtechnik GmbH, Riedstadt, Germany), weighed, and ground to pass a 2-mm sieve (SM 1, Retsch GmbH, Haan, Germany).

Samples of offered and refused diets, and of feces were analyzed in duplicate according to official analytical methods in Germany (VDLUFA, 2007). All samples were analyzed for concentrations of DM (method 3.1), crude ash (method 8.1), neutral detergent fiber (NDF) (method 6.5.1), and N (method 4.1.1). The CP concentrations were calculated from the N

concentrations (CP = N × 6.25). Samples of the offered diets were analyzed for crude lipid (method 5.1.1), acid detergent fiber (method 6.5.2), and acid detergent lignin (method 6.5.3). The NDF, acid detergent fiber, and acid detergent lignin concentrations are presented including residual ash. Sodium sulfite and heat-stable α-amylase (ANKOM Technology, Macedon, NY, USA) were used for all NDF analyses. Additionally, starch was analyzed in samples of offered diets (method 7.2.5) using an enzymatic kit (Test-Combination Nr. 10 207 748 035, R-Biopharm AG, Darmstadt, Germany). Concentrations of sugar were analyzed in one sample per offered diet pooled across all four periods (method 7.1.1). Samples of offered diets were also incubated in triplicate *in vitro* for 24 h on two days each following procedures by Menke and Steingass (1988) to estimate metabolizable energy (ME) concentrations from *in vitro* gas production and proximate nutrient concentrations. The ME concentrations of the offered diets were estimated according to the 14f equation of Menke and Steingass (1988). Additionally, samples of offered diets were incubated in triplicate in two runs using the modified Hohenheim *in vitro* gas test (Steingass and Südekum, 2013), and the ammonium concentrations in the incubation medium after 24 h was used to estimate duodenal uCP supply from the different diets. The FA profile of the diets was determined in duplicate by analyzing individual FA in samples of the offered diets. For this, glyceride fats and oils were converted into FA methyl esters by trans-esterification of the lipid extract with trimethylsulphonium hydroxide, followed by quantification using gas chromatography, according to the procedure of Högy et al. (2010).

Table 4.3 Chemical composition of the dietary ingredients (arithmetic mean; n = 2[1]).

Parameter (g/kg DM)	Corn silage	Grass silage	Grass hay	Second-cut grass hay	Barley straw	Faba bean grain	RaPass	SoyPass	Rapeseed cake	Barley grain	Sugar beet pulp molasses chips
DM (g/kg fresh matter)	400	420	923	925	936	870	891	880	920	870	882
Crude ash	29.5	111	86.2	81.0	63.0	38.3	82.7	69.9	66.8	28.1	75.1
CP	73	157	73	76	43	297	392	521	362	113	127
NDF	342	595	654	559	824	249	362	339	270	215	358
ADF	172	349	371	338	514.4	125	201	68	201	55	210
Starch	335	ND	ND	ND	ND	268	ND	ND	ND	445	ND

ND, not determined.

[1]Individual diet ingredients were sampled before and after the experiment except of corn silage and grass silage, which were sampled only once after the experiment.

Table 4.4 Chemical composition of the four offered experimental diets varying in rumen nitrogen balance (RNB) and protein source fed as a total mixed ration to the lactating dairy cows (arithmetic mean ± one standard deviation; n = 4).

	Protein sources			
	Faba bean grain		SoyPass	
Variable	RNB0[1]	RNB-[1]	RNB0	RNB-
Chemical composition (g/kg DM[2])				
DM (g/kg fresh matter)	408 ± 2.9	414 ± 5.1	412 ± 3.3	415 ± 4.0
Crude ash	74 ± 1.4	71 ± 0.6	75 ± 2.8	72 ± 3.8
Crude protein	159 ± 3.4	128 ± 2.2	174 ± 1.7	150 ± 3.1
Crude lipid	21 ± 1.8	19 ± 0.8	36 ± 1.9	28 ± 0.7
Starch	123 ± 4.5	121 ± 5.5	83 ± 3.2	112 ± 9.3
Neutral detergent fiber	405 ± 5.6	388 ± 9.5	420 ± 5.8	407 ± 7.1
Acid detergent fiber	216 ± 7.0	203 ± 3.7	220 ± 3.9	208 ± 7.4
Acid detergent lignin	27 ± 2.0	20 ± 1.4	35 ± 1.1	28 ± 1.5
Sugar[3]	53	109	67	85
Metabolizable energy (MJ/kg DM)	10.5 ± 0.07	10.5 ± 0.10	10.7 ± 0.11	10.7 ± 0.07
Theoretical uCP[2,4]	157	143	177	169
Theoretical RNB[4]	-0.03	-3.20	-0.03	-3.18
uCP – Total mixed ration[5]	160 ± 3.0	146 ± 2.2	174 ± 1.7	167 ± 2.6
RNB – Total mixed ration[5]	-0.3 ± 0.24	-2.9 ± 0.11	-0.2 ± 0.16	-2.7 ± 0.16
Fatty acids (g/100 g of fatty acids)				
C12:0	0.09 ± 0.02	0.11 ± 0.02	0.08 ± 0.01	0.09 ± 0.01
C14:0 (myristic acid)	0.32 ± 0.04	0.37 ± 0.01	0.26 ± 0.06	0.29 ± 0.02
C16:0 (palmitic acid)	17.9 ± 1.11	19.5 ± 0.54	13.8 ± 0.95	15.5 ± 0.27
C16:1	0.90 ± 0.17	0.79 ± 0.02	0.71 ± 0.06	0.69 ± 0.05
C18:0 (stearic acid)	2.26 ± 0.14	2.16 ± 0.11	2.06 ± 0.14	2.14 ± 0.04
C18:1 cis-9 (oleic acid)	28.7 ± 0.44	22.1 ± 0.70	36.5 ± 1.86	32.1 ± 0.34
C18:2 *cis*-9, *cis*-12 (linoleic acid)	34.9 ± 2.46	39.6 ± 1.38	32.9 ± 0.90	36.3 ± 0.81

C18:3 *cis*-9, *cis*-12, *cis*-15 (α-linolenic acid)	11.3 ± 0.64	12.3 ± 0.28	9.81 ± 0.56	9.91 ± 0.37
C20:0	0.67 ± 0.08	0.63 ± 0.01	0.61 ± 0.11	0.54 ± 0.02
C20:1	0.47 ± 0.09	0.43 ± 0.03	0.64 ± 0.12	0.64 ± 0.01
C22:0	0.60 ± 0.07	0.56 ± 0.01	0.50 ± 0.09	0.47 ± 0.02
C24:0	0.67 ± 0.07	0.65 ± 0.12	0.55 ± 0.26	0.52 ± 0.11
Saturated fatty acids[6]	23.2 ± 2.62	24.6 ± 0.69	18.3 ± 2.94	20.1 ± 0.42
Mono-unsaturated fatty acids[7]	30.4 ± 0.69	23.4 ± 0.69	38.2 ± 1.84	33.6 ± 0.33
Poly-unsaturated fatty acids[8]	46.3 ± 3.47	51.9 ± 1.28	42.8 ± 2.00	46.3 ± 0.54

[1]RNB0, RNB in the diet at 0 g/kg DM; RNB-, RNB in the diet at -3.2 g/kg DM.
[2]DM, dry matter; uCP, utilizable crude protein.
[3]Offered diets from all the four periods were pooled and analyzed for sugar concentrations.
[4]Theoretical uCP (g/kg DM) and RNB (g/kg DM) calculated during ration formulation.
[5]Total mixed rations were incubated (n = 24; 4 periods x 3 triplicates x 2 incubation runs). The uCP (g/kg DM) was determined after incubation at a passage rate of k = 8%/h, as described by Steingass and Südekum (2013) to further calculate RNB (g/kg DM) according to GfE (2001).
[6]Sum of all fatty acids without double bonds.
[7]Sum of all fatty acids with one double bond.
[8]Sum of all fatty acids with more than one double bond.

Milk samples (~ 100 ml per cow) were collected during milking at 16:00 h on d 14, 16, 18, and 20 of the experimental periods, and at 5:00 h on d 15, 17, and 19 as well as on d 1 of the subsequent adaptation period just before providing the new dietary treatments to the animals. Afternoon and subsequent morning milk samples were pooled per cow to one milk sample (~ 45 ml). For pooling, 20 ml of afternoon milk was taken, whereas the amount of morning milk equaled to the ratio between morning and afternoon MY (kg/cow) multiplied by 20 ml. The pooled milk samples were homogenized, conserved by adding 150 µl of Bronysolv BL (90.051.001, ANA.LI.TIK. Austria, Vienna, Austria), and refrigerated at 4°C until milk composition analysis. The conserved milk samples were analyzed by Milchprüfring Baden-Württemberg e. V. (Kirchheim unter Teck, Germany) for CP, fat, and lactose according to ASU L 01.00-78, 2002-05 (DIN ISO 6922), for urea according to

05022100.QMD, 2011-03, and for milk FA using Fourier-transform infrared spectroscopy (Bentley FTS, Bentley Instruments, Chaska, MN, USA). Milk FA were expressed as g/100 g total FA.

The chewing activity (*i.e.*, chewing related to eating and ruminating) of three randomly chosen cows per treatment group was recorded by noseband pressure sensors (RumiWatch System, ITIN + HOCH GmbH, Liestal, Switzerland) during the sample collection period. The three selected cows in each treatment group had a mean MY, days in milk, lactation number, and BW (arithmetic mean ± standard deviation) of 34.6 ± 5.6 kg/d, 144 ± 91 d, 2.5 ± 1.6 lactations, and 693 ± 44 kg BW, respectively, at the beginning of the experiment. Noseband sensors were attached to the cows starting from d 10 of each experimental period, allowing for two days of adaptation and eight days of recording of chewing activity. The noseband sensors were checked every day for correct fit and functioning. Raw data were converted to daily eating time and ruminating time (both, in min/cow and d), eating and ruminating chews (both, in n/cow and d), and the number of rumination boli (n/cow and d) using a converter software (V 0.7.3.2, ITIN + HOCH GmbH, Liestal, Switzerland). Total chewing activity was calculated as the sum of daily eating and ruminating chews or eating and ruminating time. Additionally, the number of eating and ruminating chews and the daily eating and ruminating time as well as the total number of chews and chewing time were expressed relative to the DM and NDF intakes (n/kg DM or NDF intake; min/kg DM or NDF intake).

4.3.3 Calculations

For estimating the daily DM intake of individual cows, the DM concentration of the actually ingested feed was calculated as the difference in the amount of feed offered and refused by each group of six cows (on DM basis) divided by the total FM intake of the entire group of

six cows multiplied by 1000. By multiplying these DM concentrations of the ingested feed by the daily FM intake of each individual cow recorded by the feed trough, the daily DM intake was estimated and the mean value across the eight days within a sampling period was calculated. Similarly, the concentrations of organic matter (OM), CP, and NDF in ingested feed were calculated as the difference in the respective concentrations in offered and refused feed (on DM basis) for each group of cows and then divided by their total DM intake. By multiplying these nutrient concentrations in ingested feed by the DM intakes of individual cows, the mean OM, CP, and NDF intakes across the sampling periods were estimated.

