Impact of Physically Effective Fiber on Protein Metabolism in Dairy Cows

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To my beloved mother, my sisters, and my brother

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TABLE OF CONTENTS

Table of Contents

SUMMARY

Summary

Fiber is a prerequisite in the nutrition of dairy cows, because it promotes rumen motility and mat formation, while stimulating chewing activity (i.e. eating and ruminating). Chewing and salivation ensure a stable rumen environment for the microbial symbionts by providing an even release and recycling of nutrients as well as by buffering the end products of rumen fermentation, and thus reducing the risk of acidosis. The concept of physically effective neutral detergent fiber (peNDF) has been introduced to evaluate the adequacy of fiber supply to dairy cows. The peNDF combines both, the physical and the chemical characteristics of fiber, namely the dietary particle size and the neutral detergent fiber concentration. Various studies have been conducted to understand the effects of peNDF, particularly those concerning rumen health issues and the prevention of acidosis. Although some studies have looked at the effect of peNDF on nitrogen (N) metabolism, no studies are known that have evaluated the impact of peNDF on the partitioning of N excretion in dairy cows. Given that the peNDF can be considered as that fraction of the feed that stimulates chewing and salivation in ruminants, it may enhance recycling of circulating N and therefore increase the efficiency of N use by the animal.

In this doctoral project, it was hypothesized that increasing the peNDF concentration of a diet stimulates chewing activity in dairy cows, thereby promoting salivation up to a certain peNDF concentration, after which chewing activity declines as a result of lower dry matter (DM) intake. It was further hypothesized that greater chewing activity and salivation may stimulate rumen N recycling and microbial protein synthesis (MPS), compensating for potential negative effects of reduced rumen-degradable crude protein supply on feed intake, nutrient digestibility, and performance of dairy cows. The present

dissertation uses the rumen N balance (RNB) to evaluate the availability of degradable crude protein in the rumen. Overall, three *in vivo* studies were conducted to investigate the proposed hypotheses.

The first study tested the effects of four graded peNDF concentrations of a total mixed ration (TMR) on the chewing activity of eight lactating dairy cows to evaluate whether the selected peNDF concentrations have an effect on chewing activity. The peNDF concentrations were adjusted by simply varying the mixing time of the feed mixer wagon (i.e. 28, 43, 58, and 73 min), thus affecting neither the ingredients in the diet, nor the nutrient concentrations. Results showed that marginal increases in peNDF concentrations increased linearly eating and total chewing time without limiting the DM intake or performance of the cows. Two contrasting mixing times tested in the first study were selected to be further evaluated in the second study.

The second study evaluated the effects of different combinations of two dietary peNDF concentrations and two RNB (RNB0: 0 g/kg DM and RNB–: – 1.5 g/kg DM) in a TMR fed to twenty lactating dairy cows. The peNDF concentrations were adjusted by using two mixing times of 28 and 58 min and the effects measured were chewing behavior, protein metabolism, and performance. Here, increasing dietary peNDF concentration decreased DM intake of cows independent of RNB. Although greater peNDF concentration promoted total chewing time, apparent total tract digestibility (ATTD) of nutrients and MPS concomitantly decreased with increasing peNDF concentration for RNB–. However, no difference between peNDF concentrations was observed for RNB0. Also, increasing peNDF concentration resulted in a greater proportion of ingested N being excreted via feces than via urine for RNB–, whereas no difference was again observed

for RNB0. Despite differences in intake and ATTD of nutrients, milk production and composition were similar across the four treatment combinations. Overall, the results showed that the effects of peNDF were more pronounced for RNB– than RNB0 and hence, the third study was conducted at a lower RNB.

The third study tested the effects of four different peNDF concentrations in a TMR fed to four rumen-fistulated lactating dairy cows. The peNDF concentrations were adjusted by using different mixing times (i.e. 15, 30, 45, and 60 min) and all TMR had a low RNB (– 2.1 g/kg DM). The effects measured were feed intake, ATTD, chewing activity, rumen fermentation, protein metabolism, digesta passage, and performance. Quadratic effects of peNDF concentration on nutrient intake and ATTD, total chewing time, and yield and efficiency of MPS were observed, which were all greater for both medium peNDF concentrations. Also, the proportion of ingested N excreted via urine was lower, and that secreted via milk was greater in diets with high, compared to low or medium peNDF concentrations. As in the second study, the performance of dairy cows was not affected by the change in peNDF concentration.

Overall, increasing the peNDF concentration did not always result in a reduction of DM intake by cows, which could be related to their stage of lactation and energy requirement. Total chewing time and total chews per kilogram of DM intake increased linearly with increasing peNDF concentration; however, the increment in chewing activity may have resulted only in a small increase in salivation and hence, in N recycling in the animal. Although, high peNDF concentration negatively affected the yield and efficiency of MPS and ATTD of nutrients at low RNB, there were no indications that these parameters had any effect on the yield or composition of milk produced by the cows.

There was also an interaction between the peNDF concentration and the RNB in the diets of dairy cows, with the effects being more pronounced at low RNB. Therefore, recommended peNDF concentration for dairy cows will vary with RNB and vice versa. The minimum RNB needed to sustain rumen functioning may differ depending on the dietary peNDF concentration. In this regard, more studies are needed for a better understanding of the interaction between RNB and peNDF concentration. Finally, offering dairy cows a slightly negative RNB diet at an optimum peNDF concentration may be a means of reducing the N emission from dairy milk production and hence, may provide economical and environmental benefits.

ZUSAMMENFASSUNG

Strukturwirksames Futter ist eine Grundvoraussetzung bei der Fütterung von Milchkühen, da es die Pansenmotilität und die Bildung der Faserschicht („Pansenmatte“) im Pansen fördert und gleichzeitig die Kauaktivität (Fressen und Wiederkäuen) der Kühe stimuliert. Das Kauen und der Speichelfluss sorgen für ein stabiles Pansenmilieu für die mikrobiellen Symbionten durch eine gleichmäßige Freisetzung und Wiederverwertung von Nährstoffen sowie für eine Pufferung der Endprodukte der Pansenfermentation. Somit reduziert sich das Risiko einer Pansenazidose bei Wiederkäuern. Das Konzept der „physikalisch effektiven Neutralen Detergentienfaser“ (peNDF) wurde in der Vergangenheit eingeführt, um bei Milchkühen eine ausreichende Versorgung mit strukturwirksamem Futter zu gewährleisten. Die peNDF kombiniert die chemischen und physikalischen Eigenschaften der Faser, nämlich die Konzentration und die Partikelgröße der Neutralen Detergentienfaser. Es wurden verschiedene Studien zur Wirkung der peNDF, insbesondere in Bezug auf die Pansengesundheit und zur Verhinderung einer Pansenazidose durchgeführt. Obwohl einige Studien den Effekt der peNDF-Konzentration auf den Stickstoff (N)-Stoffwechsel untersuchten, sind keine Studien bekannt, die den Einfluss von peNDF auf die Partitionierung der N-Ausscheidung bei Milchkühen erfassten. Da die peNDF als diejenige Fraktion des Futters identifiziert wurde, die bei Wiederkäuern das Kauen und den Speichelfluss stimuliert, könnte die peNDF auch die Verwertung von zirkulierendem N und damit die Effizienz der N-Nutzung durch das Tier steigern.

In der vorliegenden Arbeit wurde die Hypothese aufgestellt, dass eine Erhöhung der peNDF-Konzentration des Futters die Kauaktivität von Milchkühen stimuliert. Bis zu einer bestimmten peNDF-Konzentration wird dadurch der Speichelfluss gefördert, wird

diese überschritten, sinkt die Kauaktivität aufgrund einer geringeren Trockenmasse (TM)-Aufnahme. Darüber hinaus wurde postuliert, dass eine stärkere Kauaktivität und ein erhöhter Speichelfluss die N-Wiederverwertung im Pansen und die mikrobielle Proteinsynthese (MPS) stimulieren könnten. Dadurch könnte die Effizienz der N-Verwertung gesteigert und potenziell negative Auswirkungen einer reduzierten Zufuhr von im Pansen abbaubarem Rohprotein auf die Futteraufnahme, die Nährstoffverdaulichkeit und die Milchleistung von Milchkühen kompensiert werden. Die vorliegende Dissertation verwendet die ruminale N-Bilanz (RNB), um die Verfügbarkeit von abbaubarem Rohprotein im Pansen darzustellen. Insgesamt wurden drei *In-vivo*-Studien durchgeführt, um die aufgestellten Hypothesen zu untersuchen.

In der ersten Studie wurden die Auswirkungen von vier abgestuften peNDF-Konzentrationen einer totalen Mischration (TMR) auf die Fress- und Kauaktivität von laktierenden Milchkühen untersucht, um zu zeigen, dass die erhaltenen Konzentrationen einen Effekt auf die Kauaktivität bewirken können. Die peNDF-Konzentration wurde durch eine Änderung der Mischzeit des Futtermischwagens (28, 43, 58 und 73 min) variiert, wodurch weder die Inhaltsstoffe, noch die Nährstoffkonzentration im Futter beeinflusst wurden. Die Ergebnisse zeigten, dass geringfügige Erhöhungen der peNDF-Konzentrationen die Fress- und Gesamtkauzeit linear erhöhten, ohne die TM-Aufnahme und die Leistung von Milchkühen einzuschränken.

Daraufhin wurden zwei kontrastierende Mischzeiten für die zweite Studie ausgewählt, in welchen die Wirkung von zwei peNDF-Konzentrationen (angepasst über die Mischzeit: 28 und 58 min) und zwei RNB-Stufen (RNB0: 0 g/kg TM und RNB–: – 1,5 g/kg TM) in einer TMR auf das Kauverhalten, den Proteinstoffwechsel und die

Milchleistung bei zwanzig laktierenden Milchkühen untersucht wurde. Hier verringerte eine Erhöhung der peNDF-Konzentration des Futters die TM-Aufnahme der Kühe unabhängig von der RNB. Obwohl eine höhere peNDF-Konzentration die Gesamtkauzeit förderte, nahm für RNB– die scheinbare Gesamttraktverdaulichkeit (ATTD) von Nährstoffen und die MPS mit steigender peNDF-Konzentration ab. Kein Unterschied konnte zwischen den peNDF-Konzentrationen für RNB0 beobachtet werden. Auch führte eine steigende peNDF-Konzentration dazu, dass bei RNB– ein größerer Anteil des aufgenommenen N über den Kot als über den Urin ausgeschieden wurde, wobei für RNB0 wiederum kein Unterschied zu beobachten war. Trotz unterschiedlicher Aufnahme und ATTD der Nährstoffe waren die Milchproduktion und die Milchzusammensetzung bei den vier Behandlungskombinationen nicht signifikant unterschiedlich. Insgesamt zeigten die Ergebnisse, dass die Effekte von peNDF bei RNB– ausgeprägter waren als bei RNB0. Daher wurde die dritte Studie bei einer niedrigeren RNB durchgeführt.

Die dritte Studie untersuchte die Auswirkungen von vier peNDF-Konzentrationen einer TMR, angepasst über die Mischzeit (15, 30, 45 und 60 min) und bei einer niedrigen RNB (– 2,1 g/kg TM), auf die Futteraufnahme, ATTD, Kauaktivität, Pansenfermentation, Proteinstoffwechsel, Verdauungspassage und Leistung von vier laktierenden pansenfistulierten Milchkühen. Es wurden quadratische Effekte der peNDF-Konzentration auf die Aufnahme und ATTD der Nährstoffe, die Gesamtkauzeit, sowie die Menge und die Effizienz der MPS beobachtet. Hier wurden für die beiden mittleren peNDF-Konzentrationen die höchsten Werte bestimmt. Der Anteil des aufgenommenen N, der über den Urin ausgeschieden wurde, war niedriger und der Anteil des aufgenommenen N, der über die Milch ausgeschieden wurde, war höher bei hoher als bei

niedrigen und mittleren peNDF-Konzentrationen. Ähnlich wie bei der zweiten Studie wurde die Milchleistung von Milchkühen durch die Veränderung der peNDF-Konzentration nicht beeinflusst.

Insgesamt führte eine Erhöhung der peNDF-Konzentration nicht immer zu einer Verringerung der TM-Aufnahme der Kühe, was mit ihrem Laktationsstadium und dem Energiebedarf zusammenhängen könnte. Gesamtkauzeit und Gesamtanzahl der Kaubisse pro Kilogramm TM-Aufnahme stiegen mit steigender peNDF-Konzentration linear an. Die Zunahme der Kauaktivität führte möglicherweise nur zu einer geringen Zunahme des Speichelflusses und der N-Wiederverwertung durch das Tier. Obwohl eine hohe peNDF-Konzentration die Menge und die Effizienz von MPS und die ATTD von Nährstoffen bei niedriger RNB negativ beeinflusste, gab es keine Auswirkungen auf die Milchleistung und die Milchzusammensetzung der Kühe.

Zudem bestand eine Wechselwirkung zwischen der peNDF-Konzentration mit der RNB der Ration, wobei die Effekte der peNDF-Konzentration bei niedriger RNB ausgeprägter waren. Daher variiert die empfohlene peNDF-Konzentration für Milchkühe je nach RNB und umgekehrt. Die minimale RNB im Futter von Milchkühen, um die Pansenfunktion zu erhalten, könnte je nach peNDF-Konzentration im Futter variieren. Diesbezüglich sind weitere Studien für ein besseres Verständnis der Interaktion zwischen RNB und peNDF-Konzentration erforderlich. Eine leicht negative RNB-Ration mit einer optimalen peNDF-Konzentration bei Milchkühen kann zur Verringerung der N-Emission aus der Milchproduktion beitragen und somit wirtschaftliche und ökologische Vorteile bieten.

CHAPTER 1

GENERAL INTRODUCTION

1.1 GENERAL BACKGROUND

High-yielding dairy cows require a diet that supplies great amounts of readily degradable carbohydrates and protein for high milk production. These diets are usually provided as total mixed rations (TMR) in a chopped form to reduce the selection of single ingredients by the animals and to increase digesta passage rate and dry matter (DM) intake.

Due to the complexity of protein metabolism in ruminants and the economic risk associated with underfeeding protein, diets of dairy cows are still formulated with a considerable safety margin in the supply of total and rumen-degradable crude protein (CP) (VandeHaar and St-Pierre, 2006). Nonetheless, this leads to inefficiencies in the metabolism of protein and subsequently to high emissions of nitrogen (N) in feces and urine in dairy cows (Calsamiglia et al., 2010). Overall the milk N use efficiency (MNUE; g N in milk/kg N intake) is low in dairy cows and typically averages around 250 – 280 g milk N/kg N intake (Huhtanen and Hristov, 2009). It is well established in the literature that high-yielding dairy cows use N inefficiently by excreting around 72% of ingested N via urine and feces (Castillo et al., 2000). Overfeeding of protein leads to excessive excretion of urine N, which is a more labile form of N, as it is more susceptible to leaching and ammonia volatilization compared to fecal N (Huhtanen et al., 2008). The poor N utilization of dairy cows is a major contributor to N pollution of the environment (Castillo et al., 2000) and can result in increased feed costs and reduced profits. Excessive N intake has also been associated with impaired reproduction and may contribute to lameness in dairy cows (Sinclair et al., 2014).

As stated above, besides high amounts of protein, dairy cows' diets contain high amounts of readily degradable carbohydrates. This can lead to a variety of metabolic disorders such as acute or subacute ruminal acidosis (SARA), depressed fiber digestion, and depression of milk fat (NRC, 2001). Chewing plays a key role in increasing saliva secretion of dairy cows, and thus has an important role in buffering rumen pH, promoting proper rumen functioning for microbial digestion, and reducing the risk of SARA while maintaining high levels of feed intake in dairy cows (Beauchemin, 2018). In the context of formulating energy-dense diets for highly productive dairy cows, the concept of physically effective neutral detergent fiber (peNDF) as proposed by Mertens (1997) has received increasing attention in the past decades as a potential tool to evaluate the adequacy of fiber in dairy cows' diets. The peNDF amalgamates the chemical (i.e. neutral detergent fiber (NDF)) and the physical (i.e. particle size (PS)) characteristics of a feed or a diet because both variables, besides affecting the stratification of digesta in the reticulorumen, promote rumination and salivation in ruminants (Mertens, 1997).