Fecal DM excretion was calculated from the average intake of TiO_2 (g/animal and d) divided by the TiO_2 concentration (g/kg DM) in the feces of the respective cow in each period, assuming a fecal recovery of TiO_2 of 100% (Glindemann et al., 2009). The apparent total tract digestibility (ATTD) of DM, OM, N, and NDF were then calculated from the cows' daily DM, OM, N, and NDF intakes and their daily fecal DM, OM, N, and NDF excretions, respectively.

The energy-corrected MY was calculated using the equation from Spiekers et al. (2009): Energy-corrected MY (kg/d) = MY (kg/d) × (0.038 × milk fat (g/kg) + 0.021 × milk protein (g/kg) + 1.05) / 3.28.

4.3.4 Statistical analysis

Statistical analyses were conducted using the software SAS 9.4 (SAS Institute Inc. Cary, NC, USA). For nutrient intakes, ATTD, MY, and milk composition variables, mean values per animal and period were used and the dataset comprised 96 observations (*i.e.*, 4 periods × 4 treatments × 6 animals). The main effects of RNB level, protein source, and their interaction were tested using the following mixed model (PROC MIXED):

$$Y_{ijklm} = \mu + G_i + R_j + P_k + T_l + (RP)_{jk} + (RT)_{jl} + (PT)_{kl} + (RPT)_{jkl} + A_{mi} + e_{ijklm}$$

where: Y_{ijklm} = dependent variable; μ = overall mean; G_i = effect of the animal group during feeding (i = 1 – 4); R_j = effect of the RNB level (j = 1 – 2); P_k = effect of the protein source (k = 1 – 2); T_l = effect of the period (l = 1 – 4); $(RP)_{jk}$ = the interaction effect of j^{th} RNB level and the k^{th} protein source; $(RT)_{jl}$ = the interaction effect of j^{th} RNB level and l^{th} period; $(PT)_{kl}$ = the interaction effect of k^{th} protein source and l^{th} period; $(RPT)_{jkl}$ = the interaction effect of j^{th} RNB level, k^{th} protein source, and l^{th} period; A_{mi} = random effect of the m^{th} animal in i^{th} group; and e_{ijklm} = residual random error of experiment.

For chewing activity variables, covariance type AR (1) by day as a repeated measure with cow in each period as a subject was used, and the dataset comprised 384 observations (*i.e.*, 4 periods × 4 treatments × 3 animals × 8 d). The main effects of RNB level, protein source, and their interaction were tested using the following mixed model (PROC MIXED):

$$Y_{ijklmn} = \mu + G_i + R_j + P_k + T_l + D_m + (RP)_{jk} + (RT)_{jl} + (PT)_{kl} + (RPT)_{jkl} + A_{ni} + e_{ijklmn}$$

where: Y_{ijklmn} = dependent variable; μ = overall mean; G_i = effect of the animal group during feeding (i = 1 – 4); R_j = effect of the RNB level (j = 1 – 2); P_k = effect of the protein source (k = 1 – 2); T_l = effect of the period (l = 1 – 4); D_m = effect of day (m = 1 – 8); $(RP)_{jk}$ = the interaction effect of j^{th} RNB level and the k^{th} protein source; $(RT)_{jl}$ = the interaction effect of j^{th} RNB level and l^{th} period; $(PT)_{kl}$ = the interaction effect of k^{th} protein source and l^{th} period; $(RPT)_{jkl}$ = the interaction effect of j^{th} RNB level, k^{th} protein source, and l^{th} period; A_{ni} = random effect of the n^{th} animal in i^{th} group; and e_{ijklmn} = residual random error of experiment.

Least squares means in SAS at a significance level of $P < 0.05$, whereas tendencies were declared at $P \geq 0.05$ to < 0.10. Linear contrast comparisons of least squares means between the RNB level for each protein source and between protein sources for each RNB level were conducted using ESTIMATE statement. As the interactions of RNB x period, protein x period,

and RNB x protein x period were not significant, the above-mentioned statistical models were re-run without these effects. Moreover, the fixed effect of group and day was not significant and is thus not reported in the results.

4.4 Results

4.4.1 Nutrient intake and digestibility

An interaction between RNB level and protein source was observed for DM and OM intakes, with lower intakes for RNB- than for RNB0 in diets containing FB ($P \leq 0.01$; Table 4.5), but not in the diets containing SP ($P \geq 0.65$). Furthermore, there was an interaction between RNB level and protein source for intakes of CP and NDF as the intakes decreased for RNB- compared to RNB0 for both protein sources ($P \leq 0.01$ for both variables), with greater absolute differences between the RNB levels in FB than in SP diets. Intakes of CP and NDF were lower for the FB compared to the SP diets for both RNB levels ($P \leq 0.01$ for both variables) with greater absolute differences between the protein sources in RNB- than in RNB0 diets. Neither RNB level nor protein source affected ATTD of DM and OM ($P \geq 0.23$), but the ATTD of NDF was lower for RNB- compared to RNB0 diets ($P < 0.01$), and greater for SP than for FB diets ($P < 0.01$). An interaction of RNB level and protein source was found for ATTD of CP as the ATTD of CP decreased for RNB- compared to RNB0- diets for both protein sources ($P < 0.01$), with greater absolute differences between RNB levels in FB than in SP diets. Moreover, ATTD of CP differed between the protein sources only for RNB- level ($P < 0.01$).

Table 4.5 Effects of rumen nitrogen balance (RNB) levels and protein source on nutrient intake and apparent total tract digestibility (ATTD) in lactating dairy cows (least square means, n = 24 cows).

Variable	Protein source				SEM[1]	*P*-value		
	Faba bean grain		SoyPass			RNB	Protein	RNB x Protein
	RNB0[2]	RNB-[2]	RNB0	RNB-				
Intake (kg/cow and d)								
Dry matter	24.4^{a}	23.5^{bB}	24.3	24.2^{A}	0.51	< 0.01	0.16	0.03
Organic matter	22.5^{a}	21.6^{bB}	22.3	22.3^{A}	0.48	0.01	0.17	0.03
Crude protein	3.90^{aB}	3.01^{bB}	4.23^{aA}	3.65^{bA}	0.081	< 0.01	< 0.01	< 0.01
Neutral detergent fiber	9.85^{aB}	9.04^{bB}	10.2^{aA}	9.80^{bA}	0.208	< 0.01	< 0.01	0.02
Fecal excretion (kg/cow and d)								
Dry matter	8.96	8.82	8.82	8.90	0.272	0.85	0.83	0.48
Organic matter	7.74	7.63	7.60	7.72	0.242	0.95	0.83	0.42
ATTD (g/100 g)								
Dry matter	63.5	62.5	63.6	63.3	0.69	0.23	0.44	0.60
Organic matter	66.2	65.8	66.6	66.2	0.67	0.40	0.44	0.94
Crude protein	63.1^{a}	55.0^{bB}	64.4^{a}	60.0^{bA}	0.67	< 0.01	< 0.01	< 0.01
Neutral detergent fiber	49.1	44.9	51.9	48.5	1.19	< 0.01	< 0.01	0.63

^{a,b}Means with different superscripts in the same row for both RNB levels within the same protein source differed significantly at $P < 0.05$.
^{A,B}Means with different superscripts in the same row for both protein sources within the same RNB level differed significantly at $P < 0.05$.
[1]SEM, standard error of the mean.
[2]RNB0, RNB in the diet at 0 g/kg dry matter; RNB-, RNB in the diet at -3.2 g/kg dry matter.

4.4.2 Milk yield and composition

The MY of cows was lower for RNB- compared to RNB0 for both protein sources ($P < 0.01$; Table 4.6), and greater for the SP compared to the FB diets for both RNB levels ($P < 0.01$). An interaction between RNB level and protein source was found for energy-corrected MY, with lower energy-corrected MY for RNB- than for RNB0 in diets containing FB ($P < 0.01$), but not in diets containing SP ($P = 0.42$).

Milk CP, fat, and lactose concentrations were greater in diets containing FB than in diets containing SP for both RNB levels ($P < 0.01$ for all variables). An interaction and a tendency for interaction between RNB level and protein source were observed for milk fat yield and milk protein yield, respectively, where both the yields were lower for RNB- than for RNB0 in diets containing FB ($P \leq 0.02$), but not when diets contained SP ($P \geq 0.28$). Moreover, milk protein yield differed between the protein sources for both RNB levels ($P < 0.01$).

There was an interaction between RNB level and protein source for milk urea nitrogen concentrations as the concentrations decreased for RNB- compared to RNB0- diets for both protein sources ($P < 0.01$), with greater absolute differences between the RNB levels in FB than in SP diets. Moreover, the milk urea nitrogen concentrations were greater for the SP than for the FB diets for both RNB levels ($P < 0.01$) with greater absolute differences between the protein sources in RNB- than in RNB0 diets. Feed conversion ratio (kg energy-corrected milk/kg OM intake) was lower for RNB- than for RNB0 in the diets containing FB ($P = 0.02$), and were greater for the SP compared to the FB diets for both RNB levels ($P \leq 0.01$), without any interaction between main effects.

Table 4.6 Effects of rumen nitrogen balance (RNB) levels and protein source on milk yield and composition in lactating dairy cows (least square means, n = 24 cows).

Variable	Protein source				SEM[1]	*P*-value		
	Faba bean grain		SoyPass			RNB	Protein	RNB x Protein
	RNB0[2]	RNB-[2]	RNB0	RNB-				
Yield (kg/cow and d)								
Milk	30.4	28.2	32.7	31.5	1.55	< 0.01	< 0.01	0.17
ECM[1]	30.6^{aB}	28.6^{bB}	31.7^{A}	31.3^{A}	1.45	< 0.01	< 0.01	0.04
Protein	1.06	0.99	1.11	1.09	0.041	< 0.01	< 0.01	0.06
Lactose	1.43	1.32	1.55	1.50	0.074	< 0.01	< 0.01	0.12
Fat	1.22^{a}	1.15^{bB}	1.22	1.23^{A}	0.062	0.08	< 0.01	0.03
Feed conversion ratio (kg ECM/kg OM[1] intake)	1.33	1.28	1.38	1.37	0.045	0.03	< 0.01	0.27
Milk composition (g/kg milk yield)								
Protein	35.5	35.5	34.4	34.9	0.63	0.26	< 0.01	0.26
Lactose	46.9	46.9	47.3	47.4	0.48	0.77	< 0.01	0.77
Fat	40.3	40.8	37.5	39.1	0.82	< 0.01	< 0.01	0.13
Milk urea nitrogen (mg/dl)	11.3^{aB}	5.65^{bB}	13.4^{aA}	8.74^{bA}	7.0	< 0.01	< 0.01	< 0.01

[a,b]Means with different superscripts in the same row for both RNB levels within the same protein source differed significantly at $P < 0.05$.
[A,B]Means with different superscripts in the same row for both protein sources within the same RNB level differed significantly at $P < 0.05$.
[1]SEM, standard error of the mean; ECM, energy-corrected milk; OM, organic matter.
[2]RNB0, RNB in the diet at 0 g/kg dry matter and RNB-, RNB in the diet at -3.2 g/kg dry matter.