Therefore, a primary challenge in dairy cows' feeding is to provide energy- and protein-dense diets without compromising animal health and performance by ensuring at the same time adequate fiber in dairy cows' diets.

1.1.1 The Concept of Physically Effective Neutral Detergent Fiber

Characteristics of Fiber

Fiber is described as the fraction of feed that is slowly digestible or indigestible (Mertens, 1997) and represents "the filling effect" of diets (Hall and Mertens, 2017). According to van Soest (1994), dietary fiber includes polysaccharides and associated

plant cell wall substances resistant to mammalian digestive enzymes. Fiber can be further classified into insoluble and soluble fiber (van Soest, 1994). Insoluble fiber includes hemicellulose, cellulose, lignin, and Maillard products, whereas soluble fiber includes pectin, gums, and galactans, which are water-soluble and are usually rapidly and completely fermented by microorganisms in the digestive tract (van Soest, 1994). Historically, the first method to determine fiber was the crude fiber, developed by (Henneberg and Stohmann, 1860, 1864) and included in the Weende or proximate system. Crude fiber describes the fraction of feed that is indigestible and includes cellulose, hemicellulose, and lignin (McDonald et al., 2011). The crude fiber was later further distinguished by detergent analyses for acid detergent fiber, acid detergent lignin, and NDF (van Soest, 1961; van Soest and Marcus, 1964). The NDF consists mainly of cellulose, hemicellulose, lignin, cutin, and ashes, the acid detergent fiber of cellulose, lignin, cutin, and ashes, and the acid detergent lignin of crude lignin and cutin (McDonald et al., 2011).

Balch (1971) and Sudweeks et al. (1981) proposed the total chewing time per kilogram of DM intake as a quantitative measure of the physical characteristics of feeds. Later on, the concept of effective NDF was developed for feeds that would maintain milk fat production (Mertens, 1997). Mertens (1997) further evolved the concept of effective NDF to peNDF; whereas effective NDF is related to the total ability of feed to replace forage or roughage in a ration while maintaining the milk fat content of cows, the peNDF is related to the physical characteristics of fiber that influence chewing activity and the biphasic nature of ruminal contents.

Principle of Physically Effective Neutral Detergent Fiber

Generally, dairy cows often show one or more types of metabolic disorder when the minimum fiber requirement is not met. This includes reduced total DM digestibility and milk fat content, displaced abomasum, and an increase in the incidence of ruminal parakeratosis, laminitis, and acidosis (Sudweeks et al., 1981). Moreover, cows fed diets with a sufficient amount of dietary NDF but lacking in coarse forage can also display similar metabolic disorders as those fed a diet deficient in dietary NDF (Weston and Kennedy, 1984; Fahey and Berger, 1988). Hence, it is important to ensure both, an adequate amount of NDF and coarse forage to ensure the cow's health. With this in mind, the concept of peNDF was developed to combine the physical and chemical characteristics of the fiber (Mertens, 1997). Applying this concept in diet formulation is believed to provide a potential tool to assess fiber adequacy in dairy cows' diets.

The peNDF of a feedstuff is defined as the product of its NDF concentration and its physical effectiveness factor (pef) (Mertens, 1997). The pef can range between 0, indicating that the NDF is not fully effective to 1, indicating that the NDF is fully effective in stimulating the chewing activity of ruminants (Mertens, 1997). Both factors are critical for retaining fiber in the rumen, affecting the dynamics of ruminal fermentation and passage, and stimulating rumination (Mertens, 1997). It was suggested that feedstuffs should be compared against a hypothetical standard that would result in a maximum duration of chewing per kilogram of NDF and for this Mertens (1997) suggested long grass hay containing 100 g NDF/100 g DM which was assigned the pef of 1.0. The pef of a feed or a diet can be calculated in various ways based on the method used.

Methods to Determine Physically Effective Neutral Detergent Fiber

There are several methods to determine the peNDF of feedstuffs which can be determined either by using animal response (i.e. chewing time) in combination with regression analysis or by using laboratory or on-farm sieve methods. The concept of peNDF is based on the hypothesis that only feed particles with a higher resistance to leaving the rumen can stimulate the chewing activity of animals and thus should be related to peNDF (Mertens, 1997). Poppi et al. (1985) suggested that particles longer than 1.18 mm have greater resistance to passing out of the rumen. Hence, Mertens (1997) proposed a system to estimate the peNDF in the laboratory from the dietary NDF concentration and the particles which are retained on a 1.18-mm sieve of the Ro-Tapp dry sieving device with vertical shaking. Here, pef was defined as the proportion of particles retained on the 1.18-mm sieve ($pef_{>1.18;}$ wt/wt). This method is based on three assumptions: (i) that the NDF concentration is uniform for all PS, (ii) that the chewing activity is similar for all particles retained on a 1.18-mm sieve, and (iii) that the fragility (i.e. ease of PS reduction) is similar among sources of NDF (Mertens, 1997).

Lammers et al. (1996) developed an on-farm method to determine peNDF of feed to mimic the laboratory-scale separator for forage PS that was specified by Standard S424 of the American Society of Agricultural Engineers. For Lammers et al. (1996), the larger particles were of interest, and thus a simple separator with two sieves (19.0- and 8.0-mm apertures), and a bottom pan was designed. Later this separator evolved into the Penn State Particle Separator (PSPS) with three sieves (19.0-, 8.0-, and 1.18-mm apertures) and a bottom pan (Kononoff et al., 2003) and in 2013, a 4.0-mm sieve replaced the 1.18-mm sieve (Jones and Heinrichs, 2016). The measurement of the peNDF using the PSPS has

been widely adopted on-farm, as it is simple and cost-effective. The pef of a feed by the PSPS method can be calculated as $pef_{>8.0}$, $pef_{>4.0}$, or $pef_{>1.18}$. accordingly (Jones and Heinrichs, 2016):

- $pef_{>8.0}$ = is the sum of material (wt/wt) retained on 19.0-and 8.0-mm sieves;
- $pef_{>1.18}$ = is the sum of material (wt/wt) retained on 19.0-, 8.0-, and 1.18-mm sieves (equals to $pef_{>1.18}$ according to Mertens (1997)); and
- $pef_{>4.0}$ = is the sum of material (wt/wt) retained on 19.0-, 8.0-, and 4.0-mm sieves.

The $peNDF_{>8.0,}$ $peNDF_{>4.0}$, and $peNDF_{>1.18}$ concentrations of a feed or a diet are defined as the product of its dietary NDF concentration and its $pef_{>8.0}$, $pef_{>4.0}$, or $pef_{>1.18}$, respectively (Jones and Heinrichs, 2016). The PS distribution obtained using the PSPS method can also be used to estimate the geometric mean (X_{gm}) of PS and the geometric standard deviation (S_{gm}) of PS (Kononoff et al., 2003). The PS of forages or total mixed rations (TMR) does not follow a normal distribution pattern but rather can be plotted as a straight-line distribution on a log-probability paper (Jones and Heinrichs, 2016). The X_{gm} of particle size is the particle diameter through which 50% cumulative weight of sample passes (ASABE, 2017).

To ensure the reproducibility of PSPS measurements, a protocol was made available for the use of PSPS which controls the shaking frequency (1.1. Hz or greater/min) and stroke length (17 cm) to ensure the reproducibility of measurements (Kononoff et al., 2003). According to Kononoff et al. (2003), small moisture loss due to drying may affect PSPS measurements, but these differences were small. Differences in measurements may

be due to decreased adhesion of small particles to large particles or, with advanced drying, due to shattering of brittle material during shaking (Kononoff et al., 2003). Moreover, complete drying before sieving will result in large differences in measurements (Kononoff et al., 2003). For practical reasons, it is not recommended to measure forage or TMR on constant sample moisture during field measurement (Kononoff et al., 2003). Nevertheless, partial drying of wet TMR may be useful to reduce the adhesion of small particles to large ones, to obtain the actual peNDF amount fed to animals (Kononoff et al., 2003).

Other methods to measure the PS of feedstuffs include the Z-Box (developed at the William H. Miner Agricultural Research Institute, Chazy, United States (Cotanch and Grant, 2006)) and the non-portable Wisconsin separator that is mechanically operated and uses a horizontal shaking motion (ASABE, 2017). The Z-Box uses 3.18- and 2.39-mm sieves and was designed to correlate with the proportion of particles retained on the 1.18-mm sieve of the PSPS (Beauchemin, 2018). Nonetheless, the PSPS has been accepted as the standard particle separation technique used in the dairy cattle nutrition industry.

Potential and Limitations of the Physically Effective Neutral Detergent Fiber Concept

Although the peNDF concept combines both the physical and chemical properties of a diet, the use of this concept as a routine method to determine the fiber adequacy of diets has not been established and current feed tables do not consider the physical characteristics of feeds (GfE, 2001; NRC, 2001). Different methods in determining the peNDF and in feed preparation have made it difficult to compare studies and to evaluate and compare the effects of peNDF on different animal responses.

A limitation of the peNDF concept is the different methods used to measure peNDF. Most studies use the PSPS, either with two or three sieves (original with 1.18-sieve or new with 4.0-mm sieve). For all these cases a uniform distribution of NDF throughout the particle fractions across sieves is assumed. Hence, to increase the precision, the NDF concentration of each particle fraction can be measured separately on each sieve of the PSPS and included in the calculation of the peNDF. Nevertheless, some studies (Rustomo et al., 2006; Zebeli et al., 2008b) have shown that both these methods gave similar rankings. Thus, given that the measurements of $peNDF_{>8.0}$ and $peNDF_{>1.18}$ are more practical and cost-effective than the measurements of $peNDF_{>8.0\text{-}NDF}$ ($peNDF_{>8.0}$ considering the fractional NDF concentration of particles on 19.0- and 8.0-mm sieves) and $peNDF_{>1.18\text{-}NDF}$ ($peNDF_{>1.18}$ considering the fractional NDF concentration of particles on 19.0-, 8.0-, and 1.18-mm sieves), the two former have been recommended to be sufficient in feed formulation.

Another reason is the varied mixing procedures used to prepare the TMR which gives rise to difficulties in including peNDF in ration-balancing software. Heinrichs et al. (1999) reported that the PS of forages before preparing the TMR differs from the PS of the TMR containing the same forage. The actual PS of the fed TMR depends on various factors related to processing, mixing, and delivery of TMR to cows and the grain PS (Heinrichs et al., 1999). Lammers et al. (1996) and Heinrichs et al. (1999) proposed the use of the PSPS to fractionate the TMR into various particle fractions and to measure feed samples on an as-fed basis. Nonetheless, a standardized method of mixing the TMR is important for a better comparison between studies especially as it is assumed that the sieving results are equal when expressed on an as-fed or DM basis. Dietary moisture

content, however, affects the PS distribution of the PSPS across sieves, as observed by Ranathunga et al. (2010).

Moreover, the inconsistency in results observed across different studies (Beauchemin et al., 2003; Kononoff and Heinrichs, 2003a; Yang and Beauchemin, 2007, 2009), makes it hard to find strong evidence of the relationship between the peNDF and rumen pH, risk of SARA, and digestibility of fiber and hence to draw conclusions on the effects of peNDF in dairy cows. This may be explained by the differences in the composition of concentrates and various degrees of fermentability of the grains included in the concentrate mixture (Zebeli et al., 2006; Tafaj et al., 2007). The fermentability of forages and the proportion of forage in the diet are also factors affecting the effect of peNDF on rumen fermentation (Yang and Beauchemin, 2009). As summarized by Zebeli et al. (2012), the concept of peNDF does not take into account the fermentability of forages and concentrates. This is regardless of the fact that the digestibility and the fragility of forages are known to affect rumination (Mertens, 1997).

Via eating and ruminating, the peNDF is believed to increase the salivary buffer production which affects the ruminal pH and fermentation pattern with this also being an indicator for SARA (Mertens, 1997). Nevertheless, as different researchers use different pH threshold values to define SARA (pH 5.6, 5.8, or 6.0) and as the time below this threshold value to cause SARA has not been clearly defined, it is difficult to quantitatively characterize the effects of peNDF on ruminal fermentation and thus make it a tool to prevent SARA (Zebeli et al., 2010). This could account for researchers' reluctance to make recommendations on the optimal dietary peNDF concentrations for dairy cows.

Recommendations for Physically Effective Neutral Detergent Fiber

Mertens (1997) recommended a minimum dietary peNDF concentration of 220 g/kg DM, as calculated from total chewing time associated with NDF intake of various feedstuffs, to maintain a rumen pH of 6.0, and a dietary peNDF of 200 g/kg DM to maintain a milk fat content of 34 g/kg milk in early to mid-lactating Holstein cows. In contrast, Zebeli et al. (2006) estimated a minimum $peNDF_{>1.18}$ concentration of 190 g/kg DM to be sufficient to maintain an average rumen pH of 6.0. Furthermore, Zebeli et al. (2012) proposed an amount of 312 g $peNDF_{>1.18}$/kg DM or 148 g $peNDF_{>8.0}$/kg DM (using PSPS technique) to minimize health disturbances in dairy cows though a concentration beyond these values in the diet may potentially lower the DM intake of dairy cows (Zebeli et al., 2008a). Thus, the concentration of $peNDF_{>8.0}$ between 149 – 185 or $peNDF_{>1.18}$ between 300 – 330 g/kg DM is regarded as optimal to minimize the risk of SARA while maintaining production responses in high-yielding dairy cows (Zebeli et al., 2008a).

Estimates of peNDF concentrations of diets differ depending on the techniques used for their determination. Several studies have also demonstrated that factors such as forage to grain ratio (Rode et al., 1985; Yang and Beauchemin, 2009), grain source (Beauchemin and Rode, 1997; Beauchemin et al., 1997), and grain processing (Krause and Combs, 2003; Yang and Beauchemin, 2004) as well as the amount and fermentability of starch contained in the grain affect the requirements of cows for peNDF (Silveira et al., 2007).

Physiological Effects of Physically Effective Neutral Detergent Fiber in Dairy Cows

The effects of peNDF with regard to both its chemical and physical characteristics on the animal are complex and involve the intake and feeding behavior (i.e. feeding rate and sorting), chewing behavior (i.e. eating and ruminating), ruminal mat formation, and rumen motility (Zebeli et al., 2012). These in turn affect the passage rate of solid and liquid digesta, ruminal fermentation, nutrient intake and absorption, and the extent to which they all contribute to the animal's health and performance (Zebeli et al., 2012). When Mertens (1997) first defined the concept of peNDF, he related the peNDF to the characteristics of fiber that affect the chewing activity and the ruminal mat formation, with the ruminal mat being a critical factor for the selective retention of fiber in the rumen. Furthermore, the peNDF determines the passage of liquid and solids and the dynamic of ruminal fermentation as well as rumination. and with this, optimizes the ruminal microenvironment (Zebeli et al., 2012). For example, increasing dietary PS resulted in a greater fractional passage rate of liquid (i.e. proportion of liquid that leaves the rumen per unit time) due to increased saliva flow and rumen motility (Krause et al., 2002). Increasing the PS also decreases solid passage rate which increases retention time in the rumen and promotes microbial degradation of feedstuff and greater digestion by the animals (Pino et al., 2018).