4.4.3 Fatty acid composition of diets and milk

Total saturated FA, mono-unsaturated FA, and poly-unsaturated FA proportions in the diets consisted pre-dominantly of C16:0, C18:1cis-9, and C18:2 *cis*-9, *cis*-12 (linoleic acid), respectively (Table 4.4). Moreover, saturated FA proportions were greater and mono-unsaturated FA proportions were lower in FB than in SP diets.

There was an interaction between RNB level and protein source for *de novo* synthesized milk FA, where the proportions of milk FA (with an exception of C16:0) were lower for RNB0 compared to RNB- in diets containing SP ($P < 0.01$ for all variables; Table 4.7), but not in the diets containing FB (tendency seen in C6:0 and C8:0, $P \geq 0.07$ for all variables).

There was an interaction between RNB level and protein source for total unsaturated FA proportion with lower proportion of unsaturated FA for RNB- than for RNB0 diets for both protein sources ($P < 0.01$), with greater absolute differences between the RNB levels in the SP compared to the FB diets. Moreover, total unsaturated FA proportions were lower for the FB compared to the SP diets for both RNB levels ($P < 0.01$) with greater absolute differences between the protein sources in RNB0 than in RNB- diets. Besides, there was an interaction between RNB level and protein source for total saturated FA proportion with a greater proportion of saturated FA for RNB- compared to RNB0 for both protein sources ($P < 0.01$), and with greater absolute differences between the RNB levels in the SP compared to the FB diets. The proportion of total unsaturated FA was lower in the SP compared to the FB diets ($P < 0.01$) with greater absolute difference between the protein sources in RNB0 compared to RNB- diets. Finally, a tendency for an interaction between RNB level and protein source was observed for the proportion of odd- and branched-chain FA (OBFA), because the proportion decreased for RNB- compared to RNB0 for both protein sources ($P < 0.01$), with greater absolute differences between the RNB levels for the FB than for the SP

diets. Moreover, the proportion of OBFA was lower for the FB compared to the SP diets for both RNB levels ($P < 0.01$) with greater absolute differences between the protein sources for RNB- than for RNB0 diets.

Table 4.7 Effects of rumen nitrogen balance (RNB) levels and protein source on milk fatty acid (FA) profile and groups in lactating dairy cows (least square means, n = 24 cows).

Variable	Protein source				SEM[1]	*P*-value		
	Faba bean grain		SoyPass			RNB	Protein	RNB x Protein
	RNB0[2]	RNB-[2]	RNB0	RNB-				
FA (g/100 g of total FA)								
C4	2.42^{B}	2.46	2.49^{A}	2.43	0.043	0.64	0.31	0.04
C6	1.79^{A}	1.81^{A}	1.62^{bB}	1.73^{aB}	0.021	< 0.01	< 0.01	< 0.01
C8	1.24^{A}	1.27^{A}	1.07^{bB}	1.19^{aB}	0.017	< 0.01	< 0.01	< 0.01
C10	3.13^{A}	3.18^{A}	2.51^{bB}	2.95^{aB}	0.059	< 0.01	< 0.01	< 0.01
C12	4.01^{A}	4.05^{A}	3.20^{bB}	3.77^{aB}	0.077	< 0.01	< 0.01	< 0.01
C14 (myristic acid)	12.8^{A}	13.0^{A}	11.1^{bB}	12.2^{aB}	0.15	< 0.01	< 0.01	< 0.01
C16 (palmitic acid)	31.9^{bA}	33.2^{aA}	27.0^{bB}	30.0^{aB}	0.36	< 0.01	< 0.01	< 0.01
C17	0.60^{aB}	0.58^{bB}	0.63^{aA}	0.60^{bA}	0.008	< 0.01	< 0.01	0.02
C18 (stearic acid)	8.84^{B}	8.75^{B}	10.6^{aA}	9.58^{bA}	0.155	< 0.01	< 0.01	< 0.01
C14:1	1.15^{A}	1.13^{A}	0.99^{bB}	1.08^{aB}	0.023	0.01	< 0.01	< 0.01
C16:1 (total *cis*)	1.54	1.51	1.61	1.55	0.030	0.01	0.01	0.42
C18:1 cis-9 (oleic acid)	16.7^{aB}	15.5^{bB}	21.2^{aA}	18.3^{bA}	0.44	< 0.01	< 0.01	< 0.01
C18:1 (total *cis*)	17.8^{aB}	16.9^{bB}	23.0^{aA}	19.9^{bA}	0.46	< 0.01	< 0.01	0.01
C18:1 (total *trans*)	2.40^{aB}	2.00^{bB}	3.39^{aA}	2.66^{bA}	0.067	< 0.01	< 0.01	< 0.01

C18:2 *cis*-9, *cis*-12 (linoleic acid)	1.39	1.43	1.42	1.44	0.023	0.02	0.24	0.53
C18:2 *cis*-9, *trans*-11 (conjugated linoleic acid)	0.40^{aB}	0.24^{bB}	0.67^{aA}	0.45^{bA}	0.028	< 0.01	< 0.01	0.04
C18:3 *cis*-9, *cis*-12, *cis*-15 (α-linolenic acid)	0.39^{aB}	0.35^{bB}	0.49^{aA}	0.41^{bA}	0.009	< 0.01	< 0.01	< 0.01
Total saturated FA[3]	70.5^{bA}	71.7^{aA}	63.7^{bB}	67.7^{aB}	0.51	< 0.01	< 0.01	< 0.01
Total unsaturated FA[4]	26.4^{aB}	24.9^{bB}	33.2^{aA}	28.8^{bA}	0.52	< 0.01	< 0.01	< 0.01
Odd chain FA	3.58^{aB}	3.43^{bB}	3.64^{aA}	3.58^{bA}	0.047	< 0.01	< 0.01	0.03
Branched chain FA	2.00	1.91	2.12	2.05	0.031	< 0.01	< 0.01	0.31
Odd branched chain FA	5.57	5.34	5.76	5.63	0.075	< 0.01	< 0.01	0.07
Omega 3	0.46^{aB}	0.43^{bB}	0.59^{aA}	0.49^{bA}	0.010	< 0.01	< 0.01	< 0.01
Omega 6	2.28^{B}	2.25^{B}	2.51^{aA}	2.37^{bA}	0.030	< 0.01	< 0.01	< 0.01

[a,b]Means with different superscripts in the same row for both RNB levels within the same protein source differed significantly at $P < 0.05$.
[A,B]Means with different superscripts in the same row for both protein sources within the same RNB level differed significantly at $P < 0.05$.
[1]SEM, standard error of the mean.
[2]RNB0, RNB in the diet at 0 g/kg dry matter and RNB-, RNB in the diet at -3.2 g/kg dry matter.
[3]Sum of all FA without double bonds.
[4]Sum of all FA with double bond.

4.4.4 Eating and ruminating activity

There was an interaction between RNB level and protein source for eating chews relative to NDF and eating time relative to DM or NDF intakes with greater number of eating chews and longer daily eating time for RNB- than for RNB0 in diets containing FB ($P < 0.01$ for all the variables; Table 4.8), but not in the diets containing SP ($P \geq 0.17$). Additionally, number of eating chews and eating time when expressed relative to NDF intakes were greater for FB compared to SP for only RNB- diets ($P < 0.01$ for both variables).

An interaction between RNB level and protein source was found for ruminating time, with lower ruminating time for RNB- compared to RNB0 in diets containing FB ($P < 0.01$), but not in diets containing SP ($P = 0.72$). Number of ruminating chews and ruminating time relative to NDF intake were greater for RNB- compared to RNB0 for both protein sources ($P < 0.01$ for both variables), and for the FB compared to the SP diets for RNB levels ($P < 0.01$ for both variables), without any interaction between main effects.

Total number of chews and total chewing time per kg DM intake differed and tended to differ, respectively, between the RNB levels with lower total number of chews and chewing time per kg DM intake for RNB0 than for RNB- in diets containing FB ($P < 0.01$), but not in the diets containing SP ($P \geq 0.94$), without any interaction between the main effects. There was also an interaction observed between RNB level and protein source in total number of chews and chewing time relative to NDF intake with greater total number of chews and chewing time per kg NDF intake for RNB- compared to RNB0 for both protein sources ($P < 0.01$ for both variables), and with greater absolute differences between the RNB levels for the FB than for the SP diets. Moreover, total number of chews and chewing time per kg NDF intake were greater for the FB compared to the SP diets for both RNB levels ($P \leq 0.04$ for both variables) with greater absolute differences between the protein sources in RNB- than

in RNB0 diets. Finally, differences were observed between protein sources for feed trough visits with lower number of trough visits in SP compared to FB for RNB- diet ($P < 0.01$), without any interaction between the main effects.

Table 4.8 Effects of rumen nitrogen balance (RNB) levels and protein source on chewing activity in lactating dairy cows (least square means, n = 12 cows).

Variable	Protein sources				SEM[1]	*P*-value		
	Faba bean grain		SoyPass			RNB	Protein	RNB x Protein
	RNB0[2]	RNB-[2]	RNB0	RNB-				
Bolus								
n/cow and d	594[a]	562[b]	589	590	16.1	< 0.01	0.04	< 0.01
Eating								
n/cow and d	28471	28321	29080	27967	1630	0.17	0.78	0.31
n/kg DM[1] intake	1180	1229	1201	1179	85	0.49	0.44	0.08
n/kg NDF[1] intake	2930[b]	3179[aA]	2863	2903[B]	212	< 0.01	< 0.01	0.04
min/cow and d	377	379	390	372	14.8	0.17	0.60	0.11
min/kg DM intake	15.6[b]	16.4[aA]	16.1	15.6[B]	0.80	0.44	0.41	0.01
min/kg NDF intake	38.8[b]	42.5[aA]	38.3	38.5[B]	1.99	< 0.01	< 0.01	< 0.01
Ruminating								
n/cow and d	38339	37540	38732	38796	813	0.29	0.02	0.23
n/kg DM intake	1595	1640	1613	1652	65	0.02	0.38	0.85
n/kg NDF intake	3960	4234	3851	4058	159	< 0.01	< 0.01	0.48
min/cow and d	566[a]	547[bA]	565	568[B]	10.2	0.07	0.02	0.02
min/kg DM intake	23.4	23.8	23.5	24.0	0.72	0.07	0.68	0.78

min/kg NDF intake	58.3	61.6	56.1	58.9	1.80	< 0.01	< 0.01	0.75
Total								
n/cow and d	66734	65819	67766	66726	1945	0.13	0.13	0.93
n/kg DM intake	2780	2872	2816	2824	132.6	0.04	0.82	0.11
n/kg NDF intake	6897^{b}	7423^{a}	6722^{a}	6938^{b}	328	< 0.01	< 0.01	0.03
min/cow and d	943	925	955	941	16.6	0.04	0.06	0.84
min/kg DM intake	39.1	40.3	39.5	39.6	1.27	0.08	0.69	0.10
min/kg NDF intake	97.1^{bA}	104^{aA}	94.4^{bB}	97.3^{aB}	3.17	< 0.01	0.01	0.03
Trough visits								
n/cow and d[3]	66.7	67.2	63.3	58.1	5.14	0.25	< 0.01	0.16
min/cow and d[4]	274	254	277	259	14.3	< 0.01	0.38	0.74

^{a,b}Means with different superscripts in the same row for both RNB levels within the same protein source differed significantly at $P < 0.05$.
^{A,B}Means with different superscripts in the same row for both protein sources within the same RNB level differed significantly at $P < 0.05$.
[1]SEM, standard error of the mean; DM, dry matter; NDF, neutral detergent fiber.
[2]RNB0, RNB in the diet at 0 g/kg DM and RNB-, RNB in the diet at -3.2 g/kg DM.
[3]Number of trough visits (n/d).
[4]Feeding time at the trough (min/d).