Aside from the fiber concentration of a diet, the dietary PS can hamper DM intake as a result of distension of the reticulorumen or other compartments of the gastrointestinal tract in high-producing dairy cows fed high forage diets (Allen, 2000). Hence, decreasing the dietary PS may increase DM intake if the density of ingested particles or rumination increases (Allen, 2000). The effect of dietary PS on the DM intake is also affected by the

forage proportion in the diet (Beauchemin et al., 1994). Numerous studies have evaluated the effects of peNDF on intake with inconsistent observations reported. Feeding greater dietary PS decreased feed intake of dairy cows in some studies (Kononoff and Heinrichs, 2003a; b), but not in others (Krause et al., 2002; Krause and Combs, 2003; Maulfair et al., 2011). This supports the findings by Allen (2000) that in high-producing dairy cows, the physical ruminal fill is not the only limiting factor of intake when fed large amounts of concentrate (> 500 g/kg diet DM).

Similar to the effect of peNDF on intake, there is no clear consensus among the published literature on its effects on digestibility. While some studies (Kononoff and Heinrichs, 2003a; Yang and Beauchemin, 2005) reported that increasing PS increased the digestibility of DM, others (Kononoff and Heinrichs, 2003b; Maulfair et al., 2011) observed a decline in DM digestibility with increasing dietary PS, or no effect of dietary PS on DM digestibility (Krause et al., 2002; Yang and Beauchemin, 2006). Differences among these studies are attributable to various dietary factors such as forage level and source, concentrate source, and fermentability.

Although variable effects have been reported for the effects of peNDF on intake and digestibility, most published literature (Yansari et al., 2004; Yang and Beauchemin, 2006) confirms that forage PS does not affect the milk yield of dairy cows. Some studies (Kononoff and Heinrichs, 2003a; Yansari et al., 2004) observed an increase in DM intake; however, an expected increase in milk yield could not be observed. Zebeli et al. (2012) attributed this to the short experimental period (21 days) of studies on peNDF. It is known that long dietary PS increases the risk of potential selective feeding by dairy cows, as

these long dietary PS are easily discernible and often rejected in favor of short particles (Miller-Cushon and DeVries, 2017). Thus, reducing the dietary PS has been shown to improve daily distribution and intake of peNDF, which was discerned in greater milk protein and fat content (Zebeli et al., 2008b). A greater milk fat content indicates an improved environment of rumen microbes resulting in improved efficiency of fiber degradation, one of the major factors that affect milk composition, particularly the milk fat content of dairy cows (Mertens, 1997).

Although some studies evaluated the effects of peNDF either varied by forage to concentrate ratio and/or PS on the N metabolism in dairy cows, the focus of these studies was on the rumen microbial protein synthesis (MPS). Although peNDF was reported to affect the MPS yield, inconsistencies were found on its effect on the efficiency of MPS (Yang et al., 2002; Krause and Combs, 2003; Li et al., 2020). Increasing peNDF concentrations have been shown not to affect (Yang and Beauchemin, 2005; Li et al., 2020) or increase (Yang et al., 2002) MPS efficiency (g/kg organic matter (OM) truly digested in the rumen), or to decrease (Krause and Combs, 2003) MPS efficiency (g/kg digestible OM intake). The X_{gm} of particles in the study by Krause and Combs (2003) were much smaller compared to the other reported studies and it was concluded that reducing the dietary PS increased the passage of small particles and with it attached microbes from the rumen.

Overall, the peNDF concept has been mainly discussed with respect to rumen health issues, especially to preventing SARA and to the impact on rumen carbohydrate

metabolism. Despite its links to rumen N turnover and protein metabolism, the effect of peNDF on the partitioning of N excretion in dairy cows has not been researched so far.

1.1.2 Nitrogen Utilization in Dairy Cows

Optimizing Nitrogen Supply of Dairy Cows' Diets

As previously discussed, the MNUE is particularly low in high-yielding dairy cows. Approaches to reducing N emission from dairy production associated with nutrition includes (i) reducing dietary N concentration by feeding animals according to their physiological stage (precision feeding), (ii) decreasing ruminal protein degradation, and (iii) synchronizing fermentable energy and N availability in the rumen to improve the efficiency of MPS (Reynolds and Kristensen, 2008). As pointed out by Vyas and Amaro (2020), improving the N efficiency to reduce urinary and fecal N losses and to enhance urea-N entry into the rumen and usage by microbes are the primary goals of the ruminant nutritionist. Huhtanen and Hristov (2009) concluded that the CP concentration of the diet is the major dietary factor influencing MNUE, and so reducing dietary CP is an important means to increase the efficiency of N use. Broderick (2003; 151, 167, and 184 g CP/kg DM) and Ruiz et al. (2002; 94, 111, and 141 g CP/kg DM) observed a lower partitioning of N excretion towards urine and greater towards feces in dairy cows with reduced dietary N concentration, which would decrease the environmental burden of dairy farming. Indeed, in the face of increasing production and environmental costs with increasing dietary N supply, reducing the CP concentration of dairy cows' diets presents the ideal approach.

The German and the French Protein Evaluation Systems include the recycled-N from the rumino-hepatic cycle to estimate N supply in the rumen (INRA, 1989; GfE, 2001), whereas the British and the Dutch Protein Evaluation Systems (AFRC, 1993; Tamminga et al., 1994) do not take recycled N from the rumino-hepatic cycle into consideration Accordingly, the German Protein Evaluation System considers up to 20% of microbial synthesized protein originating from recycled urea-N, with the French System including up to 9% of microbial synthesized protein to be derived from N recycling (INRA, 1989; GfE, 2001).

Besides considering an optimal supply of utilizable CP (uCP) at the duodenum, the GfE (2001) also considers the rumen N balance (RNB) in its feed formulation, with the RNB defined as the difference between dietary CP intake and uCP intake divided by 6.25 (GfE, 2001). The RNB is a measure which indicates whether the N supply to rumen microorganisms is adequate to ensure maximal MPS and fermentation of OM without overfeeding protein (GfE, 2001). It is calculated by the difference of feed CP entering the rumen and uCP leaving the rumen and should be in the range of 0 to 50 g/d. Moreover, uCP is defined as the sum of microbial CP and undegraded feed protein at the duodenum (Lebzien and Voigt, 1999). To avoid potential shortcomings in the amount of recycled N at low N supply from the diet, the GfE (2001) recommends a balanced RNB of 0 g/kg DM up to an RNB of 50 g/kg DM. Nevertheless, a positive RNB excludes the possibility of using recycled urea-N of the rumino-hepatic pathway, and thus high emission of N is inevitable.

To exploit the inherent ability of the ruminant to recycle unused-N and to increase the N use efficiency of dairy cows, several studies have investigated the potential in providing

dairy cows diets at negative RNB (König et al., 2005; Lebzien et al., 2006). According to Lebzien et al. (2006), negative effects of N deficiency on rumen fermentation and MPS in dairy cows occur at an RNB below – 0.3 g/MJ ME, whereas an RNB of – 0.3 g/MJ ME complies with the GfE (2001) with 20% of microbial protein derived from recycled urea-N and the efficiency of MPS amounting 10.1 g/MJ ME (equal to 1.6 g/MJ ME). The greatest amount of protein at the duodenum was reported at balanced RNB (0 g/MJ ME), with no further increase in MPS with a more positive RNB (Lebzien et al., 2006). Other studies have reported no negative effects on intake and milk yield for an RNB of – 28 g/d (– 1.4 g/kg DM; van de Sand et al., 2006) and – 37 g/d (– 1.9 g/kg DM; König et al., 2005). Moreover, Holthausen et al. (2000) observed no negative effect of reduced RNB of – 94 g/d (– 5 g/kg DM) on intake, milk production and milk protein percentage compared to balanced RNB (0 g/kg DM). Other studies, however (Riemeier, 2004; Steinwidder et al., 2009), reported depressed DM intake and milk production in dairy cows when offered diets with an RNB between – 0.5 to – 7.0 g/kg DM (– 11 to – 106 g/d).

Rumen Nitrogen Recycling

The recycling of N to the rumen is an advantage ruminants have evolved to cope with asynchronous carbohydrate and protein supply or absorbable protein supply to animals in times of protein deficiency (Reynolds and Kristensen, 2008). Around 40 to 80% of urea produced in the liver may enter the gastrointestinal tract in both cattle and sheep (Harmeyer and Martens, 1980). Recycling of N to the rumen occurs in two ways, via saliva and by diffusion from the blood through the rumen wall (Reynolds and Kristensen, 2008). The amount of urea-N recycled into the rumen via salivation and the rumen wall

is dependent on the amount of urea synthesized in the liver, and thus on the amount of N ingested and the digestibility of dietary N (Piccione et al., 2006).

During the process of eating and rumination, urea-N can re-enter the rumen and contribute to the rumen urea-N pool (Maltby et al., 2005). Urea accounts for 60 to 70% of the total N in the saliva (Somers, 1961), with the amount of salivary urea-N delivered into the rumen is dependent on the plasma urea concentration and the total saliva production (Huntington and Archibeque, 2000). In ruminants, saliva may account for 15 to 94% of the urea-N recycled in the rumen (Huntington and Archibeque, 2000) and may vary with the amount depending on dietary factors such as the forage level and dietary source (Stewart and Smith, 2005). Salivary urea transfer to the rumen, calculated as the difference between total splanchnic flux and urinary excretions rate as a percent of total hepatic urea-N production, was observed to be greater in steers (72%) fed high forage diets than when fed high concentrate diet (21%), which may be explained by reduced saliva production for the latter (Huntington, 1989). Nevertheless, saliva production was not investigated in the study by Huntington (1989). Diets high in roughage stimulate chewing and therefore promote saliva secretion into the rumen.

Therefore at reduced dietary CP concentration, the delivery of urea into the rumen via saliva is thought to be of increased importance (Hobson et al., 1982). Additionally, greater saliva secretion due to chewing may increase the liquid passage rate. Krause et al. (2002) observed a greater ruminal liquid outflow rate, presumably due to increased saliva secretion as a result of higher chewing activity. An increase in liquid outflow rate may lower ruminal ammonia concentration and thus increase the demand for recycled urea-N.

In general then, increasing the total chewing time (i.e. eating and rumination) may contribute to increased N recycling and the provision of more N for microbial maintenance and growth. As dietary peNDF is defined as the fraction of feed that stimulates chewing behavior, it would be worth investigating whether increasing dietary peNDF to stimulate chewing activity (i.e eating and ruminating) while simultaneously reducing rumen N supply may be a tool to increase the N use efficiency in ruminants and especially in dairy cows.

1.2 APPROACH TO INCREASE NITROGEN EFFICIENCY IN DAIRY COWS

Ensuring optimal fiber and protein supply to dairy cows is important to meet their requirements and to maximize milk production while maintaining rumen health and function. With the increasing concern for reducing N emissions from milk production which has arisen in the past couple of decades, increasing the N use efficiency of dairy cows remains one of the major goals for dairy nutritionists. As environmental concerns force reductions in dietary CP concentration, the salvage of recycled urea-N by rumen microbes for synthesis may play a more critical role in efforts to enhance N use efficiency and concomitantly reduce N emissions from dairy farming.

The main approach proposed in this thesis is to increase the efficiency of use of N by cows by maximizing the salvage of recycled urea-N through the stimulation of their chewing activity and salivation. Since dietary peNDF apparently stimulates the chewing activity of ruminants, it would be worth investigating how a change in a cow's chewing activity as a result of varying dietary peNDF concentrations may alter the N metabolism of ruminants. The concept of peNDF has been widely explored with regard to its effect

on rumen health, but no study has yet explored any possible interaction between dietary peNDF concentration and the rumen N supply as a potential tool to increase the MNUE of dairy cows.

This thesis aims to examine how chewing behavior (i.e. chewing time and number of chews) affects the N metabolism of dairy cows, specifically their N use efficiency and partitioning of N excretion. Potentially confounding effects that may have been caused by varying the nutrient composition of any proposed diet were avoided. Hence, when changing the peNDF concentration of a diet, only the dietary PS was varied while the nutrient composition (i.e. NDF concentration) was kept constant. Additionally, the question arises whether the effect of chewing behavior on N metabolism is dependent on the rumen N supply, with the RNB being the parameter used in this thesis to represent the availability of degradable CP in the rumen. Under slightly rumen N limiting conditions (negative RNB), the effect of chewing activity on the N metabolism may be more pronounced. Moreover, it may compensate for the possible negative effects of low RNB on animal's performance.

Thus, the two major hypotheses of this doctoral dissertation are:

I. Increasing peNDF concentration of a diet stimulates chewing activity (i.e. total chewing time and chews) and thus promotes salivation up to a certain peNDF concentration after which chewing activity declines as a result of lower DM intake.

II. Greater chewing activity and salivation may stimulate rumen N recycling and MPS, and compensate for potential negative effects of reduced rumen N supply on intake, digestibility, and performance of dairy cows.

The dissertation is divided into three major parts:

1) Pre-studies related to the techniques used.
2) and 3) Two *in vivo* studies both aimed at investigating the research questions posed by this thesis.

In short, a pre-study on the effect of PSPS operation and dietary moisture content on measurements of peNDF was evaluated and are described in **Chapter 2A.** The effects of the mixing time of a total mixed ration in a feed mixer wagon on dietary peNDF concentration and consequently on chewing behavior and performance were tested in a small *in vivo* study with eight lactating dairy cows and is described in **Chapter 2B**.

Chapter 3 evaluates the interaction between dietary peNDF concentration and RNB (i.e. balanced and low RNB) and its effect on chewing behavior, protein metabolism, and performance in an *in vivo* study with twenty lactating dairy cows.

Finally, the effects of graded dietary peNDF concentration under N limiting conditions on chewing behavior, ruminal fermentation, passage rate, protein metabolism, and performance were investigated in an *in vivo* study with four rumen-fistulated lactating dairy cows and is described in **Chapter 4**.

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CHAPTER 2A

EFFECTS OF OPERATOR, SAMPLE MOISTURE CONTENT, AND METHOD OF DETERMINATION ON PENN STATE PARTICLE SEPARATOR MEASUREMENTS

2.1 ABSTRACT

The aim of this study was to validate the technique of measuring physically effective neutral detergent fiber (peNDF) with the Penn State Particle Separator (PSPS). The peNDF concentrations of a total mixed ration (TMR) were determined using two ($peNDF_{>8.0}$ particle separators fitted with 19.0- and 8.0-mm sieves) and three ($peNDF_{>1.18}$ particle separators fitted with 19.0-, 8.0-, and 1.18-mm sieves) sieves. The effects of the operator (i.e. which person was operating the machine), the sample moisture content, and some aspects of the methodology used (i.e. uniform vs. actual distribution of neutral detergent fiber (NDF) concentration throughout particle fractions) on PSPS measurements were evaluated. Two different moisture contents (i.e. as collected vs. 2 h drying) and three different PSPS operators were evaluated. Also two mixing times of TMR in the feed mixer wagon (i.e. 35 and 55 min) were used to achieve two different particle size (PS) distributions. Of one operator, material retained on each sieve was sampled to determine its NDF concentration followed by the calculation of $peNDF_{>8.0\text{-NDF}}$ and $peNDF_{>1.18\text{-NDF}}$, which are modified $peNDF_{>8.0}$ and $peNDF_{>1.18}$ concentrations which takes into account the NDF concentration of particle fractions (i.e. fractional NDF) of the two and three sieves of the PSPS, respectively. The peNDF concentrations were then compared between the two methodologies, i.e. uniform vs. fractional NDF concentration of the TMR. Data were analyzed using a MIXED model.

The operator affected the measurements leading to a difference in PS distribution within the PSPS. The $peNDF_{>8.0}$ concentration of the TMR differed between operators, but the $peNDF_{>1.18}$ concentration was similar between operators. Reducing the moisture content of the sieved TMR shifted the PS distribution of the diet leading to less material

retained on the top sieve and more material retained on the lower two sieves and in the bottom pan. Although the peNDF$_{>8.0}$ concentration decreased with drying, the peNDF$_{>1.18}$ concentration remained unaffected. This may be due to the fact that peNDF$_{>1.18}$ includes a greater proportion of particles in the diet making this parameter less likely to be affected. Furthermore, both the peNDF$_{>8.0}$ and peNDF$_{>1.18}$ concentrations calculated using the uniform NDF concentration of the TMR were slightly underestimated in comparison to the peNDF$_{>8.0\text{-NDF}}$ and peNDF$_{>1.18\text{-NDF}}$ calculated using the fractional NDF concentration. Nonetheless, the differences were small.

According to present results, it is recommended to use only one operator when conducting a study or for on-farm use. It would also be advisable to specify the moisture content at sieving when the diet or feedstuff is dried prior to sieving for a better comparison between studies. The peNDF method assuming a uniform distribution of NDF throughout particle fractions would be the more practical and cost-effective method as it omits additional sampling of material retained on each sieve and in the pan and their subsequent determination of NDF.

2.2 INTRODUCTION

Physically effective neutral detergent fiber (peNDF) is important in the diet of dairy cows in order to maintain and foster chewing activity (i.e. eating and rumination), rumen function, and overall animal health (Mertens, 1997). It combines the concept of the physical and chemical properties of fiber in feedstuffs, such as the particle size (PS) with the overall neutral detergent fiber (NDF) concentration (Mertens, 1997). The Penn State Particle Separator (PSPS) has been widely used to measure the peNDF of forages and total mixed rations (TMR). For on-farm measurements, it has been recommended for

practical reasons that samples should be measured using the PSPS as offered to animals, and thus without previous drying (Kononoff et al., 2003).

Wet feed samples may result in the retention of more material on the upper sieves, resulting in a higher estimated peNDF concentration of the feedstuff, attributable to the adhesion of small particles to larger ones. It has been observed that moisture loss in alfalfa haylage samples due to drying (moisture content from 574 to 356 g/kg fresh matter (FM)) affects neither the PS distribution measurements nor the estimated geometric mean (X_{gm}) of particle length (X_{gm} = 17.7 – 17.9 mm; Kononoff et al., 2003). Conversely, moisture loss in corn silage samples due to drying (moisture content from 580 to 344 g/kg FM) affected the determination of PS distribution and consequently the X_{gm} of particles, although these differences were small (X_{gm} = 12.1 – 11.2 mm; Kononoff et al., 2003). Complete drying (moisture content from 580 to 0 g/kg FM) for both, alfalfa haylage and corn silage, was observed to cause large differences in PS distribution associated with shattering of brittle particles during shaking and lastly affected the estimated X_{gm} of particles (X_{gm} = 17.7 – 10.3 mm for alfalfa haylage and X_{gm} = 12.1 – 8.6 mm for corn silage; Kononoff et al., 2003).

Kononoff et al. (2003) also tested the effects of three different sieving frequencies (0.9, 1.1, and 1.6 Hz) at a stroke length of 17 cm on PS measurements and reported differences among tested frequencies. While reducing shaking frequency from 1.1 to 0.9 Hz significantly increased material retained on the top sieve, no differences were observed between 1.1 and 1.6 Hz shaking frequency. Thus, Kononoff et al. (2003) recommended a shaking frequency of 1.1 Hz with the operator advised to calibrate the frequency of

movement over a distance of 17 cm prior to actual measurements. As the PSPS operator may change in the course of a study or on the farm, it should be evaluated whether different operators may result in differences in measurements and influence the obtained dietary peNDF values.

Often the peNDF concentration is estimated by assuming a uniform distribution of the NDF concentration throughout the different particle fractions retained on the sieves of the PSPS. To overcome this limitation, the NDF concentration of each particle fraction can be measured separately and considered in the calculation of dietary peNDF. Some studies (Rustomo et al., 2006; Yang and Beauchemin, 2006; Zebeli et al., 2008) have reported similar rankings of dietary peNDF calculated by the uniform or the varied distribution (i.e. fractional NDF) method. Therefore, as the former method using the uniform NDF of a feed is more practical on-farm and less costly than using the fractional NDF, this method is recommended (Zebeli et al., 2012).

The present study was conducted to establish the conditions for the subsequent *in vivo* studies (Chapters 2B, 3, and 4). The first aim was to evaluate whether more than one PSPS operator can be used during a study and whether the samples needed to be dried prior to sieving. For this the effects of different PSPS operators and sample moisture contents (i.e. as collected vs. with drying) on the PSPS measurements were evaluated. The second aim was to evaluate the different peNDF methods (i.e. uniform or fractional NDF) in order to find the most suitable measurement method for the subsequent *in vivo* studies. Importantly, the third aim was to evaluate whether the peNDF concentration of a diet can be adjusted by altering the mixing time in the feed mixer wagon.

2.3 MATERIALS AND METHODS

2.3.1 Diet and Sampling

The study was performed on a standard TMR offered to high-producing lactating dairy cows based on grass and corn silages (Table 2.1) with a forage to concentrate ratio of 55:45 (dry matter (DM) basis). The PS distributions were varied by using different mixing times (i.e. 35 and 55 min) of the TMR in the feed mixer wagon (Power Champ L, Marmix GmbH & Co. KG, Unterwachingen, Germany; Figure 2.1) which was equipped with a horizontal stir wing mixer fitted with blades. The ingredients were loaded into the mixer wagon in the following order: grass silage 1st cut, grass silage 2nd cut, concentrate and mineral mixtures, corn silage, grass hay 1st cut, barley straw, and grass hay 2nd cut. Detailed information on the TMR mixing is reported in the Appendix (Table A.1).

Samples for the PSPS measurements were taken on two alternate days. During each mixing time, six times 1.6 kg of sample material (as-fed basis) was collected in plastic bags which corresponded to two moisture contents (i.e. as collected vs. 2 h drying) per mixing time (i.e. 35 and 55 min) with each of the three operators determining the PS distribution of each combination (total of 12 treatments). Simultaneously, 300 g samples of the TMR as-fed were taken directly after 35 and 55 min of mixing time for chemical analyses of the TMR and were stored at – 20°C until further processing.

Figure 2.1 Feed mixer wagon Power Champ L (Marmix GmbH & Co. KG, Unterwachingen, Germany) (own source).

Table 2.1 Ingredient and chemical composition of the total mixed ration.

Variable	Total mixed ration
Ingredients, g/kg dry matter	
Corn silage	200
Grass silage, 1st cut	45.4
Grass silage, 2nd cut	104
Grass hay, 1st cut	99.8
Grass hay, 2nd cut	72.6
Barley straw	27.2
Concentrate mixture[1]	427
Beta-concentrate[2]	2.27
Sodium chloride[3]	3.63
Calcium carbonate[2]	8.17
Mineral-vitamin mixture[2,4]	9.07
Monosodium phosphate[2]	0.91
Magnesium oxide[2]	0.45
Chemical composition,[5] *g/kg dry matter*	
Dry matter, g/kg fresh matter	438
Organic matter	924
Crude protein	155
Neutral detergent fiber	380
Acid detergent fiber	198

[1]Composition (per kg dry matter): 250 g corn grain, 250 g rapeseed cake, 200 g winter barley grain, 200 g field bean grain, 100 g pea grain.
[2]Bergophor Futtermittelfabrik, Kulmbach, Germany.
[3]K + S Minerals and Agriculture GmbH, Kassel, Germany.
[4]Concentrate GM134 composition as-fed, according to manufacturer information: 200 g Ca, 40 g Mg, 50 g Na, 7 g Zn, 5 g Mn, 1.1 g Cu, 35 mg Se, 60 mg I, 20 mg Co, 250,000 IU Vitamin A, 65,000 IU Vitamin D, 5000 mg Vitamin E, and 120 mg Vitamin B7.
[5]Chemical composition: n = 2, except crude protein with n = 1.

2.3.2 Operation of Penn State Particle Separator

The PSPS with three sieves of 19.0-, 8.0-, and 1.18-mm aperture sizes was used in this study (Figure 2.2) and operated according to Kononoff et al. (2003) with slight modifications. The three sieves were stacked on top of each other on the bottom pan with the greatest aperture sieve on top and the least aperture sieve at the bottom.

Figure 2.2 Penn State Particle Separator with 19.0-, 8.0-, and 1.18-mm sieves and a bottom pan (own source).

To determine the PS distribution of each treatment combination, each sample material (i.e. as collected vs. 2 h drying) was divided into four subsamples of equal weight, where each subsample was spread out on the top sieve so that big chunks could be untangled. On a flat surface, the PSPS was then shaken horizontally five times in one direction with a stroke length of 17 cm. A forward and backward motion was considered as one shake. Then, the PSPS was rotated one-fourth turn and again shaken five times. This process was repeated for a total of 8 sets of five shakes resulting in a total of 40 shakes. After shaking of PSPS was completed, the material on each sieve and in the bottom pan was

weighed and the amounts recorded. The PSPS process was repeated another three times with the rest of the subsamples.

2.3.3 Operator and Sample Moisture Content

To test the effect of moisture content, samples were tested with or without prior drying. For oven-dried samples, three collected 1.6 kg (as-fed basis) TMR samples of each mixing time were spread out on individual aluminum pans and placed in a forced-air oven for 2 h at 45°C. The weight before and after drying was recorded to determine initial and final moisture content. After cooling at room temperature, the TMR samples were directly subjected to PSPS measurements.

To test the effect of the operator on the PSPS measurements, three operators (1 male and two females) were selected. Prior to shaking, the operator of the device calibrated the frequency of movement at a stroke length of 17 cm. The number of full movements divided by the time in seconds equals the shaking frequency value, which should be ideally 1.1 Hz (Kononoff et al., 2003). Each of the three operators determined the PS distribution of samples with and without oven-drying for both mixing times.

2.3.4 Method of Determination

To test the results of different peNDF calculations (i.e. uniform or fractional NDF), feed material retained on each sieve and in the bottom pan used by the male operator was further sampled for both moisture contents (i.e. as collected vs. 2 h drying) and feed mixing times (i.e. 35 and 55 min). Samples were stored at – 20°C until further processing when their DM and NDF concentrations were determined.

2.3.5 Chemical Analyses

Frozen samples of TMR and particle fractions obtained from the sieves were lyophilized for 48 h (LYO GT2 Basis, SRK Systemtechnik GmbH, Riedstadt, Germany) and ground to pass a 1-mm screen (SM 100, Retsch GmbH, Haan, Germany). Samples of TMR were analyzed in duplicate for DM, crude ash, nitrogen, NDF, and acid detergent fiber concentrations. Particle fractions were analyzed in duplicate for DM and NDF concentrations. The DM and crude ash concentrations were determined according to VDLUFA (2007; methods 3.1 and 8.1). The organic matter concentration (g/kg DM) was calculated by subtracting the crude ash concentration (g/kg DM) from 1000. The crude protein (CP) concentrations were estimated by multiplying the nitrogen concentrations by 6.25, which were determined by Dumas combustion using the vario MAX CN Element Analyzer (Elementar Analysensysteme GmbH, Hanau, Germany) according to VDLUFA (2007; method 4.1.2). The NDF and acid detergent fiber concentrations were determined using an Ankom^{200} Fiber Analyzer (Ankom Technology, Fairport, United States) following VDLUFA (2007; methods 6.5.1 and 6.5.2) with heat-stable α-amylase (Ankom Technology, Fairport, United States) and sodium sulfite (Merck KGaA, Darmstadt, Germany), expressed inclusive of residual ash.

2.3.6 Calculations

The recorded amount of material on each sieve and in the bottom pan after shaking was used to calculate the proportion of material retained on each sieve (wt/wt). The physical effectiveness factors (pef) were calculated by adding the proportion of material (wt/wt) retained on two ($pef_{>8.0}$; 19.0- and 8.0-mm) or three sieves ($pef_{>1.18}$; 19.0-, 8.0-, and 1.18-mm) (Jones and Heinrichs, 2016). The $peNDF_{>8.0}$ and $peNDF_{>1.18}$

concentrations were defined as the NDF concentration of the TMR multiplied by $pef_{>8.0}$ and $pef_{>1.18}$, respectively. To calculate $peNDF_{>8.0\text{-}NDF}$, the proportion of DM (wt/wt) retained on the top and middle sieves were each multiplied by the NDF concentration of the TMR retained on the respective sieves of the PSPS and then summed. Similarly, the $peNDF_{>1.18\text{-}NDF}$ was calculated by multiplying the proportion DM (wt/wt) retained on the top, middle, and lower sieves by the NDF concentration of the TMR retained on the respective sieves of the PSPS and summed. The X_{gm} of the PS of the TMR was then calculated for both, the uniform and the fractional NDF method, according to Jones and Heinrichs (2016).

2.3.7 Statistical Analyses

All data were analyzed using the general linear model procedure of SAS (V9.4, SAS Institute Inc., Cary, United States). To evaluate the effect of operator (i.e. 1 male and 2 females), moisture content (i.e. as collected vs. 2 h drying), and mixing times (i.e. 35 and 55 min), the model included operator, drying, mixing time, and day as fixed effects. To evaluate the effect of mixing time and drying on the two peNDF determination methods (i.e. uniform vs. fractional NDF), the model included drying, mixing time, and day as fixed effects. The effects were declared significant at $P < 0.05$, and trends were recognized at $0.05 \leq P < 0.10$. The differences between operators were evaluated with the Tukey's test at a significance level of $P < 0.05$. Descriptive statistics were used to compare the two peNDF determination methods (i.e. uniform vs. fractional NDF). All means are presented as least squares means.

2.4 RESULTS AND DISCUSSION

2.4.1 Effect of Operator

Kononoff et al. (2003) recommended a shaking frequency of 1.1 Hz with the operator advised to calibrate the frequency of movement over a distance of 17 cm prior to actual measurements. The PSPS operator often varies during a study or on-farm and thus the present study aimed at testing whether the measurements of the same TMR differ with operators. The PS distribution differed between the operators ($P \leq 0.02$, Table 2.2), resulting in a difference in the peNDF$_{>8.0}$ concentration ($P < 0.01$), but not in the peNDF$_{>1.18}$ concentration ($P = 0.42$). The peNDF$_{>1.18}$ concentration is less likely to be affected, as it considers the most particles in a diet (particles > 1.18 mm). Interestingly, one operator (i.e. operator 1 = male) stood out the most, with more material retained on the top sieve, and less material retained in the lower sieves and the pan. This resulted in higher values of pef$_{>8.0}$ and pef$_{>1.18}$, peNDF$_{>1.18,}$ and X_{gm} of PS for the respective operator ($P \leq 0.02$, for all variables). The force and the rate of shaking motion must be sufficient to allow for particles of small enough size to fall through (Kononoff et al., 2003).

Kononoff et al. (2003) reported an increased amount of material falling through the 1.18-mm sieve for corn silage when increasing the frequency from 1.1 to 1.6 Hz, without differences in the X_{gm} of PS observed, which might be attributable to the small size of corn silages. The increase in the shaking force increases the likelihood of particles falling through (Kononoff et al., 2003). It was observed in the present study, however, that if too much force was applied or the distance assigned for each movement was not complied with by the operator, longer forage particles only slid over the sieve surface and did not

fall through the apertures. This may have occurred for one operator (i.e. operator 1 = male) in the present study, as the other two operators did not show similar results.

Observations by the present author found that the greater force applied by the male operator resulted in less material falling through the upper sieve. Thus, small particles trapped in between or lying on top of the larger forage particles in the tested TMR may have been unable to pass through the sieves. Therefore, operators should be trained properly before conducting PSPS measurements, and only one operator should be assigned to the PSPS within one research study.

Table 2.2 Effects of operator, sample drying, and TMR[1] mixing time on Penn State Particle Separator measurements.