4.5 Discussion

4.5.1 Composition of the experimental diets

In the present study, dietary concentrations of CP, starch, and RUP (Kand and Dickhoefer, 2021), as well as their uCP supply (all in g/kg DM) were varied to achieve the desired RNB levels of 0 and -3.2 g/kg DM. The uCP supply and RNB level of the diets estimated from the *in vitro* technique were very similar to the targeted values during ration formulation. The uCP supply from all diets exceeded the uCP requirements of the cows for maintenance and lactation of 3,040 g/d according to GfE (2001). Therefore, no effects of a deficit in the uCP supply to cows are expected.

The NDF concentrations were similar across the diets. Additionally, the particle size distribution in the offered diets was determined thrice with four replicates of each diet in period 3 and period 4 using Penn State Particle Size Separator (Nasco, Fort Atkinson, WI, USA) according to Jones and Heinrichs (2017). No differences in the geometric mean of particle size ($P \geq 0.19$; data not shown) between the offered diets were observed. Across the diets, the proportion of particles retained (means ± standard deviation, as-fed basis) were 37 ± 4.0 g/100 g for 19 mm, were 17 ± 2.2 g/100 g for 8 mm, were 42 ± 2.1 g/100 g for 1.18 mm, and were 4 ± 1.7 g/100 g for the bottom pan. Hence, varying dietary particle size across the offered diets is not expected to be responsible for differences in nutrient intake and ATTD as well as chewing activity.

4.5.2 Interaction effects of rumen nitrogen balance and protein source on nutrient intake, nutrient digestibility, and milk yield and composition

An interaction effect was observed between RNB level and protein source for DM intake. Contrary to our expectations, that dietary treatments would not affect DM intake, DM intake

was lower in the RNB- compared to RNB0 diets containing FB, whereas no differences were seen between both SP diets. The lower DM intake was likely due to the lower ATTD of NDF in the FB diet, as both these variables showed similar differences between RNB levels for FB diet. A lower ATTD of NDF may increase rumen fill and thus ultimately reduce feed intake by the animal (Oba and Allen, 1999).

The reduced DM intake and pronounced differences in ATTD of NDF and CP in diet containing FB compared to SP reflected an impaired rumen microbial activity in response to the low N availability in the rumen. In this line, rumen MCP synthesis was also reduced at RNB- in the FB diets, but was similar for both RNB levels for the SP diets (Kand and Dickhoefer, 2021). The lower yields of energy-corrected milk, milk fat, and milk protein were at least partly related to the lower intakes and ATTD of nutrients observed for RNB- diet containing FB and thus the lower digestible OM intake (Kand and Dickhoefer, 2021), CP intake, and NDF intake. Results observed in the FB diets confirm those of previous studies analyzing the effects of dietary CP and RDP supply on feed intake and diet digestibility (Lee et al., 2011), rumen fermentation and MCP synthesis (Lebzien et al. 2006), and MY and composition (Schiavon et al., 2015) in lactating dairy cows.

The RNB is an indicator for N supply in the rumen system and is calculated as the difference between the consumed N and the estimated N leaving the rumen as RUP and MCP (GfE, 2001). However, under a condition of sufficient energy supply relative to the requirements of rumen microbes, rumen MCP synthesis can be realized only, if N would not be limiting for its incorporation into the microbial mass (Bach et al., 2005). Although RNB (in g/kg DM and g/d) were similar for each RNB level across both protein sources, the amount of N available to rumen microbes did not appear to be enough to meet their requirements and thus to sustain microbial activity in the RNB- diet containing FB. Indeed, milk urea

concentrations in RNB- diet containing FB were below the range of 15 – 25 mg/100 ml (*i.e.*, recalculated milk urea for RNB- (FB) 11.9 mg/100 ml) that indicate a sufficient N availability to rumen microbes (Steingass et al., 2001). Additionally, estimated urinary N excretions were lower for this diet compared to those containing SP (Kand and Dickhoefer, 2021), supporting the explanation that N supply to the rumen microbes was indeed lower in FB diets. The lower N supply to rumen microbes relative to their N requirements would decrease the activity of cellulolytic and proteolytic microbes (Belanche et al., 2012), explaining the effects of RNB on ATTD of CP and NDF in FB diets as well as in SP diets. The lack of differences in ATTD of DM and OM may be related to the fact that sugar and starch, who inherently have a greater ATTD than CP and NDF, were found in greater concentrations for RNB- than for RNB0 in the FB and SP diets (see Table 4.5). Additionally, as amylolytic bacteria are suggested to have relatively low ammonia saturation constant (Belanche et al., 2012), the activity of these bacteria was likely not hampered at low N concentrations in the rumen and therefore helped to maintain ATTD of DM and OM at RNB- level in both FB and SP diets.

No effects of RNB level on DM intake in the SP diets were observed, despite lower ATTD of CP and NDF in RNB- compared to RNB0. These differences in ATTD of CP and NDF with SP were, however, smaller than those in the FB diets and maybe too small to impair feed intake. Nevertheless, it cannot be precluded that, although uCP supply from all diets exceeded the uCP requirements of the cows for maintenance and lactation, it was greater with SP compared to FB diets, which may have likely contributed to increased endogenous N supply to the rumen microbes as detailed in Kand and Dickhoefer (2021). This greater uCP supply in SP diets may have also at least partly compensated for the negative RNB- effects on nutrient intake, ruminal fermentation, ATTD of NDF, and milk protein yields.

Similarly, previous research by Giallongo et al. (2015) and Mutsvangwa et al. (2016) on dietary treatments varying in CP and RDP supply have also shown no difference in the feed intake,-rumen fermentation and MCP synthesis, and MY and composition in lactating dairy cows. Variable responses, with some studies showing no and others negative effects (see above) of reduced dietary N supply may be related to dietary energy and protein sources.

4.5.3 Interaction effects of rumen nitrogen balance and protein source on eating and ruminating activity

In the present study, rumination time (min/d) was lower for RNB- compared to RNB0 for FB but not for SP diets, which was likely due to lower DM intake in the RNB- diet. Moreover, when expressed relative to the NDF intake of cows, total number of chews were greater for RNB- than RNB0 for both, FB and SP diets; however, with more pronounced differences in FB diets. The enhanced chewing activity of cows fed the RNB- diets confirms findings of Schiavon et al. (2015) who observed a tendency for a lower DM intake, but longer rumination times relative to the feed intake (*i.e.*, min/kg DM intake) of lactating dairy cows when the CP concentration in their diets decreased from 153 to 124 g/kg CP. In this regard, intensified eating activity and ruminating activity (in min/kg DM or NDF intake) in the RNB- diets containing either FB or SP in the present study may be assumed to stimulate saliva secretion and thereby N recycling. Nevertheless, this recycling of N was not sufficient to sustain ATTD of NDF, and MY in the FB diets, probably due to the high level of production of the cows, as suggested by Calsamiglia et al. (2010). The greater differences in the number of chews relative to the NDF intake of cows between the RNB levels in the diets with FB compared to those with SP might be due to the less pronounced effects of RNB level in the SP diets on

DM intake, rumen fermentative activity (as indicated by a smaller decline in ATTD of NDF), and possibly on retention time of the digesta in the rumen.

4.5.4 Interaction effects of rumen nitrogen balance and protein source on milk fatty acids

In the present study, milk sampling was performed from d 14 onwards, resulting in less days for adaptation of the animals to the experimental diets than in previous studies (Shingfield et al., 2006; He and Armentano, 2011), examining dietary effects on milk FA composition in lactating dairy cows. In comparison to the aforementioned studies, mean dietary crude lipid concentrations were low in the present study, ranging from 19 to 36 g/kg DM, and therefore might not have greatly impacted milk FA profile. In contrast, other studies analysing dietary effects on milk FA composition (Schiavon et al., 2016; Gaillard et al., 2017; Schulz et al., 2018) with similar dietary crude lipid concentrations of 20 − 31 g/kg DM also allowed for only 13 or 14 days of adaptation. Therefore, the 14-d-adaptation period before milk sampling in the present study appears sufficient for milk composition and FA profile to adjust to the new diets.

The lower fiber degradation at RNB- compared with RNB0 in FB diets possibly reduced production of acetate, the main precursor of milk fat, and therefore inhibited milk fat synthesis (Chilliard et al., 2000). Changes in the concentrate feed of the cows were reflected by variations in milk FA proportions, as also highlighted by Chilliard et al. (2000). For example, total saturated FA proportions in milk were greater in FB than in SP diets for both RNB levels, following the same pattern of the FA proportions in the diets. However, total unsaturated FA proportions in milk were lower in RNB- than in RNB0 for both protein sources and in FB than in SP diets for both RNB levels. However, the differences in milk unsaturated FA were more pronounced than those between the dietary FA. The observed differences in

milk unsaturated FA proportions indicate that bio-hydrogenation of unsaturated FA could have been greater for RNB- than RNB0 and in FB than in SP diets. The above may be a result of an increased ruminal retention time of the digesta, as could be implied from the lower DM intake and from an increased chewing activity per unit of DM intake in the RNB- diet containing FB. Nevertheless, more evidence would be needed to conclude on the effects of RNB on ruminal bio-hydrogenation of dietary FA.

It is known that feeding palmitic acid increases milk fat concentration due to pre-formed FA (Piantoni et al., 2013). However, those effects have been observed in diets in which palmitic acid is the sole or main source of lipids with a difference in palmitic acid concentrations between the control and treatment diets of about ~13 − 19 g/kg DM (Piantoni et al., 2013; de Souza et al., 2017). In the present study, the differences in palmitic acid concentrations between, for instance, RNB0 (5.0 g/kg DM) and RNB- diets (4.3 g/kg DM) were only 0.63 g/kg DM and thus rather small. It is therefore unlikely that palmitic acid had a significant impact on the milk fat concentration and milk FA profile in the present study.

Different to other milk FA, OBFA are of microbial origin and have been proposed as indicators of microbial growth in the rumen (Vlaeminck et al., 2015) , which was, irrespective of protein source, negatively affected by RNB- diets in the present study,. Interestingly, differences in milk OBFA proportions were in accordance with those observed for urinary purine derivatives excretion and estimated rumen MCP synthesis in our experiment (Kand and Dickhoefer, accepted). However, a conclusion on the relation between rumen MCP synthesis and OBFA proportions in milk should be made with caution, because several studies did not observe any correlations between OBFA proportions and rumen MCP synthesis (Castro-Montoya et al., 2016; Westreicher-Kristen et al., 2020).