Variable	Operator (n = 4)			Drying (n = 6)		Mixing time (n = 6)		SEM[1]	*P*-value		
	1	2	3	0 h	2 h	35 min	55 min		Operator	Drying	Mixing time
Moisture, g/kg FM[1]	54.1	54.2	53.9	55.9	52.3	54.2	53.9	0.43	0.90	<0.01	0.60
Particle size distribution, g/100 g material retained on sieves											
19.0 mm	68.4[a]	45.2[b]	46.1[b]	58.1	48.4	54.9	51.5	2.66	<0.01	<0.01	0.18
8.0 mm	6.9[a]	14.1[b]	14.6[b]	11.0	12.8	11.4	12.4	0.85	<0.01	0.08	0.27
1.18 mm	20.0[a]	33.1[b]	32.1[b]	25.9	30.9	28.0	28.9	1.43	<0.01	<0.01	0.40
Pan	4.6[a]	7.6[b]	7.2[b]	4.9	8.0	5.8	7.2	0.55	0.02	<0.01	0.09
$pef_{>8.0}$[2]	0.75[a]	0.59[b]	0.61[b]	0.69	0.61	0.66	0.64	0.019	<0.01	0.17	<0.01
$pef_{>1.18}$[2]	0.95[a]	0.92[b]	0.93[b]	0.95	0.92	0.94	0.93	0.006	0.02	0.19	<0.01
$peNDF_{>8.0}$,[3] g/kg DM[1]	291[a]	229[b]	234[b]	267	236	261	241	7.6	<0.01	0.01	<0.01
$peNDF_{>1.18}$,[3] g/kg DM	368	357	358	367	355	373	350	3.9	0.42	0.01	0.14
X_{gm}[4] of particle size, mm	15.9[a]	10.1[b]	10.5[b]	13.7	10.7	12.7	11.7	0.70	<0.01	0.13	<0.01

[a,b] Means with different superscripts differed significantly at $P < 0.05$ between operators.
[1]DM = dry matter; FM = fresh matter; SEM = standard error of means; TMR = total mixed ration.
[2]$pef_{>8.0}$ and $pef_{>1.18}$ = physical effectiveness factor determined as the proportion of material retained on two (19.0- and 8.0-mm) and three (19.0-, 8.0-, and 1.18-mm) sieves of the Penn State Particle Separator, respectively.
[3]$peNDF_{>8.0}$ and $peNDF_{>1.18}$ = physically effective neutral detergent fiber determined as the dietary neutral detergent fiber concentration multiplied by $pef_{>8.0}$ and $pef_{>1.18}$, respectively.
[4]X_{gm} = geometric mean of particle size calculated according to Jones and Heinrichs (2016).

2.4.2 Effect of Sample Moisture Content

Measurement of the dietary PS of the feed offered to animals on an as-fed basis can be used to obtain the actual peNDF concentration. Thus it has been recommended that samples should not be chemically or physically altered prior to sieving (Kononoff et al., 2003). Nevertheless, small particles may stick to longer particles, especially with wet feed, resulting in more material being retained on the top sieve and preventing smaller particles from passing through. The moisture content of a feedstuff or diet may affect the PSPS measurements with regard to the PS distribution across the different sieves. Thus, the present study aimed to test how drying affects the PSPS measurement and consequently the dietary $peNDF_{>8.0}$ and $peNDF_{>1.18}$ concentrations.

Interestingly, a moisture loss of around 54 g/kg FM of the original sample due to 2 h of drying in the present study resulted in a shift in the PS distribution with less material retained on the top sieve and more on the 1.18-mm sieve and in the bottom pan ($P < 0.01$, Table 2.2). This shift in PS distribution resulted in a reduction in $pef_{>1.18}$, $pef_{>8.0}$, and consequently, dietary $peNDF_{>8.0}$ concentration ($P < 0.01$). Furthermore, drying resulted in a reduction in X_{gm} of PS from 13.7 to 10.7 mm in the present study ($P < 0.01$, Table 2.2). Kononoff et al. (2003) tested the effect of sample moisture on alfalfa haylage and corn silage by drying samples at 55°C for five drying times within 48 h on PSPS measurements. The authors observed that a moisture loss from 574 to 356 g/kg FM (2 h drying) for alfalfa haylage resulted in no significant difference in PSPS measurements, whereas a moisture loss from 580 to 344 g/kg FM (3 h drying) for corn silage significantly increased particles < 1.18 mm and decreased the X_{gm} of PS. However, these differences

were small, so Kononoff et al. (2003) concluded that moisture loss of about 400 g/kg FM of the original sample will only result in small differences in PSPS measurements which is in line with the results in the present study.

The addition of water during the mixing of TMR resulted in increased adhesion of concentrate and small corn silage particles to longer forage particles and also promoted clumping of smaller particles. Based on personal observations in the present study, the decrease in the amount of material retained on the top sieve and the increase in the amount of material retained in the bottom sieve and the pan with drying was attributable to the lower adhesion of smaller particles to longer ones rather than increased fragility of TMR particles to shattering. Thus, the reduced $pef_{>1.18}$ and $pef_{>8.0}$ due to moisture loss in the samples could have been the actual $pef_{>1.18}$ and $pef_{>8.0}$ of the TMR fed to the cows. Lahr et al. (1983) increased the moisture content of a TMR by adding water but found this had no effect on the total chewing time or total chews when it was fed to dairy cows. Unfortunately, no dietary peNDF concentration was reported by Lahr et al. (1983) but it can probably be assumed that the TMR with the least addition of water represented the actual peNDF concentration fed to the cows.

Although moisture content of the sample may affect sieving properties, it has been recommended for practical reasons not to conduct the PSPS measurements at a standard moisture content (Kononoff et al., 2003). Nevertheless, short drying of samples to dismantle small particles from larger ones, without increasing the fragility of the sample may increase the accuracy of measurements especially for forages with high moisture content or TMR in which water was added. Regardless of whether drying was performed

before PSPS measurements or not, it would be advisable to specify the moisture content at sieving for better comparison between studies.

2.4.3 Effect of Mixing Time

Increasing the mixing time of the TMR in the feed mixer wagon from 35 to 55 min decreased the $peNDF_{>8.0}$ and $peNDF_{>1.18}$ concentrations of the TMR ($P < 0.01$, for both variables, Table 2.2). Although not a statistically significant amount, less material was retained on the top sieve and more on the lower sieves and in the bottom pan leading to numerically lower $pef_{>8.0}$ and $pef_{>1.18}$ values. Interestingly, when analyzed only for operator 1, increasing mixing time reduced $peNDF_{>8.0}$ ($P = 0.04$, Table 2.3), whereas $peNDF_{>1.18}$ was not affected ($P = 0.10$). This underlined the fact that management factors are more likely to affect $peNDF_{>8.0}$ than $peNDF_{>1.18}$ concentration.

Leonardi and Armentano (2003) tested the effect of two different PS of alfalfa hay (chopped vs. long) fed as a TMR to dairy cows. The chopped alfalfa hay was obtained by processing it solely for 15 min in a TMR wagon. Subsequently, the rest of the TMR ingredients were added and all mixed together in the TMR wagon. For the TMR containing long alfalfa hay, the alfalfa hay was added to the feed mixing wagon last and only mixed for 2 to 3 min to minimize the number of small particles. The study by Leonardi and Armentano (2003) used a different particle separator with five sieves and a pan, and the results showed that fewer particles were retained on the top two sieves (particles > 18.0 mm) and more retained on the lower two sieves (particles ≤ 5.61 mm) and in the pan. In comparison, the present study added water into the TMR after the last

forage component was added into the feed mixer wagon, which added another 9 min to the total duration of TMR mixing (see Appendix Table A.1). Thus, it is difficult to make direct comparisons between the mixing times of the two studies.

The mixing of a TMR varies with the forage ingredients and the desired dietary PS needed to achieve a homogeneous diet. A more prolonged mixing time than in the present study may not be possible as the TMR otherwise becomes too slushy and reducing the mixing time may result in a less uniform distribution of the feed components or particles of greater length. Thus, another possibility would be to change the order in which the ingredients were added into the feed wagon. Also, the time spent mixing after the addition of each ingredient affects the resulting peNDF$_{>8.0}$ and peNDF$_{>1.18}$ concentrations of the diet. Results in this study show that it is possible to achieve different dietary peNDF$_{>8.0}$ concentrations by varying the mixing time employed in the feed mixer wagon. Nonetheless, varying the peNDF$_{>1.18}$ concentration via the mixing time may only be possible if mixing times are further prolonged. This in turn will increase the risk of the TMR becoming too slushy to be fed to the animals. Even so, the following additional options may be used to vary the mixing time (i) changing the order of loading the ingredients into the feed mixer wagon, (ii) increasing the roughage proportion in the diet, or (iii) varying the theoretical chopping length of individual forages.

2.4.4 Method of Determination

One of the limitations of the peNDF concept is the assumption of a uniform distribution of NDF throughout the different particle fractions of a feed (Mertens, 1997). Various studies (Rustomo et al., 2006; Zebeli et al., 2008) evaluated the differences

between the two methods either by estimating the peNDF using the total NDF concentration of the TMR or forage, or using the fractional NDF concentration of the TMR or forage. Although the latter increases the precision in estimating the peNDF concentration of a TMR or forage, various studies have demonstrated that both methods gave similar rankings of peNDF concentration (Rustomo et al., 2006; Zebeli et al., 2008).

The present study aimed at re-evaluating the suitability of determining the $peNDF_{>8.0}$ and $peNDF_{>1.18}$ concentrations either by assuming a uniform distribution of NDF throughout the particle fractions or by taking into account the fractional NDF concentration of each particle fraction (i.e. $peNDF_{>8.0\text{-NDF}}$ and $peNDF_{>1.18\text{-NDF}}$). These differences were, however, small, and in the case of $peNDF_{>1.18}$, negligible. Similar to the $peNDF_{>1.18}$ concentration, the $peNDF_{>1.18\text{-NDF}}$ concentration was not affected by mixing time ($P = 0.18$, Table 2.3). Interestingly, in contrast to $peNDF_{>8.0}$ concentration ($P = 0.04$), $peNDF_{>8.0\text{-NDF}}$ concentration was unaffected by mixing time ($P = 0.13$), which may be attributable to a non-homogeneous sampling of the total TMR or the particle fractions affecting the subsequent NDF determination.

The lower concentrations of $peNDF_{>8.0}$ compared to $peNDF_{>8.0\text{-NDF}}$ may be due to the fact that the former method underestimated the NDF concentration of the particles greater than 8 mm, which is in agreement with observations made by Zebeli et al. (2008). Particles greater than 8 mm include mainly forages, and thus, the NDF concentration of this particle fraction is greater than the NDF concentration of the whole TMR. In agreement with the other studies (Rustomo et al., 2006; Zebeli et al., 2008), both methods gave similar rankings of the TMR on the $peNDF_{>8.0}$ and $peNDF_{>1.18}$ concentrations. Furthermore, compared to the peNDF method using fractional NDF, the peNDF

concentration with uniform NDF is more practical as it omits the additional sampling of material after PSPS sieving and its subsequent NDF analysis. Hence, for practical reasons, for on-farm use and for reducing costs, the method assuming uniformity of NDF concentration throughout particle fractions is more applicable compared to using the fractional NDF concentration.

Table 2.3 Effect of sample drying, method of determination, and TMR[1] mixing time on Penn State Particle Separator measurements (n = 4).

Variable	Mixing time				SEM[1]	*P*-value	
	35 min		55 min			Drying	Mixing time
Drying	0 h	2 h	0 h	2h			
Moisture, g/kg FM[1]	561	531	557	515	8.2	<0.01	0.60
$pef_{>8.0}$[2]	0.83	0.71	0.8	0.68	0.027	<0.01	0.15
$pef_{>1.18}$[2]	0.99	0.94	0.96	0.93	0.009	0.03	0.16
$peNDF_{>8.0}$,[3] g/kg DM[1]	325	279	302	257	11.3	<0.01	0.04
$peNDF_{>8.0\text{-NDF}}$,[4] g/kg DM	339	297	310	270	11.0	0.05	0.13
$peNDF_{>1.18}$,[3] g/kg DM	389	372	361	350	7.0	0.28	0.10
$peNDF_{>1.18\text{-NDF}}$,[4] g/kg DM	388	373	362	358	6.6	0.50	0.18

[1]DM = dry matter; FM = fresh matter; NDF = neutral detergent fiber; peNDF = physically effective neutral detergent fiber; SEM = standard error of means; TMR = total mixed ration.
[2]$pef_{>8.0}$ and $pef_{>1.18}$ = physical effectiveness factor determined as the proportion of material retained on two (19.0- and 8.0-mm) and three (19.0-, 8.0-, and 1.18-mm) sieves of the Penn State Particle Separator, respectively.
[3]$peNDF_{>8.0}$ and $peNDF_{>1.18}$ = peNDF determined as the dietary NDF concentration multiplied by $pef_{>8.0}$ and $pef_{>1.18}$.
[4]$peNDF_{>8.0\text{-NDF}}$ and $peNDF_{>1.18\text{-NDF}}$ = peNDF based on fractional NDF for the proportion of material retained on two (19.0- and 8.0-mm) and three (19.0-, 8.0-, and 1.18-mm) sieves of the Penn State Particle Separator, respectively.

2.5 CONCLUSIONS

Despite some minimal prior practice, the operator affected the PSPS measurements. The PSPS operator needs to be trained to maintain force and frequency while sieving.

Thus, it would be advisable to maintain one operator when conducting a study or for on-farm use. The tested moisture content affected the PSPS measurements, with a reduction in $peNDF_{>8.0}$ and $peNDF_{>1.18}$ concentrations with lower moisture content at sieving. So for better comparison between studies, the moisture content at sieving needs to be kept constant (when possible) and be specified when reporting. Mixing time can be used to achieve small differences in $peNDF_{>8.0}$ concentration. Greater differences in $peNDF_{>8.0}$ and $peNDF_{>1.18}$ concentrations might be achieved by changing the forage level or the order of loading the feed ingredients into the feed mixer wagon. Importantly, the PSPS operator and drying had a greater impact on the PSPS measurements than mixing time. As $peNDF_{>1.18}$ includes the most particle fractions in a diet or feed, this parameter is less likely to be affected by management factors. Thus, dietary $peNDF_{>8.0}$ concentration is recommended for evaluating the effect of management factors on the physical properties of a diet. Estimating the $peNDF_{>8.0}$ and $peNDF_{>1.18}$ concentrations using the total NDF rather than the fractional NDF concentration has been shown to slightly underestimate these values; however, these differences are rather small. The method that assumes a uniform NDF distribution is therefore regarded as the preferable method.

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CHAPTER 2B

THE EFFECT OF FEED MIXING TIME ON CHEWING BEHAVIOR AND PERFORMANCE OF LACTATING DAIRY COWS

2.7 ABSTRACT

The aim of this study was to test the effect of mixing time of a total mixed ration on the concentration of dietary physically effective neutral detergent fiber (peNDF) and subsequently on feed intake, chewing and feeding behavior, and milk production in lactating dairy cows. Eight Holstein cows were used in a 4 x 3 Youden square design during 3 periods of 4 days of adaptation followed by 4 days of data collection. Cows received a total mixed ration (forage to concentrate ratio of 54:46 on a dry matter (DM) basis) that was mixed in a feed mixer wagon for four different mixing times (28, 43, 58, and 73 min), to create four diets with varying peNDF concentrations. The following data were recorded from all animals: individual DM intake, milk yield, chewing behavior (eating and rumination times), and feeding behavior (trough visits/day and duration (min/trough visit)). All data were analyzed using a MIXED model and were tested for the linear and quadratic effects of mixing time.

Increasing mixing time linearly reduced the concentration of dietary peNDF. The DM intake, milk yield, and milk composition were similar across all diets. Eating and total chewing time (min/d and min/kg DM intake) as well as number of daily trough visits linearly decreased, whereas the rate of DM intake (g/min of trough visit) increased with decreased peNDF concentration due to a prolongation of mixing time. Such differences in chewing and feeding behavior may affect the diurnal nutrient availability to rumen microbes. Further research is needed to understand the effects of peNDF concentration on feed intake and behavior of cows and how these factors will affect nutrient utilization at rumen and animal level when neither ingredients nor proportions are changed.

2.8 INTRODUCTION

Dietary fiber plays a key role in maintaining rumen function and health, and subsequently, the performance of dairy cows. Nevertheless, cows offered diets rich in neutral detergent fiber (NDF), but low in long forage particles may develop the same metabolic disorders as cows offered diets deficient in NDF (Heinrichs and Konokoff, 1996). The concept of physically effective neutral detergent fiber (peNDF) amalgamates the physical (i.e. particle size) and chemical (i.e. NDF) characteristics of a diet (Mertens, 1997).

Besides the ingredient composition of the diet (e.g. forage species, forage to concentrate ratio), the type and state of equipment used for total mixed ration (TMR) mixing and distribution (Heinrichs et al., 1999) as well as the mixing time of TMR (Marchesini et al., 2020) largely dictate dietary particle size and thus peNDF concentration. Various studies have investigated the effects on dry matter (DM) intake, chewing behavior, and performance in cows when peNDF concentrations were modified by varying forage particle size and forage amount in the diet (Beauchemin et al., 2003; Yang and Beauchemin, 2007). Only a few studies evaluated solely the impact of different dietary particle size (Beauchemin and Yang, 2005; Marchesini et al., 2020), especially by varying the feed mixing time (Marchesini et al., 2020). Adjusting the peNDF concentration by changing the chemical composition of the diet (forage level and/or source) will add more factors which could mask the effects of dietary particle size on the tested parameters. The previous study (Chapter 2A) demonstrated that mixing time was an effective method for changing peNDF concentration while maintaining both the ingredients and their proportions in the diet and therefore its chemical composition.

The aim of the present study was to evaluate the linear or quadratic effects of different peNDF concentrations on DM intake, chewing and feeding behavior, and milk production in high-yielding dairy cows, the peNDF concentrations being changed simply by adjusting the dietary particle size by manipulation of the mixing time. It was hypothesized that increasing peNDF concentrations would increase chewing activity (eating and rumination time), but possibly decrease the animals' feed intake, resulting in a quadratic response of measured variables to dietary peNDF concentration.

2.9 MATERIALS AND METHODS

2.9.1 Animals and Housing

The present study was performed at the Meiereihof experimental farm of the University of Hohenheim (48°42'50.6"N and 9°13'03.0"E) between July and August 2018. Eight lactating Holstein cows (1 primi- and 7 multi-parous) were used with an average (arithmetic mean ± standard deviation) milk yield of 40.9 ± 5.60 kg/d, body weight of 703 ± 36.2 kg, and 197 ± 67.7 days in milk on day 1 of the study. Animals were assigned to four treatment groups equalizing mean milk production and days in milk. Cows were housed together in a free-stall barn, milked twice daily (05:00 and 16:00 h) in an auto-tandem milking parlor (Westfalia, GEA Farm Technologies, Bönen, Germany) and daily milk yield was recorded by in-parlor milk meters (Metatron P21, GEA Farm Technologies, Bönen, Germany).

Body weight was recorded once daily after morning milking using an automated walk-over-weighing system (GEA Farm Technologies, Bönen, Germany). A trough system (Waagen Döhrn GmbH & Co. KG, Wesel, Germany) automatically registered fresh

matter intake of each animal by a transponder system.

All procedures were performed in compliance with the ethical and animal welfare legislation (No. 401181016).

2.9.2 Study Design and Diets

The study was performed as a 4 x 3 Youden square with three periods of 4 days of adaptation followed by 4 days of data and sample collection (i.e. sampling period). Cows were offered a TMR based on corn silage and grass silage with a forage to concentrate ratio of 54:46 (on DM basis). The TMR was formulated to meet the utilizable crude protein (CP) and energy requirements of high-producing dairy cows according to the recommendations of GfE (2001) assuming 650 kg of body weight, 30 kg/d of milk yield, and 24 kg/d of DM intake.

Ingredient composition (Table 2.4) was the same as the diet normally offered to the lactating dairy cows of the experimental farm; thus, no prolonged adaptation time to the diet was included at the beginning of the study. Animal groups were assigned to one of the four TMR that were chemically identical but differed in particle size distribution due to the following different mixing times in the feed mixer wagon (Power Champ L, Marmix GmbH & Co. KG, Unterwachingen, Germany): 28, 43, 58, and 73 min. The mixer wagon was equipped with a horizontal stir wing mixer with blades that reduced dietary particle size with prolonged mixing times.

The ingredients were loaded into the mixer wagon in the following order: grass silage 1st cut, grass silage 2nd cut, molasses, concentrate and mineral-vitamin mixtures, corn silage, grass hay 1st cut, grass hay 2nd cut, and barley straw. The mixing protocol is given

in the Appendix (Table A.2). Diets were freshly prepared every morning and offered once daily for *ad libitum* consumption at 09:00 h. Cows had *ad libitum* access to fresh drinking water.

Table 2.4 Ingredients of the total mixed ration varied by feed mixing time.

Ingredients, g/kg dry matter	Total mixed ration
Corn silage	226
Grass silage, 1st cut	86.9
Grass silage, 2nd cut	37.7
Grass hay, 1st cut	88.5
Grass hay, 2nd cut	74.5
Barley straw	27.6
Concentrate[1]	420
Molasses[2]	14.9
Beta-concentrate[2]	2.30
Sodium chloride[3]	3.74
Calcium carbonate[2]	8.00
Mineral-vitamin mixtures[2,4]	8.92
Monosodium phosphate[2]	0.94
Magnesium oxide[2]	0.47

[1]Composition (per kg dry matter): 250 g corn grain, 250 g rapeseed cake, 200 g winter barley grain, 200 g field bean grain, 100 g pea grain.
[2]Bergophor Futtermittelfabrik Dr. Berger GmbH & Co. KG, Kulmbach, Germany.
[3]K + S Minerals and Agriculture GmbH, Kassel, Germany.
[4]Composition as-fed, according to manufacturer information: 200 g Ca, 40 g Mg, 50 g Na, 7 g Zn, 5 g Mn, 1.1 g Cu, 35 mg Se, 60 mg I, 20 mg Co, 250,000 IU Vitamin A, 65,000 IU Vitamin D, 5000 mg Vitamin E, and 120 mg Vitamin B7.

2.9.3 Diet and Milk Sampling

Samples of offered and refused diets were collected every morning immediately after and before feed distribution, respectively. Samples were collected from the two feed troughs of each treatment group, mixed, and one subsample (~ 300 g; as-fed basis) was stored at – 20°C until further processing. Two additional samples of offered diet (400 g each; as-fed basis) were collected from every feed trough every morning after feed

distribution and stored at 4°C until analysis of particle size distribution. Milk samples were taken on day 3 (afternoon) and day 4 (morning) of each sampling period. Samples were pooled per animal and period weighted by the respective milk yields at each milking, preserved with Bronysolv (ANA.LI.TIK Austria, Vienna, Austria), and stored at 4°C until analysis.

2.9.4 Chewing and Feeding Behavior

Chewing behavior of animals was recorded throughout the sampling period using automatic jaw movement recorders (RumiWatch System, Itin & Hoch GmbH, Liestal, Switzerland). Data was converted by the 24-h-resolution option using the RumiWatch conversion software V0.7.3.2 to obtain daily eating and rumination time (min/d) with their sum being defined as total chewing time (min/d). Daily data from each animal were averaged per period. During the study, data of some cows was lost due to malfunction of the RumiWatch devices, resulting in the following number of observations per treatment: n = 4 for 28 and 43 min, and n = 5 for 58 and 73 min of mixing time. Daily number of visits (visits/d) and duration of each visit (min/visit) of individual animals were registered by the trough system.

2.9.5 Determination of Particle Size Distribution

Samples of the offered diet were dried in a forced-air oven at 45°C for 2 h prior to sieving, a procedure that had been found necessary during a pre-study to reduce the adherence of smaller particles to bigger particles. Samples were weighed before and after drying, to determine the DM concentration at sieving. The particle size distribution was determined in quadruplicate using the Penn State Particle Separator (PSPS; Nasco, Fort

Atkinson, United States) device with three sieves (19.0-, 8.0-, and 4.0-mm) and a bottom pan (Figure 2.3). This procedure was done by just one PSPS operator. After shaking of the PSPS was completed, the material on each sieve and in the bottom pan was weighed and the amounts recorded.

Figure 2.3 Penn State Particle Separator (Nasco, Fort Atkinson, United States) with 19.0-, 8.0-, and 4.0-mm sieves and a bottom pan (own source).

2.9.6 Chemical Analyses

Frozen samples of offered and refused diets were lyophilized (LYO GT2 Basis, SRK Systemtechnik GmbH, Riedstadt, Germany) for 48 h and ground to pass a 1 mm screen (SM 100, Retsch GmbH, Haan, Germany). Samples were pooled by treatment and period by taking the same amount from each daily sample and analyzed for their chemical composition according to VDLUFA (2007). The DM, crude ash, and crude lipid concentrations were determined in duplicate (methods 3.1, 8.1, and 5.1.1, respectively). The organic matter concentration (g/kg DM) was calculated by subtracting crude ash concentration (g/kg DM) from 1000. The CP concentrations were estimated by

multiplying the nitrogen concentrations by 6.25, which were determined in duplicate by Dumas combustion (method 4.1.2) using the vario MAX CN Element Analyzer (Elementar Analysensysteme GmbH, Hanau, Germany). The NDF and acid detergent fiber were analyzed in duplicate using an Ankom^{200} Fiber Analyzer (Ankom Technology, Fairport, United States) and the amounts expressed inclusive of residual ash (methods 6.5.1 and 6.5.2). Heat-stable α-amylase and sodium sulfite were used for NDF analysis. Starch was analyzed with an enzymatic kit (Test-Combination No. 10 207 748 035, R-Biopharm AG, Darmstadt, Germany).

Digestible organic matter, metabolizable energy, and net energy of lactation contents were estimated in triplicate for 24 h in two *in vitro* incubation runs according to Menke and Steingass (1988). The utilizable CP concentrations in offered diet samples were estimated in triplicate using the modified Hohenheim gas test method from the ammonium concentrations in the inoculum according to Steingass et al. (2001). For this, diet samples were incubated for 24 h and two runs.

Milk fat, protein, lactose, and urea-nitrogen were analyzed in duplicate using Fourier-transform infrared spectroscopy (Bentley FTS, Bentley Instruments, Chaska, United States) 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.

2.9.7 Calculations

The total duration (min/d) of trough visits per day was calculated by multiplying the daily number of visits (visits/d) by the duration of each visit (min/visit). The feeding rate

(g DM intake/min) was then calculated by dividing the daily DM intake (kg/d) by the daily duration of trough visits (min/d) of each animal and 1000.

The proportion of material retained on each sieve (wt/wt) of the PSPS was calculated. Two physical effectiveness factors (pef) were calculated by adding the proportions of material retained on two ($pef_{>8.0}$; 19.0- and 8.0-mm) or three sieves ($pef_{>4.0}$; 19.0-, 8.0-, and 4.0-mm) according to Jones and Heinrichs (2016). The $peNDF_{>8.0}$ and $peNDF_{>4.0}$ concentrations were defined as the dietary NDF concentration multiplied by $pef_{>8.0}$ and $pef_{>4.0}$, respectively. The geometric mean (X_{gm}) of particle size of the TMR was calculated according to Jones and Heinrichs (2016).

The *in vitro* digestible organic matter was estimated by the equation 43f of Menke and Steingass (1988). The metabolizable energy and net energy of lactation contents were estimated using equation 14f of Menke and Steingass (1988). The rumen nitrogen balance was calculated as the difference between dietary CP intake and utilizable CP intake divided by 6.25 (GfE, 2001). Energy-corrected milk yield was calculated according to Spiekers et al. (2009). The feed conversion ratio was calculated as energy-corrected milk yield (kg/d) divided by the DM intake (kg/d) of each animal.

2.9.8 Statistical Analyses

For all variables, arithmetic means were calculated per animal and period and used for statistical analysis by the MIXED procedure of SAS (V9.4, SAS Institute Inc., Cary, United States). The model included mixing time and period as fixed effects and cow within group as a random effect. The interaction between mixing time and period was insignificant and so not included in the final model. Linear and quadratic effects of mixing

time were tested by orthogonal polynomial contrasts using the CONTRAST statement. Effects were declared significant at $P < 0.05$ and tendencies were declared for $P \geq 0.05$ and < 0.10. All means are presented as least squares means.

2.10 RESULTS AND DISCUSSION

2.10.1 Particle Size Distribution and Physically Effective Fiber

Prolonged mixing time linearly decreased X_{gm} of particle size from 14.6 to 11.1 mm ($P < 0.01$, Table 2.6). The proportion of material retained on the top 19.0 mm sieve declined with increasing mixing time ($P < 0.01$), whereas the proportions of material retained on the other two sieves (8.0-mm and 4.0-mm) and in the pan increased linearly ($P < 0.01$, for all variables), as a result, $pef_{>8.0}$ and $pef_{>4.0}$ decreased linearly with increasing mixing time ($P < 0.01$, for all variables). Consequently, due to the similar NDF concentrations in all diets (Table 2.5), concentrations of dietary $peNDF_{>8.0}$ and $peNDF_{>4.0}$ decreased linearly ($P < 0.01$, for all variables) with prolonged mixing time, which confirms our expectations of significantly changing the dietary peNDF concentration by changing the mixing time in the wagon. Beauchemin and Yang (2005) studied the effects of forage particle size in a corn-silage-based TMR on its $peNDF_{>8.0}$ concentration as well as the feed intake and chewing activity of dairy cows. Tested $peNDF_{>8.0}$ concentrations (89, 103, and 115 g/kg DM) in their study were lower than those in the present study (237, 249, 267, and 283 g/kg DM). Yet, according to Beauchemin and Yang (2005), chewing time of cows increased linearly with increasing peNDF concentrations, therefore differences in peNDF concentrations in the present study appear big enough to lead to expectations of effects on animal behavior.

Table 2.5 Chemical composition of the total mixed rations varied by feed mixing time (arithmetic mean ± standard deviation; n = 3).

Chemical composition, g/kg DM[1]	Mixing time			
	28 min	43 min	58 min	73 min
DM, g/kg fresh matter	432 ± 6.0	432 ± 4.7	435 ± 6.2	438 ± 4.0
OM[1]	918 ± 1.2	919 ± 5.3	923 ± 1.0	922 ± 0.8
Crude protein	150 ± 4.5	155 ± 3.7	153 ± 2.4	153 ± 2.0
Crude lipid	36.1 ± 0.64	36.3 ± 0.25	36.3 ± 0.82	37.0 ± 1.34
Neutral detergent fiber	367 ± 12.3	367 ± 9.9	368 ± 7.4	361 ± 5.5
Acid detergent fiber	195 ± 4.9	199 ± 4.5	199 ± 4.0	194 ± 1.2
Starch	155 ± 18.0	146 ± 11.2	145 ± 6.0	148 ± 2.7
Digestible OM,[2] g/100 g OM	71.2 ± 1.22	71.3 ± 0.49	71.5 ± 0.45	72.9 ± 0.52
Metabolizable energy,[3] MJ/kg DM	10.5 ± 0.13	10.4 ± 0.13	10.5 ± 0.04	10.8 ± 0.11
Net energy of lactation,[3] MJ/kg DM	6.3 ± 0.10	6.3 ± 0.09	6.4 ± 0.03	6.6 ± 0.08
Utilizable crude protein[4]	147 ± 1.7	151 ± 3.1	150 ± 1.3	149 ± 0.8
RNB[5]	0.5 ± 0.62	0.6 ± 0.25	0.5 ± 0.21	0.6 ± 0.30

[1]DM = dry matter; OM = organic matter.
[2]Estimated by the *in vitro* Hohenheim gas test method (Menke and Steingass, 1988; equation 43f).
[3]Estimated by the *in vitro* Hohenheim gas test method (Menke and Steingass, 1988; equation 14f).
[4]Estimated by the modified *in vitro* Hohenheim gas test method (Steingass et al., 2001).
[5]Rumen nitrogen balance (RNB, g nitrogen/kg DM) = (crude protein (g/kg DM) – utilizable crude protein (g/kg DM))/6.25 (GfE, 2001).