4.5.5 Practical implications

The results of the present study suggest that there is considerable scope to reduce the RNB in dairy cattle diets. More so, imported, cost-intensive protein sources (*i.e.*, SP) can be substituted for local protein feeds (*i.e.*, FB) even in high-yielding cows, without negative effects on animal performance and milk quality (at sufficient RDP supply or enhanced N recycling). Against the background of rising costs of high-quality protein feeds, reducing RNB and using local feed sources may have benefits from both, an ecological and an economic perspective. However, the effects of negative RNB differed between diets with varying ingredient composition and CP concentrations in the diets. Moreover, the carbohydrate and protein concentrations as well as the rate and extent of their ruminal degradation in alternative protein feeds are highly variable. In addition, different dietary protein source and RNB may influence the amino acid composition of microbial and feed protein reaching the duodenum which may in turn affect MY and composition (Sinclair et al., 2014). Hence, consideration of the above-mentioned dietary factors in protein recommendation systems combined with more frequent feed analysis and the monitoring of nutrient intake and MY of the animals are needed to avoid possible adverse effects of negative RNB on feed intake, diet digestibility, and performance in high-yielding cows.

4.6 Conclusions

Negative RNB reduce feed intake, diet digestibility, and milk production in high-yielding dairy cows, particularly in diets containing rapidly degradable protein sources. A reduced N supply to rumen microbes might be overcome by a surplus uCP supply relative to the requirements of cows and / or by an increase in their eating and ruminating activity when offered diets containing slowly degradable protein sources; however, underlying

compensatory mechanisms need further investigation. The present results highlight the interaction between RNB level and protein source and help to explain the contrasting findings observed in previous studies with negative RNB, but also suggest the need to better understand the effect of negative RNB in diets differing in ingredient and / or chemical composition.

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Chapter 5

5. General discussion

Matching nitrogen (N) intake to actual requirements of rumen microbes and their host animals is of great importance to avoid inhibition of microbial growth and synthesis of microbial crude protein (MCP), as the unsatisfactory conditions of rumen microbes may lead to a suboptimal rumen fermentation and ultimately to negative effects on nutrient digestibility, feed intake and milk yield (MY), and milk composition. However, responses so far have been variable where some studies (Riemeier, 2004; Steinwidder et al., 2009; Aschemann et al., 2012a; Aschemann et al., 2012b) have shown negative effects of low N supply to the rumen microbes (*i.e.*, low rumen nitrogen balance; RNB), for instance, on feed intake, digestibility, and milk production, while other studies (Holthausen et al., 2000; König et al., 2005; van de Sand et al., 2008) did not detect negative effects; thereby, suggesting a need for a critical RNB threshold in diets of dairy cows. Furthermore, the above-mentioned contradictory results are likely to be affected by dietary and animal factors, such as diet composition (including carbohydrate (CHO) and N sources), extent and rate of rumen crude protein (CP) degradation, and intake and performance level of the animals. However, possible effects of interactions between RNB level and dietary N and CHO sources have so far not been investigated. Therefore, the first part of the doctoral dissertation focused on the effects of dietary RNB levels and CHO and N sources on rumen fermentation and MCP synthesis *in vitro* (**Chapter 2**). Additionally, based on the *in vitro* results, an *in vivo* study was conducted (**Chapter 3 and Chapter 4**) that examined the effects of RNB levels and dietary protein sources on the feed intake, rumen MCP synthesis, N partitioning, MY, and milk composition.

5.1 Estimation of utilizable crude protein and calculation of rumen nitrogen balance during ration formulation

5.1.1 Literature data and regression equation

The utilizable crude protein (uCP) is defined as total CP at the duodenum minus endogenous protein and is estimated as the sum of MCP synthesized in the rumen and undegraded feed protein (RUP) (Lebzien and Voigt, 1999). In the present thesis, the uCP supply (g/kg dry matter; DM) from the individual feeds was estimated following the regression equation 9 of Lebzien et al. (1996) that included dietary metabolizable energy (ME), RUP, and CP concentrations. Further, from the estimated uCP (g/kg DM), the dietary RNB was calculated (GfE, 2001). In the scientific studies (including present thesis) prior to the start of the experiment, ration formulation of the experimental diets relative to the requirements of the animals are calculated based on the feed value tables from the books like DLG (1997), Spiekers et al. (2009), or recently from the online databanks like Agroscope (2016) and DLG (2018). These feed value tables among others are widely used for RUP and ME concentrations of the feeds. Accordingly, there is a lack of first-hand information on RUP concentration of the individual feeds used in the scientific trials (including present thesis, except a few see Table 5.1) from *in situ* or digestibility trials (by performing total feces collection studies using wethers) at different passage rates of dietary protein. Besides, estimation of MCP synthesis by multiplying literature-based ME concentration of the feed and a constant efficiency of MCP synthesis of 10.1 g or 10.5 g CP/MJ ME (GfE, 2001) may lead to an over- or underestimation of uCP supply (g/d). Such differences in the theoretical and actual uCP supply and thereby RNB calculations were observed by Riemeier (2004) and Weltin and Jilg (2013). Riemeier (2004) studied the effects of four RNB levels (24 g, −21g, −63g, and −112 g/d) on digestibility, milk production, and composition in seven

fistulated lactating cows and observed differences between theoretical and measured RNB level. Riemeier (2004) observed the greatest difference in the uCP supply between the regression equation estimation and those measured at the duodenum for the lowest RNB diets (−112 g/d). Accordingly, the authors proposed that such high discrepancy (an overestimation of uCP supply from the regression equation), particularly for RNB −112 g/d was perhaps because the equation for uCP estimation does not account for shortage in rumen available N. Nevertheless, one may suggest that measured uCP in g/d depends on intake level of the animal and the rumen passage rate that may also be affected due to low RNB diets. Besides, it should also be noted that the equation of Lebzien et al. (1996) originated from *in vivo* experiments using total mixed ration (TMR); hence, may not always fit correctly to the individual feeds during ration formulation. Accordingly, diets of animals formulated based on the assumption that nutritive values of individual feeds in the TMR are additive may not hold true, since individual feeds in the rumen may have positive or negative associative effects, as observed by Westreicher-Kristen et al. (2017). Nonetheless, in **Chapters 3 and 4**, estimated uCP (g/kg DM) of the TMR based on the individual dietary nutrient composition and literature-based ME and RUP concentrations were slightly different to the uCP estimated from *in vitro* incubations using modified Hohenheim gas test (HGT) (Steingass and Südekum, 2013), indicating a nearly additive (*i.e.*, positive associative) effect on combining of individual feeds (see Table 5.1). Regardless, analysis of the nutrient composition of the feed before the study, formulation of the ration at the laboratory level, and then the estimation of uCP (g/kg DM) from *in vitro* incubations may perhaps help in scientific studies as well as in the commercial farms, to avoid considerable under- or overestimation of uCP supply to the cows (beyond their requirements), nonetheless, there exists a limitation when RNB is expressed in g/d (see section 5.1.2).

5.1.2 Laboratory techniques

In vitro technique is a cost-effective and quick technique that has proven to be useful in uCP estimations as confirmed with forages (Edmunds et al., 2012), and dried distillers' grains with solubles (Westreicher-Kristen et al., 2015). Estimation of uCP (g/kg DM) from ammonia-N concentration *in vitro*, as described by Steingass and Südekum (2013), follows a regular HGT procedure (Menke and Steingass, 1988) with a slight modification in the buffer composition. In this buffer modification, a chemical alteration is performed with a 2 g/l increase in ammonium bicarbonate and 2 g/l decrease in sodium bicarbonate in the buffer solution to avoid N from becoming a limiting factor in microbial biomass production (Edmunds et al., 2012). Nevertheless, this supplied N from the buffer medium (from both HGT and modified HGT buffer) can partly alter the estimation of RNB (g/kg DM) in the diets. Therefore, the possibility of using N-free McDougall's buffer solution (McDougall, 1948) to reduce additional N concentration in the buffered rumen inoculum was examined in the in vitro work (in **Chapter 2**).

For this, a short study (own work) was conducted by incubating various substrate mixtures (those used in the *in vitro* study, see **Chapter 2**) each with N containing buffer and without N containing buffer, Menke and Steingass (1988) and McDougall (1948) buffer, respectively, as elaborated in Camborda et al. (2018). The following three combinations of N and CHO sources: wheat gluten-corn starch, urea-corn starch, urea-cellulose, at two RNB levels: balanced RNB0 (0 g/kg DM) and negative RNB (−9 g/kg DM) using each of the buffer media were studied. Gas production and concentrations of short-chain fatty acids were measured while true degradability was calculated on treating (*i.e.*, digesting) the undegraded feed in ANKOM fiber analyzer (ANKOM Technology, Macedon, NY, USA) after 24 h of incubation. Gas production (ml/200 mg DM) increased with Menke and Steingass buffer for

all the dietary treatments (P < 0.01), indicating that fermentation was N limited with McDougall's buffer. In contrast, no difference between the buffers (P ≥ 0.11) for short-chain fatty acid concentrations (µmol/ml) with urea-corn starch and urea-cellulose diets was observed. True degradability decreased at a negative RNB level (-9 g/kg DM) in wheat gluten-corn starch and urea-corn starch diets with McDougall's buffer (P < 0.01), but not with Menke and Steingass buffer (P ≥ 0.23). The *in vitro* study indicated that Menke and Steingass buffer changed the RNB towards the positive scale providing a sufficient N to the rumen microbes. As a result, in **Chapter 2**, McDougall's buffer solution (*i.e*., N-free buffer) was considered for the *in vitro* incubation to estimate the dietary RNB to avoid additional N supply to rumen microbes.

The *in vitro* method estimates the uCP supply (g/kg DM) directly without providing separate MCP and RUP estimation. Direct estimation of uCP supply (g/kg DM) from the *in vitro* method, can be considered a better way to reduce the error caused during separate estimation of MCP and RUP (Edmunds et al., 2012). Nonetheless, as contribution of RUP may not be equal to MCP at the duodenum; it is important to understand how much of each MCP and RUP is attained at the duodenum using *in vitro* technique with different diets and RNB levels. Although *in situ* technique can be used to estimate proportions of RUP of the feed, there is still a large dependency on fistulated animals that reduces the feasibility of the technique. A laboratory method for uCP estimation by separate assessment of MCP synthesis from ME concentrations (GfE, 2001) and of RUP concentration from prediction equations of Kirchhof (2007) for forages and of Shannak et al. (2000) for concentrates may be performed. However, when estimating uCP with this method, assessment of the most accurate passage rate of the feed is equally important as differences in the passage rate may alter RNB levels considerably (see Table 5.1). The uCP (g/kg DM) comparison at k =

5%/h for forages and 8%/h for concentrates with and without *in situ* technique did not deviate markedly to indicate any large differences in the RUP concentrations between *in situ* and laboratory method for faba bean grain (FB), SoyPass (SP), and RaPass feed (see Table 5.1 footnote-8). Moreover, dietary RNB (in g/kg DM) only slightly differed between the *in vitro* method (*i.e.*, modified HGT), and the separate estimation method (of MCP from ME concentrations (GfE, 2001) and of RUP concentration) on assuming the most suitable passage rates (see Table 5.1), as likewise highlighted in **Chapter 3**. Yet, these minor differences may become relevant when expressed in g/d, particularly in high-yielding dairy cows with a high feed intake level. In this regard, a change in the dietary RNB (g/d) to a very positive or a negative level, may lead to an over- or an undersupply of dietary CP, respectively, to dairy cattle, with the consequence of unsuccessful feed protein recommendation. As a result, there is a need for validated and standardized laboratory procedures to estimate the dietary uCP supply and to optimize the RNB level in dairy cattle nutrition, particularly because highly variable nutrient composition of the local feeds may aggravate an error in uCP estimation and therefore in the RNB level.