Table 2.6 Effect of mixing time on particle size distribution and physically effective NDF[1] concentration of a total mixed ration (n = 3).

Variable	Mixing time				SEM[1]	*P*-value	
	28 min	43 min	58 min	73 min		Linear	Quadratic
Particle size distribution, g/100 g pre-dried material[2] retained on sieves							
19.0 mm	57.7	49.6	45.5	43.2	1.55	<0.01	<0.01
8.0 mm	17.4	21.9	23.2	21.9	1.14	<0.01	<0.01
4.0 mm	10.4	12.3	13.1	14.0	0.45	<0.01	0.38
Pan	14.5	16.2	18.2	20.8	0.54	<0.01	0.31
$pef_{>8.0}$[3]	0.75	0.72	0.69	0.65	0.008	<0.01	0.08
$pef_{>4.0}$[3]	0.86	0.84	0.82	0.79	0.006	<0.01	0.42
$peNDF_{>8.0}$,[4] g/kg DM[1]	283	267	249	237	4.1	<0.01	<0.01
$peNDF_{>4.0}$,[4] g/kg DM	323	313	296	288	3.1	<0.01	0.57
X_{gm}[5] of particle size, mm	14.6	13.0	12.0	11.1	0.3	<0.01	0.03

[1]DM = dry matter; NDF = neutral detergent fiber; SEM = standard error of means.
[2]DM concentrations of diet samples were (arithmetic mean ± standard deviation): 489 ± 10.9, 487 ± 8.5, 502 ± 5.4, and 507 ± 11.1 g/kg fresh matter for 28, 43, 58, and 73 min of mixing time, respectively.
[3]$pef_{>8.0}$ and $pef_{>4.0}$ = physical effectiveness factor determined as the proportion of material retained on two (19.0- and 8.0-mm) and three (19.0-, 8.0-, and 4.0-mm) sieves of the Penn State Particle Separator, respectively.
[4]$peNDF_{>8.0}$ and $peNDF_{>4.0}$ = physically effective NDF calculated by multiplying the dietary NDF concentration (g/kg DM) by $pef_{>8.0}$ and $pef_{>4.0}$, respectively.
[5]X_{gm} = geometric mean of particle size calculated according to Jones and Heinrichs (2016).

2.10.2 Feed Intake, Milk Yield, and Milk Composition

There were no treatment effects on DM and NDF intake ($P \geq 0.11$, for both variables, Table 2.7) which contradicts our hypothesis that feed intake would decline with increasing peNDF concentrations, but confirms our results with other studies that did not find any effect of dietary particle size on DM intake of dairy cows (Beauchemin et al., 2003; Beauchemin and Yang, 2005). In line with the similar DM intake and chemical composition of the diets across treatments, no effect of mixing time was observed on milk yield and composition, energy-corrected milk yield, or milk fat and protein yields ($P \geq 0.33$, for all variables). Milk lactose yield tended to decrease linearly with prolonged mixing time ($P = 0.08$).

The feed conversion ratio was similar between the diets ($P = 0.41$), suggesting that diet digestibility was also not affected by TMR mixing time. Due to the short experimental periods and the small number of animals used, data related to milk performance should be interpreted with caution. Yet other studies that also used corn silage (Yang and Beauchemin, 2005) or alfalfa silage (Krause et al., 2002; Kononoff and Heinrichs, 2003) did not observe any effect on DM intake or milk yield of cows with changing dietary particle size. Instead, smaller dietary particle sizes increased DM intake of steers solely fed barley silage (Soita et al., 2002) or of Holstein cows fed an alfalfa-based diet (Kononoff and Heinrichs, 2003), which may be attributed to a lower rumen fill. Interestingly, DM intake of dairy cows decreased with decreasing particle size in a study of Krause and Combs (2003). Diets in this study had much smaller particles, contained only 390 g/kg DM of forages, and were rich in starch.

Table 2.7 Effect of mixing time of a total mixed ration on feed intake, milk production, and milk composition of lactating dairy cows (n = 6).

Variable	Mixing time				SEM[1]	*P*-value	
	28 min	43 min	58 min	73 min		Linear	Quadratic
Dry matter intake, kg/d	24.3	24.9	24.6	24.1	0.64	0.67	0.25
NDF[1] intake, kg/d	8.9	9.1	9.0	8.7	0.23	0.31	0.11
Milk yield, kg/d	38.7	39.2	39.3	38.7	1.93	0.99	0.41
ECM[1] yield,[2] kg/d	34.6	35.5	34.9	34.8	1.55	0.99	0.51
Milk fat, g/kg milk	32.0	33.1	31.8	33.4	0.81	0.53	0.82
Milk protein, g/kg milk	31.5	32.1	31.6	31.5	0.49	0.77	0.33
Milk lactose, g/kg milk	48.7	48.9	48.5	48.2	0.33	0.24	0.48
Fat yield, kg/d	1.24	1.29	1.24	1.27	0.038	0.89	0.82
Protein yield, kg/d	1.22	1.25	1.24	1.20	0.028	0.64	0.37
Lactose yield, kg/d	1.88	1.99	1.87	1.80	0.061	0.08	0.10
MUN,[1] mg/dl	23.0	22.9	22.3	22.6	0.96	0.76	0.89
Feed conversion ratio[3]	0.69	0.71	0.71	0.70	0.033	0.73	0.41

[1]NDF = neutral detergent fiber; ECM = energy-corrected milk; MUN = milk urea-nitrogen; SEM = standard error of means.
[2]ECM calculated as milk yield (kg/d) x ((0.038 x milk fat (g/kg) + 0.021 x milk protein (g/kg) + 1.05)/3.28) according to Spiekers et al. (2009).
[3]Feed conversion ratio calculated as ECM (kg/d) divided by dry matter intake (kg/d).

Krause et al. (2002) also offered small dietary particle sizes similar to those in the diets offered by Krause and Combs (2003) but, in contrast, did not observe an effect on DM intake. They suggested that there might have been negative effects of very low feed particle sizes on rumen pH and functioning when combined with high concentrations of non-structural carbohydrates.

Hence, the inconsistent responses of DM intake and milk yield to dietary particle size observed in the literature may result from, for instance, differences in the type of concentrate feed (starch concentration and fermentability), forage type (NDF concentration and fermentability) and its particle size, and the proportion of forage in the diet. Moreover, the stage of lactation and thus performance level of cows may also alter the effects of particle size or peNDF concentration on feed intake. Kononoff and Heinrichs (2003) observed that the effect of dietary particle size on feed intake tends to be more pronounced in cows in early lactation than on those in mid-lactation. Cows in the present study were at the end of lactation and their energy requirements were fully met. Thus, smaller dietary particle sizes did not encourage them to increase their feed intake.

2.10.3 Chewing and Feeding Behavior

Daily eating time of cows decreased linearly from 334 min/d when the mixing time of their TMR was 28 min to 259 min/d when the mixing time was 73 min ($P = 0.02$, Table 2.8). Eating time expressed per kilogram of DM and NDF intake was greatest at 28 min mixing time and linearly decreased with longer mixing times ($P < 0.01$, for all variables). This is in line with other studies which offered diets with forages of long

particle size (X_{gm} = 4.1 – 6.8 mm; Kononoff and Heinrichs, 2003) to Holstein cows or with high proportions of forage (100% barley silage; X_{gm} = 4.7 – 18.8 mm; Soita et al., 2002) to steers. Despite similar DM intakes, prolonged mixing time increased feed intake rate of cows (g DM/min eating time), possibly because there was less need for additional mastication. Total time spent at feed troughs (min/d) and the number of trough visits (visits/d) decreased with increasing TMR mixing time ($P < 0.01$, for all variables). Such changes in feeding behavior of cows may alter diurnal nutrient supply to rumen microbes and thus nutrient use at rumen and animal level. Daily rumination times (in min/d and min/kg of DM and NDF intakes) were similar between treatments ($P \geq 0.11$), which contradicts other studies that have reported a quadratic response ($peNDF_{>8.0}$ = 101 – 151 g/kg DM; Kononoff and Heinrichs, 2003) or decrease ($peNDF_{>8.0}$ = 27 – 153 g/kg DM; Yansari et al., 2004) in daily rumination time of cows as their dietary particle size decreased. The effect of increasing particle size on rumination time is believed to diminish as particle size increases, with no further increase in rumination time expected after a certain particle length (Allen, 1997; Beauchemin, 2018). Allen (1997) set this threshold at a mean particle size of 10 mm, above which only moderate or no further increase in rumination time will occur.

The X_{gm} of particle size in the present study was above this threshold, which may explain the lack of effect on rumination time. In comparison, the X_{gm} in the studies by Kononoff and Heinrichs (2003) and Yansari et al. (2004) ranged from 4.1 to 6.8 mm and from 1.7 to 3.3 mm, respectively. Additionally, rumination time was strongly influenced by DM intake (Johnston and DeVries, 2018) and NDF intake (Beauchemin, 2018) which, however, was similar across treatments in the present study.

Table 2.8 Effect of mixing time of a total mixed ration on chewing and feeding behavior of lactating dairy cows.

Variable	Mixing time				SEM[1]	*P*-value	
	28 min	43 min	58 min	73 min		Linear	Quadratic
Eating[2]							
min/d	334	307	266	259	31.3	0.02	0.62
min/kg DM[1] intake	13.8	13.0	11.2	11.3	1.31	<0.01	0.33
min/kg NDF[1] intake	37.5	35.3	30.6	31.3	3.56	<0.01	0.29
g DM/min	73.2	80.5	95.1	96.8	9.80	0.07	0.76
Rumination[2]							
min/d	562	523	544	516	56.2	0.11	0.72
min/kg DM intake	22.9	21.7	23.8	22.5	2.44	0.88	0.96
min/kg NDF intake	62.1	59.6	64.8	62.4	6.66	0.65	0.99
Total chewing[2]							
min/d	884	829	819	782	86.6	0.03	0.74
min/kg DM intake	36	34.9	35.2	33.8	3.70	0.27	0.92
min/kg NDF intake	97.7	95.4	96.1	93.7	10.12	0.36	0.99
Trough visits[3]							
min/visit	6.9	8.6	7.1	7.5	0.68	0.90	0.13
min/d	250	225	216	202	15.6	<0.01	0.27
visits/d	40.9	31.3	30.4	29.2	2.82	<0.01	0.14

[1] NDF = neutral detergent fiber; DM = dry matter; SEM = standard error of means.
[2] Chewing parameters: n = 4 for 28 and 43 min, n = 5 for 58 and 73 min.
[3] Trough data: n = 6.

According to Beauchemin (2018), the maximum physiological capacity for chewing of cows is around 16 h/d. Cows in the present study chewed between 782 to 884 min/d (13.0 to 14.7 h/d), and were thus near their physiological maximum.

Nevertheless, similar to eating time, total chewing time increased linearly with prolonged mixing time ($P = 0.03$), which confirms results obtained by Kononoff and Heinrichs (2003). Hence, as DM intake of cows was not affected by treatment, the anticipated quadratic response in total chewing time of cows to increasing peNDF concentrations was not observed. Yet, confounding factors related to diet composition, animal characteristics, and possibly feeding management should be accounted for to improve the accuracy of the PSPS method in predicting changes in the chewing activity of cows and to capture their overall responses in feeding behavior.

2.11 CONCLUSIONS

Prolonging mixing time in feed mixer wagons effectively reduces particle size and thus dietary peNDF concentrations of TMR. Small increases in peNDF concentrations increase linearly total chewing time, number of trough visits, and feeding rate in lactating dairy cows, without limiting their feed intake and performance. Further research is needed to elucidate the relationship between peNDF concentration, feed intake, chewing behavior, and performance of dairy cows and to understand how changes in feeding behavior may affect rumen nutrient turnover.

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CHAPTER 3

EFFECTS OF PHYSICALLY EFFECTIVE FIBER CONCENTRATION AT DIFFERENT RUMEN NITROGEN BALANCES ON CHEWING BEHAVIOR, NITROGEN METABOLISM, AND PERFORMANCE OF LACTATING DAIRY COWS

3.1 ABSTRACT

This study aimed at analyzing the interactions of physically effective neutral detergent fiber (peNDF) concentration and differing rumen nitrogen balances (RNB) in the diets of lactating dairy cows with regard to their intake, chewing and feeding behavior, rumen microbial protein synthesis (MPS), partitioning in nitrogen (N) excretion, and performance. Twenty lactating Holstein cows were randomly assigned to a 4 x 4 Latin square that consisted of four 21-days-periods with 12 days of adaptation and 9 days of sampling. Dietary treatments followed a 2 × 2 factorial arrangement with two RNB (RNB0 = 0.1 g/kg dry matter (DM) vs. RNB– = – 1.5 g/kg DM) and two peNDF concentrations adjusted via diet mixing time (high peNDF = 28 min vs. low peNDF = 58 min). The four total mixed rations were isoenergetic and isofibrous and both RNB diets were identical in their forage composition and varied solely in their concentrate components. All data were analyzed using PROC MIXED in SAS 9.4 (SAS Institute Inc., Cary, United States).

Nutrient intake was lower with high than with low peNDF concentration. Milk yield was similar for both dietary peNDF concentrations, but greater for RNB0 than RNB– diets. In terms of chewing behavior, the peNDF concentration interacted with the RNB, whereas high peNDF concentration resulted in a greater total number of chews (chews/d) and longer chewing time (min/d) of animals for RNB– but not for RNB0 diets. The observed increase in chewing was due to an increase in daily eating time and the number of eating chews without any differences in rumination activity. There was an interaction between RNB and peNDF concentration for apparent total tract digestibilities of DM, organic matter, and crude protein (aCPd); whereas, at low peNDF, no difference was

observed between RNB, but lower values were observed for RNB– than RNB0 diets at high peNDF. Accordingly, the efficiency of rumen MPS (g/kg DM intake and g/kg crude protein intake) was lower with high than with low peNDF concentration for RNB– but similar between both peNDF concentrations for RNB0 diets. The proportion of ingested N excreted via urine was lower and via feces was greater in the RNB– diet with high than with low peNDF, with no differences between the two RNB0 diets. Nonetheless, milk N use efficiency was similar between the two peNDF concentrations, but greater in RNB– than RNB0 diets.

In conclusion, the effects of peNDF concentration are more pronounced for RNB– compared with RNB0 diets and the effects of RNB are more pronounced at high than low peNDF concentration. The peNDF does not positively affect the milk N use efficiency nor the apparent total tract digestibility of nutrients; however, the peNDF positively affected total chewing time as well as the proportion of ingested N excreted via urine in dairy cows specifically for low RNB diets. Increased chewing activity may result in increased saliva secretion and increased N recycling, however, these potential effects were too small to counteract the effects of a negative RNB on milk yield.

3.2 INTRODUCTION

The concept of physically effective neutral detergent fiber (peNDF) combines the physical and chemical properties of a diet, namely the particle size (PS) and the neutral detergent fiber (NDF) concentration in order to quantify the fraction of a feed that stimulates chewing in ruminants (Mertens, 1997). Much research has been conducted on the effects of dietary peNDF concentration on chewing activity, milk performance, ruminal fermentation, and rumen microbial protein synthesis (MPS) in high-yielding

dairy cows offered total mixed rations with corn silage, alfalfa silage or hay, or a combination of these forages (Beauchemin et al., 2003; Krause and Combs, 2003; Yang and Beauchemin, 2006a, 2009). Although increased chewing activity and saliva production may have an impact on N-recycling via the rumino-hepatic pathway, the role of dietary peNDF in the efficient use of nitrogen (N) and the partitioning of N excretion in ruminants has not been studied so far.