Table 5.1 Estimation of utilizable crude protein (uCP in g/kg dry matter; DM) and calculation of rumen nitrogen balance (RNB in g/kg DM) of feeds studied in Chapter 3 and Chapter 4 using laboratory and *in situ* method.

	Protein sources			
	Faba bean grain		SoyPass	
	RNB0[1]	RNB-[1]	RNB0	RNB-
Theoretical uCP[2]	157	143	177	169
Theoretical RNB[2]	−0.03	−3.20	−0.03	−3.18
uCP – Total mixed ration[3]	160 ± 3.0	146 ± 2.2	174 ± 1.7	167 ± 2.6
RNB – Total mixed ration[3]	−0.3 ± 0.24	−2.9 ± 0.11	−0.2 ± 0.16	−2.7 ± 0.16
uCP – individual feed ingredients[4]	162	146	179	169
RNB – individual feed ingredients[4]	−0.1	−3.0	−0.1	−2.8
uCP – (RUP[5]; k = 5%/h)[6]	165	146	165	160
RNB – (RUP; k = 5%/h)[6]	−0.6	−3.0	2.2	−1.3
uCP – (RUP; k = 8%/h)[6]	177	154	190	181
RNB – (RUP; k = 8%/h)[6]	−2.6	−4.2	−1.8	−4.7
uCP – (RUP; k = 5% forages and 8%/h concentrate)[7]	163	149	183	174
RNB – (RUP; k = 5% forages and 8%/h concentrate)[7]	−0.3	−3.5	−0.6	−3.5
uCP – (RUP; k = 5% and 8%/h) + *in situ*[8,9]	165	149	178	170
RNB – (RUP; k = 5% and 8%/h) + *in situ*[8,9]	−0.7	−3.4	−0.3	−2.9

[1]RNB0, RNB in the diet is 0 g /kg DM; RNB-, RNB in the diet is -3.2 g/kg DM.

[2]Theoretical uCP (g/kg DM) and RNB (g/kg DM) calculated during ration formulation according to GfE (2001).

[3]Total mixed rations were incubated (n = 24; 4 periods x 3 triplicates x 2 incubation runs). The uCP (g/kg DM) was determined after incubation at a passage rate of k = 8 %/h, as described by Steingass and Südekum (2013) to further calculate RNB (g/kg DM) according to GfE (2001).

[4]Individual feeds containing concentrates and forages were incubated (n = 1, ingredients x 3 triplicates x 2 incubation runs). The uCP (g/kg DM) of the total mixed ration was calculated as proportion of individual feed in the diet at a passage rate of k = 8 %/h, as described by Steingass and Südekum (2013) to further calculate RNB (g/kg DM) according to GfE (2001).

[5]RUP, undegraded feed protein.

[6]The ruminal RUP concentrations for individual feed ingredients were predicted from their concentrations of crude protein fractions using the equation of Kirchhof (2007) for forages and of

Shannak et al. (2000) for concentrates, either at passage rate of 5% or at 8%/h.
[7]The ruminal RUP concentrations for individual feed ingredients were predicted from their concentrations of crude protein fractions using the equation of Kirchhof (2007) for forages and of Shannak et al. (2000) for concentrates, each at passage rate of 5% and 8%/h, respectively.
[8]The ruminal RUP concentrations for individual feed ingredients (except faba bean grain, SoyPass, and RaPass) were predicted from their concentrations of crude protein fractions using the equation of Kirchhof (2007) for forages and of Shannak et al. (2000) for concentrates, each at passage rate of 5% and 8%/h, respectively. The RUP concentration for faba bean grain, SoyPass, and RaPass were determined *in situ* at the passage rate of 8%/h.
[9]The microbial crude protein of the individual feed was determined by multiplying metabolizable energy concentration by 10.1 g crude protein/MJ metabolizable energy (GfE, 2001), where metabolizable energy concentration was estimated from proximate nutrient concentrations and gas production during *in vitro* incubation. The uCP (g/kg DM) was calculated as addition of RUP and microbial crude protein (Lebzien and Voigt, 1999) and the RNB (g/kg DM) was calculated according to GfE (2001).

5.2 Contrasting results from *in vitro* and *in vivo* studies

In vitro estimation of uCP and RNB (both g/kg DM) of the diet has various advantages including the possibility of studying a very negative RNB level that may not be tested *in vivo* (see **Chapter 2**). Nevertheless, the results of the *in vitro* batch system (**Chapter 2**) contradicted the *in vivo* findings in the present thesis (**Chapter 3 and Chapter 4**). For instance, in the *in vitro* study, the protein degradation and MCP synthesis showed pronounced differences between the RNB levels in diets containing a slowly degradable protein source, while in the *in vivo* study pronounced differences between the RNB levels (for similar variables) were observed in diets with a rapidly degradable protein source.

Despite the differences in the dietary ingredients and total uCP supply (g/kg DM) in each of the dietary treatments and methods, the following reasons may have partly contributed to contrasting *in vivo* and *in vitro* outcomes, (i) *in vitro* approach failed to consider a constant inflow of N via rumen wall and saliva (*i.e.*, urea-N recycling), accounting for approximately 20% of the extra N supply for MCP synthesis (GfE, 2001), (ii) closed batch system only depicted the effects of low RNB in the rumen, without considering the intake of fresh feed,

continuous feeding, rumen fluid generation, and continuous growth and passage of rumen microbes *in vivo*, and (iii) difficulty in cultivating and maintaining protozoa in the *in vitro* system may have shown an incomplete representation of the *in vivo* study, despite the affirmation in **Chapter 2** that protozoa contributed to bacterial predation and intra-ruminal recycling of N, and played a vital role in ruminal protein metabolism.

In **Chapter 2**, *in vitro* synthesis of MCP declined with decreasing RNB level for wheat gluten and soy protein, but not for casein diets (all containing corn starch as an energy source). A possible reason could be related to the faster degradation rate of casein as compared to the other N sources, which allowed for a sufficient supply of N compounds from dietary sources by the time starch was degraded in the rumen (see **Chapter 2**). Moreover, the estimated uCP supply (g/kg DM) was greater in casein compared to other N sources that may have contributed to the greater N reflux. Thus, the CP degradation products as well as the synthesized microbial biomass of the rapidly degradable protein sources that remain within the closed system could be used at a later stage of fermentation or even be recycled by the rumen microbes. In contrast, in the *in vivo* study (see **Chapter 3**), surplus rumen degraded protein (RDP) supply (*i.e.*, from FB diets), that was not incorporated into MCP synthesis, may withdraw from the rumen through absorption or passage, and partly lost via feces, urine, and milk of the animals, and hence, may be unavailable to rumen microbes. Likewise, the surplus RUP supply (*i.e.*, from SP diets) may by-pass the rumen to be utilized in the duodenum, and excess uCP supply may be utilized again via urea-N recycling compensating for the MCP synthesis. Overall, the results suggest that such differences in N metabolism are of particular relevance in diets containing rapidly degradable protein sources and may explain the contrasting results observed in the present thesis (in **Chapters 2 and 3**).

The *in vitro* findings were not in agreement with the *in vivo* findings for N sources. Yet, it is worth understanding, if different RNB levels and CHO sources (*i.e.*, rapidly and slowly degradable CHO source) *in vivo* would confirm *in vitro* findings (of **Chapter 2**) that is if CHO fermentation and MCP synthesis reduce at low RNB in the rapidly degradable CHO source. Various studies have tested the effects of reduced N supply and diverse CHO sources in lactating dairy cows, and have shown contrasting outcomes on intake, MCP synthesis, and milk production. For instance, Moorby et al. (1996) studied two dietary CP concentrations (162 g and 175 g/kg DM) and two CHO sources (starch source, barley; sugar source, sugar beet pulp) at a calculated RNB between −23 g and −111 g/d with uCP supply of ≥ 3,000 g/d (~ 2,194 g/d of cows' uCP requirement) and found a reduction in MCP synthesis and milk protein yield at low RNB in sugar-, but not in starch-rich diets. In-contrast, Cantalapiedra-Hijar et al. (2014) tested two dietary CP concentrations (120 g and 160 g/kg DM) and two CHO sources (starch source, corn starch; fiber source, dehydrated beet pulp) at a calculated RNB between 14 g and −37 g/d with uCP supply of ≥ 1840 g/d (~ 1,815 g/d of cows' uCP requirement). The authors found reduced DM intake, MCP synthesis, energy-corrected milk yield, and milk protein yield at low RNB in fiber-, but not in the starch-rich diets. With observed results, Cantalapiedra-Hijar et al. (2014) suggested that net absorption of essential amino acids was greater for starch- than for fiber-rich diets at low RNB. Consequently, Cantalapiedra-Hijar et al. (2014) highlighted a greater rumen protein-sparing effect (reduction in protein deamination activity in the rumen) in the presence of starch- than fiber-rich diets at low RNB level. In another study, Fanchone et al. (2013) studied two dietary CP concentrations (110 g and 140 g/kg DM) and two CHO sources (starch source, wheat; fiber source, dehydrated beet pulp) at a calculated RNB between 0 g and −80 g/d with uCP supply of ≥ 2,523 g/d (~ 2,386 g/d of cows' uCP requirement). The authors observed no change in

DM intake for both CHO sources, but a tendency for an increase in the efficiency of MCP synthesis (g N/kg fermented organic matter) in starch- compared to fiber-rich diets at low RNB level. Besides, Fanchone et al. (2013) observed a numerical increase in the flow of absorbed amino acids from fiber- to starch-rich diets, and proposed improved urea-N recycling and the use of ammonia in diets rich in starch than fiber.

Except Moorby et al. (1996), both Cantalapiedra-Hijar et al. (2014) and Fanchone et al. (2013) were not in agreement with the *in vitro* outcomes of **Chapter 2** where negative effects of reduced RNB in the studied variables were more pronounced in diets containing a rapidly degradable CHO source. Therefore, it may be proposed that the effects RNB level and CHO source *in vitro* may not confirm the *in vivo* findings. A reason as suggested previously could be that the *in vitro* closed system cannot depict the continuous removal of N from the rumen due to ammonia-N absorption and outflow of microbial and feed CP that caused N deficiency, particularly for rapidly degradable CHO source. Regardless, the *in vitro* and the *in vivo* work in the present thesis highlighted that the effect of the RNB level is influenced by dietary CHO and N sources.

5.3 Relationship between microbial crude protein synthesis and rumen nitrogen balance

A major factor that affects the efficiency MCP synthesis (g/kg CP intake) is the availability of N (relative to requirements of rumen microbes) for CHO fermentation (NRC, 2001). A positive RNB indicates an excess N in the rumen that leads to an increased urinary N excretion synthesized from ammonia-N (Reynolds and Kristensen, 2008). In contrast, a negative RNB may indicate inadequate microbial growth and synthesis of MCP. Nevertheless, rumen microbes may be able to utilize available N more efficiently under a

lack of N supply in the rumen, as this shortage of N to the rumen microbes can be compensated by urea-N recycling (Riemeier, 2004). As a result, Figure 5.1 describes a negative relationship between MCP synthesis (g/kg CP intake) and RNB (g/kg DM), suggesting that dairy cows are able to maintain their MCP synthesis even at low RNB level. Therefore, the German protein recommendation system that aims to feed diets with a RNB = 0 g/d to avoid N shortage or surplus to the rumen microbes (GfE, 2001), may be difficult to acknowledge. The fore-mentioned may be suggested because the recommendation of RNB = 0 g/d disregards the capacity of cattle to recycle dietary N and does not explore the potential of the rumen microbes and host animals in presence of reduced dietary N supply. Besides, there is certainly scope of reducing the recommendation of RNB 0 g/kg DM (also g/d) as seen in Figure 5.1 where the RNB threshold may be considered between -2 to -3 g/kg DM. Likewise, in the present thesis negative effects on intake, MCP synthesis, and MY at low RNB level (−65 g/d) were not seen in each of the dietary treatments. Not only but also, a positive RNB does not increase the degradation and synthesis capacity of the microbes in the rumen (Riemeier, 2004). Therefore, as observed in Figure 5.1 and as suggested by Huhtanen and Hristov (2009) there is a need to account for (variable) urea-N recycling in the protein evaluation systems to reduce an overestimation of RDP requirements of the cattle.

However, the potential of reducing the N supply to the rumen microbes up until the maintenance of MCP synthesis certainly needs to be explored together with dietary factors such as diet composition (*i.e.*, N and energy source, their degradation rate) and their synchronization. The fore-mentioned may be suggested because dietary factors at reduced RNB may alter rumen microbial community, which could lead to greater bacterial predation by the protozoa and therefore influence the intra-rumen N recycling. Besides, other factors

like fiber fractions that specifically increase the physically effective fiber in the diets may be explored, as increased fiber fractions may promote chewing activity (eating and ruminating time and chews) by simulating saliva secretion to enhance N recycling via the rumino-hepatic pathway and thereby support rumen fermentation and MCP synthesis at a low RNB level.

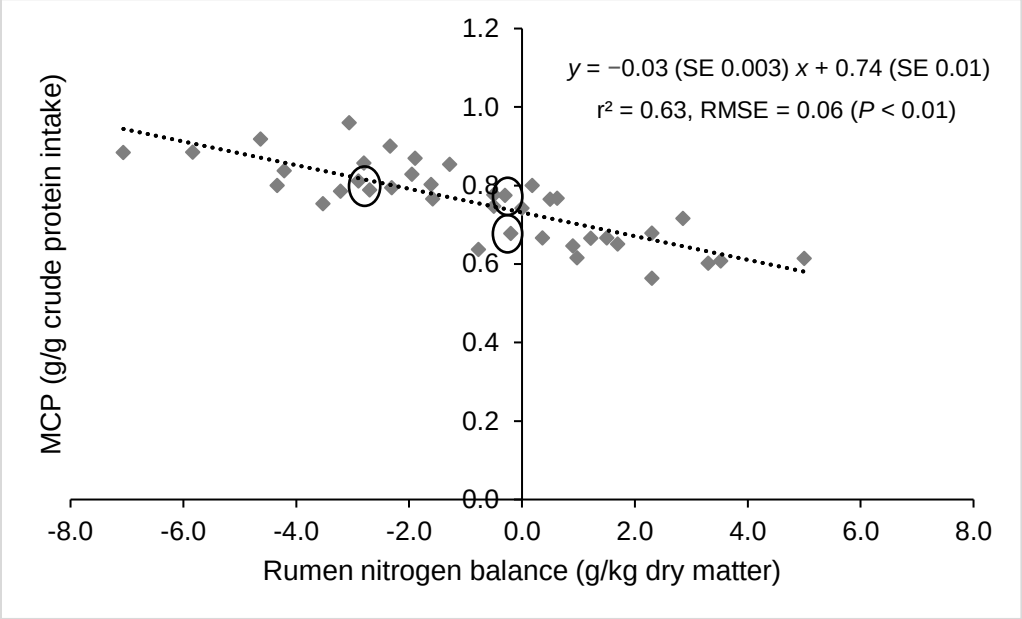

Figure 5.1 Relationship between microbial crude protein (MCP) synthesis and rumen nitrogen balance (n = 39).

SE, standard error; RMSE, root mean square error. Source: Riemeier (2004), Steinwidder et al. (2009), Afzalzadeh et al. (2010), Aschemann et al. (2012b), Chibisa and Mutsvangwa (2013), Fanchone et al. (2013), Cantalapiedra-Hijar et al. (2014), Mutsvangwa et al. (2016), and Chapter 3 and 4. Circled points represent the dietary treatments studied in Chapters 3 and 4.

5.4 Interaction effect of rumen nitrogen balance level, protein source, and lactation stage of dairy cows

In **Chapters 3 and 4**, 24 lactating cows were divided into four groups during the study. All four groups of cows for each dietary treatment had a similar (arithmetic mean ± standard

deviation) MY (35.2 ± 5.9 kg/d), days in milk (DIM; 120 ± 83 d), lactation number (2.8 ± 1.7 lactations), and body weight (681 ± 44 kg) before the start of the experiment. Depending on the group means, the dietary requirements of the cows were calculated. With an aim to incorporate almost the entire lactation stage as well as constraints in the availability of cows for the trial, cows from the different lactation stages were included in the study. As a result, the included cows in different lactation stages received similar uCP (g/kg DM) within each diet irrespective of their actual requirements. Yet, due to the variation in intake, the uCP (g/d) at the duodenum may have been different between the cows. Although the average uCP supply from all diets exceeded the average uCP requirements of the cows (average of the cows from all lactation stages) in the study for maintenance and lactation of 3,040 g/d calculated according to GfE (2001) (see **Chapter 3)**, it may be worthwhile to explore if the supplied RDP and uCP to the cows were sufficient particularly at early-mid-DIM. Therefore, to further investigate the effect of dietary treatments on satisfying the uCP requirements of the cows when in different lactation stages, these cows were grouped into early-mid-DIM (≤ 190 DIM) and late-DIM (> 190 DIM) (see Table 5.2).

Significant differences in DM (kg/d) and N intakes (g/d), and milk N and fecal N excretion (both in g/d) between early-mid-DIM compared to late-DIM were observed for dietary treatments, possibly because the uCP supplied to the late lactating cows was beyond their requirements compared to early-mid lactating cows. For instance, the DM intake was greater in cows in early-mid-DIM compared to those in late-DIM ($P \leq 0.02$), due to an increase in MY ($P < 0.01$, MY data not shown), while no difference was found in DM intake between the RNB levels and protein sources in late-DIM ($P \geq 0.09$). An effect of interaction was found between RNB, protein source, and DIM for rumen MCP synthesis (g/kg DM intake) ($P < 0.01$). The MCP synthesis reduced in RNB- compared to RNB0 level for FB diets in early-

mid-DIM; however, no difference was observed in MCP synthesis between the RNB levels for FB diets in late-DIM. Additionally, an interaction effect between RNB, protein source, and DIM was observed for milk N (P = 0.04). A comparison showed a reduced milk N (g/d) in RNB- compared to RNB0 level for the SP diet in late-DIM, but not between the RNB levels for the SP diet in early-mid-DIM. A reason for no change between RNB levels for the SP diet in early-mid-DIM cannot be well inferred, but it may be assumed that low N supply to the rumen microbes and host animals were compensated by increasing urea-N recycling and by greatly reducing urinary N excretion. Despite lower N intake in late lactation, no significant difference in the magnitude of excretion between the dietary treatments in early-mid-DIM compared to late-DIM was observed, hinting that urine N excretion expressed as a proportion of N intake was greater in late-DIM than early-mid-DIM.

From the above-described results, it may be concluded that the supplied uCP to the late lactating cows at reduced RNB (~ −65 g/d) was enough to maintain MCP synthesis even in diets containing FB source, and rather, the cows excreted surplus dietary N. Accordingly, Kluth et al. (2003) studied the effect of positive RNB (50 g/d) in high-yielding cows under a sufficient supply of uCP at the duodenum and suggested that cows in late-DIM were able to tolerate extra N deficit compared to those in early-mid-DIM. Therefore, there may be a potential to further reduce the uCP supply (g/d) and feed a negative RNB diet than those studied in the present thesis to late lactating cows. Along the line, there may be an opportunity to help farmers to reduce the costs of overfeeding the dietary protein without any compromise on MY. Besides, as the uCP requirements of the animals certainly change with DIM, a single dietary RNB threshold may not be appropriate for the lactating cows in different stages of lactation, as this may escalate economic costs and has negative animal health implications.

Table 5.2 Effects of rumen nitrogen balance (RNB), protein source (Faba bean grain and SoyPass), and days in milk (DIM) on intake, microbial protein synthesis (MCP), and nitrogen (N) excretion in lactating dairy cows studied in Chapter 3 (least square means, n = 24 cows).

Variables	Early-mid-DIM (≤ 190 d)[1]				Late-DIM (> 190 d)[1]				SEM[3]	*P*-value[4]			
	Faba bean grain		SoyPass		Faba bean grain		SoyPass			RNB	Prot[2]	DIM	RNB x Prot x DIM
	RNB0[4]	RNB-[4]	RNB0	RNB-	RNB0	RNB-	RNB0	RNB-					
DMI[2] (kg/d)	25.6^{a}	24.4^{b}	25.3^{a}	25.4^{a}	23.2	22.5^{x}	23.3^{y}	22.9	0.64	< 0.01	0.16	0.02	0.51
MCP (g/kg DMI)	129^{a}	98^{b}	125^{a}	117^{a}	122	110	111	120	5.98	< 0.01	0.36	0.79	< 0.01
N balance (g/d)													
N intake	654^{b}	501^{d}	706^{a}	615^{c}	592^{b}	463^{d}	648^{a}	553^{c}	16.1	< 0.01	< 0.01	< 0.01	0.02
Milk N	187^{a}	171^{b}	190^{a}	193^{a}	147^{bcx}	138^{cy}	158^{a}	147^{b}	7.01	< 0.01	< 0.01	< 0.01	0.04
Fecal N	243^{a}	233^{b}	244^{a}	253^{a}	218^{b}	202^{cy}	236^{a}	215^{bcx}	8.47	< 0.01	< 0.01	0.02	0.04
Urine N	215^{b}	96.1^{d}	256^{a}	160^{c}	214^{b}	112^{d}	235^{a}	178^{c}	8.48	< 0.01	< 0.01	0.73	< 0.01

^{a,b,c,d}Means with different superscripts in the same row within the same DIM differed significantly at $P < 0.05$.
^{x,y}Means with different superscripts in the same row within the same DIM tended to differ at $P \geq 0.05$ to < 0.10.
Means with no superscripts in the same row within the same DIM do not differ significantly at $P < 0.05$.
[1]Early-mid-DIM, mean ± standard deviation 107 ± 40 d; late-DIM, mean ± standard deviation 257 ± 44 d.
[2]SEM, standard error of the mean; Prot, protein; DMI, dry matter intake.
[3]The main effect of RNB level, protein source, DIM, and their interactions (n = 24 cows) were tested for the variables using mixed model (SAS; PROC MIXED) with random effect of animal in the group.
[4]RNB0, RNB in the diet at 0 g/kg dry matter; RNB-, RNB in the diet at -3.2 g/kg dry matter.

5.5 Challenges and limitation of present thesis and future prospects

5.5.1 Differences in utilizable crude protein supply across the dietary treatments

In **Chapters 3 and 4**, the uCP supply from all the diets exceeded the uCP requirements of the cow for maintenance and lactation; yet, the uCP and total dietary CP supply were greater in SP compared to FB diets. Nevertheless, as elaborated in **Chapter 3**, measures were taken during ration formulation of the *in vivo study* to assure for iso-energetic diets, identical forage to concentrate ratio, and similar RNB levels across the dietary treatments, with FB and SP accounting for a great share of the total dietary CP. Yet, due to considerable differences in CP concentration and degradability between FB and SP sources, the total CP concentration of all dietary treatments differed. Similarly, in **Chapter 2**, the basal ration contained 1 g of hay in all the diets, with similar RNB levels achieved across the diets containing various CHO and N sources. Yet, due to considerable differences in the degradability between the studied treatments, urea (particularly in cellulose diets) and casein accounted for a greater share of the total dietary CP compared to other treatments. As a result of alterations in the CP supply and therefore uCP supply (both *in vitro* and *in vivo*) between the dietary treatments, differences may have occurred in MCP synthesis (**Chapters 2 – 4**), urea-N recycling, and MY (**Chapter 3 and Chapter 4**). Overall, the results of the present thesis suggest that RNB cannot be considered exclusively for the differences in the dietary treatment, as uCP supply to the animals in proportion to their requirements is equally important when formulating the ration of the dairy cows. The above may be proposed, because, even at a similar RNB level (for example, RNB- diets with FB and SP), uCP supply (g/d) to cow depending on the dietary feed can be entirely different. Therefore, for prospective studies, similar uCP supply to the animals across the dietary treatment is

necessary to understand the detailed influence of low RNB to the rumen microbes at various dietary CHO and N sources.

5.5.2 Indirect technique for estimation of microbial crude protein synthesis

In **Chapter 3**, MCP synthesis was estimated using purine derivative (PD) excretion. The use of PD excreted in the urine is one of the most used methods to estimate microbial flow to the duodenum (Tas and Susenbeth, 2007; Ahnert et al., 2015), and is an alternative method to avoid complications and concerns associated with animal welfare (Shingfield, 2000). Besides, difficulties are also associated with *in vivo* measurements using fistulated animals for duodenal or omasal sampling (Firkins et al., 2006). For instance, variation in the digesta sampling and flow, markers for digesta flow, and concerns on the ratios of marker to N concentrations in rumen microbes are observed with the *in vivo* measurements using fistulated animals (Shingfield, 2000). Moreover, a limited number of fistulated animals always results in a compromise in the sample size of the study, with a challenge whether to consider a reduced sample size with fistulated animals or a large sample size with non-fistulated animals.

Errors associated in the synthesis of MCP estimation using PD for example, the feed purines that may escape rumen degradation, a shift in the purine concentrations in microbial mass, endogenous excretion of PD (Dickhoefer et al., 2015) and, a constant purine-N to the total-N ratio (Pérez et al., 1998) are well described in **Chapter 3**. Moreover, the measures taken to reduce possible errors during MCP estimations are also elaborated in **Chapter 3**. Yet, the response of rumen microbes to low N supply, CHO fermentation, and digesta passage rate on purine-N to total-N ratio cannot be completely ignored. As a result,

estimated MCP synthesis obtained from PD was compared with an alternative non-invasive approach of milk odd-branched chain fatty acids concentrations in **Chapter 4**, indicated as biomarkers for MCP synthesis in the rumen (Vlaeminck et al., 2015). Nevertheless, the comparison showed only a moderate relation between MCP synthesis from PD and odd-branched chain fatty acids in the present thesis and also in other studies (Castro-Montoya et al., 2016; Westreicher-Kristen et al., 2020).

Consequently, a few inaccurate assumptions associated with the PD method to estimate the microbial N flow, a moderate relation of milk odd-branched chain fatty acids concentrations and MCP synthesis, and constraints associated with direct measurements *in vivo* underline the pressing need for enhanced and reliable non-invasive methods. In this view, there is an increasing interest recently by researchers (Schwarm et al., 2009; Schuba et al., 2017; Wassie, 2019) to estimate rumen MCP synthesis through fecal metabolic N by calculating the difference between total and dietary fecal N, following the methodology of Mason (1969) developed a few decades ago. Likewise, estimation of MCP synthesis using empirical equations based on the estimation of fermentable energy available in the rumen is lately being explored by feed protein evaluation systems as described by Hristov et al. (2019) that seems promising, yet, needs further attention.

5.5.3 Long-term effects of reduced dietary rumen nitrogen balance in dairy cows

Numerous studies have assessed the effects of reduced dietary N supply to rumen microbes (*i.e.*, low RNB) on urinary N excretion, N partitioning, MY, and milk composition. Additionally, negative effects of surplus dietary CP intake on animal health particularly on the decrease in uterine pH during the luteal phase that may reduce fertility (Elrod and Butler,

1993), reduction in the pregnancy rate (Canfield et al., 1990), an increase in blood urea-N, which affects ovarian follicular and embryo development (Sinclair et al., 2000), and lameness (Sinclair et al., 2014) have been studied. Yet, far less emphasis has been placed on long-term effects of low dietary CP on reproductive parameters. A few studies on reduced dietary CP supply to the cows have been reported such as (i) increased risk of retained placenta and metabolic disease (Curtis et al., 1985), (ii) decrease in body fat mobilization in early lactation (Cadórniga and López Díaz, 1995), (iii) negative energy balance that implies a key factor driving poor fertility (Law et al., 2009), and (iv) reduction in MY as also observed in the present thesis. Despite the above-mentioned negative effects, a confirmation in contemporary high-yielding cows is necessary, where high-yielding cows may be more sensitive to N deficiency compared to low-yielding ones. Therefore, as highlighted in **Chapter 3**, long-term studies with reduced RNB on variations in urea-N recycling, health, and reproduction of dairy cows are required, to assess a minimum RNB estimate and change the dietary RNB recommendation to capture the ecological and economical implications at herd and farm system level. Accordingly, an opportunity to improve the efficiency of N use and performance of dairy cows by using a holistic approach that accounts for state-of-the-art genomic selection to improve genetic merit, herd management strategies, and their interacting factors may also be reviewed.

5.5.4 Practical implications

The results obtained from the present thesis underline that other factors such as dietary composition (namely CHO and protein sources), extent and rate of rumen CP degradation, and intake and performance level of the animals indeed affect the minimum RNB level

required for the efficient rumen fermentation particularly when a diet is fed as TMR for *ad libitum* intake to the dairy cows. However, some questions continue to remain unexplored, for instance, if observed effects of RNB level and various dietary sources (those differing in rate and extent of degradation) in the TMR based diets would be similar (as **Chapter 3 and Chapter 4**) when cows are grazing or fed only grass silage based diets or fed partial mixed rations, whilst the concentrate feed is fed in the milking parlor later during the day. One may only assume that feeding individual dietary feed and at specific time intervals may influence CHO degradation, microbial growth, and MCP synthesis and thereby minimum RNB level. However, factors such as farm and grassland management and the ability of ruminants to conserve N when deficient may play a crucial role. Additionally, persuading the farmer to account and perform changes regularly during ration formulation in the dietary RNB and uCP supply of the cows, particularly when feeding various dietary sources, remains a challenge. Furthermore, differences in the feeding behavior of the individual animal, variation within the same herd for the competition of resources, and their capacity to adapt to changing dietary conditions are difficult to understand and interpret. Therefore, instead of animal level, a holistic approach to understand the N-balance at herd or farm level could be helpful to recognize the N-input, N-output, and N-surplus, for instance, at a specific boundary system (*i.e.*, at the farm-gate level). With this approach, the farmers will also be able to maintain their farms in an entrepreneurial manner and reduce their N-surplus to remain within the permitted N usage limits according to European regulations.

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Chapter 6

6. General conclusions

Present thesis and literature support that reducing dietary rumen nitrogen balance in high- yielding dairy cows can improve nitrogen use efficiency and reduce the risk of nitrogen emissions into the environment. This can be implied, as even at a low rumen nitrogen balance level (~ −65 g/d) negative effects on dry matter intake, microbial protein synthesis, and milk production were not seen with each of the dietary treatments. Nevertheless, the maximum reduction potential of rumen nitrogen balance varies with diet composition (including carbohydrate and nitrogen sources), extent and rate of rumen crude protein degradation, and intake and performance level of the dairy cows. Therefore, outlining a single minimum rumen nitrogen balance threshold for dairy cattle diets is not reasonable. Besides, the supply of utilizable crude protein relative to the requirements of the animals should be considered, to account for differences in nitrogen recycling while deriving recommendations for optimum rumen nitrogen balance levels in diets of dairy cows.

Contrasting *in vitro* and *in vivo* findings on the effects of interaction between rumen nitrogen balance level and nitrogen source (*i.e.*, rapidly and slowly degradable protein source) emphasizes that current rumen *in vitro* system and various laboratory methods may not be sufficiently equipped to exploit the full potential of estimating dietary utilizable crude protein supply and rumen nitrogen balance. Therefore, there is a need for validated and standardized laboratory procedures, to simulate *in vivo* conditions at low rumen nitrogen supply in a closed system, particularly because the minor differences in utilizable crude protein and rumen nitrogen balance become relevant when expressed in g/d in high-yielding dairy cows.

Additional *in vivo* studies are needed to understand the detailed impact of negative rumen nitrogen balance and various dietary carbohydrate and nitrogen sources on the altered

rumen microbial community and bacterial predation by the protozoa, particularly when similar utilizable crude protein is supplied to the dairy cows. Furthermore, a long-term study would be useful to comprehend the impact of negative rumen nitrogen balance on the health and reproduction of dairy cows, as high-yielding cows may be more sensitive to reduced nitrogen supply compared to low yielding cows. Alternatively, challenges associated with individual animal variation within the same herd for the competition of resources and their capacity to adapt to changing dietary conditions may suggest adopting a holistic approach to understand the nitrogen balance at herd and farm system level to fully capture economic and ecological implications of the farm.