Dairy cows are commonly fed diets rich in crude protein (CP) and rumen-degradable crude protein (RDP) to ensure their protein requirements, as well as the RDP requirements of rumen microbes, are met (VandeHaar and St-Pierre, 2006). Current environmental concerns related to N emissions from dairy cattle farming strengthen the need to improve the N use efficiency (kg milk N/kg N intake) in dairy cows by, for example, reducing dietary CP and RDP concentrations. The German Protein Evaluation System (GfE, 2001) considers the rumen nitrogen balance (RNB) as a measure to depict whether the RDP supply to rumen microorganisms is adequate for ruminal MPS and fermentation of organic matter (OM). The RNB is calculated as the CP intake minus the sum of the microbial CP and the rumen-undegradable protein (RUP) at the duodenum, and the difference is divided by 6.25 (GfE, 2001). Up to 20% of the microbial CP in the rumen may be synthesized from urea-N recycled via the rumino-hepatic pathway (GfE, 2001). Urea-N can re-enter the rumen in two ways, via saliva and by diffusion from the blood through the rumen wall (Reynolds and Kristensen, 2008). In this regard, stimulating the chewing activity and thus saliva production of cows may present an option to improve the inherent ability of ruminants to salvage circulating urea-N to the rumen and convert it to high-quality microbial protein.

It was hypothesized that increasing dietary peNDF concentrations will stimulate chewing activity (i.e. total number of chews and chewing time (min) expressed per day and per kilogram dry matter (DM) intake) of dairy cows and along with this saliva production and N recycling. As a consequence, increasing dietary peNDF concentration may at least partially compensate for possible negative effects of negative RNB on rumen MPS (g N/d), diet digestibility, and ultimately, milk production of dairy cows, while reducing urinary N excretion. Hence, the objective of the present study was to investigate the effects of peNDF concentration as affected by dietary RNB on chewing behavior, rumen MPS and N balance, as well as milk performance of dairy cows.

3.3 MATERIALS AND METHODS

3.3.1 Animals and Housing

The present study was performed at the Meiereihof research farm of the University of Hohenheim, Stuttgart, Germany (48°42’50.6” N and 9°13’03.0” E) between January and April 2019 in accordance with the national ethical and animal welfare legislation (No. 401181021). Twenty lactating Holstein cows (10 primiparous and 10 multiparous cows) with an average (arithmetic mean ± standard deviation) milk yield of 38.7 ± 7.03 kg/d, body weight (BW) of 677 ± 58.1 kg, and days in milk (DIM) of 103 ± 59.4 just before the beginning of the study were assigned to four treatment groups of five animals each. The four groups were arranged to equalize average milk yield and DIM between groups. Animals were housed together in a free-stall barn and milked twice daily (05:00 and 16:00 h) in an auto-tandem milking parlor (GEA Farm Technologies former Westfalia, Bönen, Germany). Daily milk yield was recorded by in-parlor milk meters (Metatron P21, GEA Farm Technologies, Bönen, Germany). The BW was recorded daily directly after

morning milking using an automated walk-over-weighing system (TaxaTron, GEA Farm Technologies, Bönen, Germany). Cows had *ad libitum* access to feed and fresh drinking water. The fresh matter (FM) intake of individual cows was measured automatically by weighing troughs and recorded using a transponder system (Waagen Döhrn GmbH & Co.KG, Wesel, Germany). For this, each group was assigned to three specific feed troughs throughout the whole study.

3.3.2 Study Design and Diets

The study was performed as a 4 x 4 Latin square in a 2 x 2 factorial arrangement with four periods of 21 days each. After 12 days of adaptation to the experimental diets, data and sample collection started on day 13 of each period and lasted until day 1 of the subsequent period just before the new diets were offered after the morning milking. The two treatment factors were dietary RNB and dietary peNDF concentration. Diets were formulated to meet the utilizable CP (uCP) and net energy of lactation (NE_L) requirements of a 700 kg cow with a DM intake of 26 kg/d and milk production of 36 kg/d (GfE, 2001).

The diets were offered as a total mixed ration (TMR) with a forage to concentrate ratio of 53:47 (on DM basis; Table 3.1) and formulated to be isoenergetic and isofibrous, and similar in their uCP supply at the duodenum (Table 3.2). The two different dietary RNB were created by varying the ingredient composition of the concentrate mixture: balanced RNB (RNB0; 0 g/kg DM) and negative RNB (RNB–; – 1.5 g/kg DM; Table 3.1).

Table 3.1 Ingredient composition of the total mixed rations differing in RNB[1] (RNB0 = 0.1 g/kg DM[1]; RNB– = – 1.5 g/kg DM) and fed to lactating dairy cows.

Ingredients, g/kg DM	RNB0	RNB–
Maize silage	146	146
Grass silage 1, 1st cut	160	160
Grass silage 2, 1st cut	38.8	38.7
Grass hay, 1st cut	88.8	88.5
Grass hay, 2nd cut	80.3	80.0
Barley straw	20.1	20.1
Soybean grain	96.5	43.6
Barley grain	261	230
Post-extraction rapeseed meal[2]	84.4	92.2
Rapeseed cake[3]	.	44.4
Beet sugar[4]	.	34.9
Urea[5]	2.50	.
Beta-carotene[4]	2.50	2.50
Sodium chloride[4]	3.30	3.30
Calcium carbonate[4]	7.90	7.90
Mineral-vitamin mixture[4,7]	8.80	8.70

[1]DM = dry matter; RNB = rumen nitrogen balance.
[2]Fa. Allgaier, Allmendingen, Germany.
[3]BKK Bio-Diesel GmbH, Rudolstadt, Germany.
[4]Bergophor Futtermittelfabrik Dr. Berger GmbH & Co. KG, Kulmbach, Germany.
[5]PIARUMIN® (SKW Stickstoffwerke Piesteritz GmbH, Lutherstadt Wittenberg, Germany).
[6]K + S Minerals and Agriculture GmbH, Kassel, Germany.
[7]GM134 composition as-fed, according to manufacturer information: 200 g Ca, 40 g Mg, 50 g Na, 7 g Zn, 5 g Mn, 1.1 g Cu, 35 mg Se, 60 mg I, 20 mg Co, 250,000 IU Vitamin A, 65,000 IU Vitamin D, 5000 mg Vitamin E, and 120 mg Vitamin B7.

Two different dietary peNDF concentrations were obtained by adjusting the PS of the diets through different mixing times of the TMR in the feed mixer wagon (Power Champ L, Marmix GmbH & Co.KG, Unterwachingen, Germany): 28 min (high peNDF) and 58 min (low peNDF).

These two mixing times were selected based on the results of the *in vivo* study in Chapter 2B, where the greatest difference in total chewing time and total number of chews of dairy cows were observed when they were offered a TMR mixed for 28 min and 58 min. The feed mixer wagon was equipped with a horizontal stir wing mixer fitted with blades, which consequently reduced dietary PS with prolonged mixing times. The

ingredients of the diets were loaded into the mixer wagon in the following order: grass silages, maize silage, concentrate and mineral mixtures, barley straw, and grass hays. The mixing protocol is given in the Appendix (Table A.3).

Each animal group received each diet once across the four periods in a random order in each period. The diets were prepared freshly every morning and offered once daily for *ad libitum* consumption at 09:00 h. The amounts of offered diets were adjusted daily to allow for diet refusals of approximately 10% of the total offered amounts (on DM basis). The external fecal marker titanium dioxide (TiO_2; 60797, Kronos® 1171, Kronos Worldwide Inc., Dallas, United States) was used to estimate fecal excretion of animals and consequently, apparent total tract nutrient digestibility. For this, the TiO_2 was incorporated into the concentrate mixtures from days 10 to 21 of each period to achieve a daily TiO_2 intake of about 34 g/cow.

3.3.3 Sample Collection and Processing

Offered and Refused Diets

For analysis of chemical composition, samples of offered diets were collected every morning immediately after feeding between day 14 and day 21 of every period, whereas samples of refused diets were taken daily before feeding from day 15 until day 1 of the subsequent period. Samples were collected from three different sites (i.e. one from the left and right top corner, and one from the middle center of the trough) of each of the three troughs per treatment group (around 3.0 kg across the three troughs per diet; as-fed basis), pooled by diet, and mixed thoroughly. A subsample of around 300 g each (as-fed

basis) was taken from every pooled sample, weighed, and stored at – 20°C until further processing.

For PS determination, samples of offered diets were collected similarly as stated above on days 13, 14, 16, 18, and 20 of every period. All samples (i.e. 3 troughs x 3 sampling sites per diet) were immediately pooled by diet, mixed thoroughly, and four subsamples of 450 g each (as-fed basis) were taken for every diet. Similarly, refusal samples were collected on days 15, 17, 19, and 21 for PS determination. These fresh subsamples of offered and refused diets were directly weighed and stored at 4°C until the determination of PS distribution on the same day.

Milk

Milk samples (100 ml) were taken daily between days 14 and 21 alternating between afternoon and morning milking. Samples were pooled by animal and two consecutive days (i.e. one afternoon and one morning milking) by taking 20 ml of afternoon milk and adding an amount of morning milk calculated as the ratio between morning and afternoon milk yield (kg/cow) multiplied by 20 ml. Pooled samples (45 ml each) were then preserved with 150 µl Bronysolv (ANA.LI.TIK Austria, Vienna, Austria) and stored at 4°C for later analysis of milk fat, protein, lactose, and urea-N.

Feces and Urine

Feces and urine samples were collected simultaneously directly after milking once daily, from day 14 until day 1 of the subsequent period. Sampling was alternated between morning and afternoon. For this, animals were randomly divided into two sampling groups of ten animals each, with the first group starting in the afternoon of day 14, and

the second group in the morning of day 15. Hence, in total eight samples were collected from each cow during every sampling period. Fecal spot samples of 400 g FM were collected by rectal grab and stored at – 20°C until the end of each sampling period. The samples were then pooled by animal and period by taking the same amount (FM basis) of each daily sample and a subsample (160 g FM) was taken, weighed, and stored at – 20°C until further processing.

Urine spot samples were collected by perineal massage, immediately homogenized, and filtered through a gauze with a pore size of 0.5 mm to remove impurities. A subsample of 250 ml was then acidified using an aqueous solution of sulfuric acid (20%; vol/vol; Merck KGaA, Darmstadt, Germany) to reduce urine pH to below 3. The total amount of sulfuric acid added was recorded. From the acidified urine subsample, four 40 ml-aliquots were transferred into 50-ml-centrifuge tubes, of which two were used for N analysis and the other two for purine derivatives (PD) analysis. These aliquots were then stored at – 20°C until the end of each experimental period.

For N analysis, aliquots of individual animals were thawed, pooled by animal and period by taking the same amount from each aliquot, and thereof two subsamples of 35 ml each were stored in falcon tubes at – 20°C until further analysis. For PD analysis, the two aliquots of each animal and day were thawed, pooled by animal and two subsequent days by taking the same amount (à 10 ml) from each aliquot, generating a total of four pooled samples per animal in each period. About 160 ml of the pooled samples were then sieved through filter paper (DP 400 185, Ø 185 mm, average pore size 7–12 µm, Hahnemühle FineArt GmbH, Dassel, Germany). Twenty millimeters of the filtrate was diluted with

distilled water (1:5; vol/vol) and shaken for homogenization. Thereof, two aliquots of 12 ml each were stored at – 20°C until PD analysis.

3.3.4 Chemical Analyses

Frozen samples of the offered and refused diets were lyophilized (LYO GT2 Basis, SRK Systemtechnik GmbH, Riedstadt, Germany) for 48 h, weighed, and ground to pass a 1-mm-screen (Retsch SM 100, Retsch GmbH, Haan, Germany). Ground samples of offered and refused diets were then each pooled by diet and period, by taking the same amount from every daily sample. Frozen fecal grab samples were also lyophilized for 48 h, weighed, ground to pass a 2-mm-screen, and pooled by cow and period, by taking the same amount from each daily sample. The DM and crude ash concentrations in offered diets, diet refusals, and feces were determined in duplicate following the official analytical methods in Germany (VDLUFA, 2007; methods 3.1 and 8.1) and to subsequently estimate the OM concentration (g/kg DM). The crude lipid concentrations in offered and refused diets were determined in duplicate according to VDLUFA (2007; method 5.1.1). The N concentrations in offered and refused diets as well as in fecal and urine samples were determined in duplicate according to VDLUFA (2007; method 4.1.1) using Kjeldahl digestion (KT20 KJELDAHLTHERM®, C. Gerhardt GmbH & Co. KG, Königswinter, Germany), distillation (B324, Büchi Labortechnik GmbH, Essen, Germany), and titration (719 S Titrino, Metrohm AG, Herisau, Switzerland). The CP concentrations were estimated by multiplying the N concentrations by 6.25 (VDLUFA, 2007). The NDF and acid detergent fiber concentrations in offered and refused diet as well as in feces (NDF only) were determined in duplicate inclusive of residual ash using an Ankom200 Fiber Analyzer (Ankom Technology, Fairport, United States) following

the methods by VDLUFA (2007; methods 6.5.1 and 6.5.2). All NDF analyses were done with the use of heat-stable α-amylase (ANKOM Technology, Macedon, United States) and sodium sulfite (Merck KGaA, Darmstadt, Germany). Starch was analyzed in offered diet samples in duplicate using an enzymatic kit (Test-Combination Nr. 10 207 748 035, R-Biopharm AG, Darmstadt, Germany).

To estimate ME and NE_L contents as well as digestible OM concentrations of the offered and refused diet samples, *in vitro* incubations were carried out in triplicate for 24 h on two days according to Menke and Steingass (1988). Equation 14f was used to calculate ME and NE_L contents from proximate nutrient concentrations and gas production during *in vitro* fermentation and equation 34f for digestible OM concentrations. The uCP concentrations in offered diet samples were estimated from the ammonium concentrations in the inoculum after 24 h of *in vitro* incubation according to Steingass et al. (2001). For this, samples were additionally incubated in triplicate for 24 h on two days. The RNB was calculated as the CP concentration minus the uCP concentration divided by 6.25 (all in g/kg DM).

Fecal and diet samples were analyzed in duplicate for TiO_2 using spectrophotometry (Varian Cary 50 Bio UV-Visible Spectrophotometer, Varian Australia Pty Ltd, Australia) following the procedure of Boguhn et al. (2009) with slight modifications, whereas Kjeldahl digestion was carried out for 4 h instead of 40 min. The PD (allantoin and uric acid) concentrations of urine spot samples were determined in duplicate according to the procedures described by Balcells et al. (1992) and George et al. (2006) with minor modifications using high-performance liquid chromatography (Varian 920-LC, Palo

Alto, United States). Feed, urine, and feces analyses were repeated when the coefficient of variation between repetitions exceeded 5%.

Milk fat, protein, and lactose were analyzed in duplicate according to ASU L 01.00-78, 2002-05 and milk urea-N in duplicate according to 05022100.QMD, 2011-03 by infrared absorption with a Fourier Transform Spectrometer (Bentley FTS, Bentley Instruments, Chaska, United States) at the Milchprüfring Baden-Württemberg e.V. (Kirchheim unter Teck, Germany).

3.3.5 Chewing and Feeding Behavior

Animals were equipped with halters with automatic noseband pressure sensors (RumiWatch System, Itin & Hoch GmbH, Liestal, Switzerland) from day 13 (morning) until day 1 (morning) of the following period to measure their daily chewing behavior at 10-Hz-frequency. The RumiWatch conversion software V0.7.3.2 was used to convert the logged data into 24-h-summaries to obtain eating and rumination time (min/d) and the number of eating and rumination chews (chews/d). The sum of daily eating and rumination time and eating and rumination chews were defined as total chewing time (min/d) and the total number of chews (chews/d), respectively. Eating, rumination, and total chewing time as well as number of eating, rumination, and total chews were expressed in min/d or n/d, respectively, and min/kg DM and NDF intakes. Collected daily data were averaged per animal and period, which resulted in 18 observations for RNB– diet with high peNDF concentration and 19 observations for all other diets. Missing data was due to the failure of the pressure sensors in recording the data or related to the fact that cows stripped off their halter.

3.3.6 Particle Size Distribution and Sorting Index

Fresh samples of offered and refused diets were dried in a forced-air oven at 60°C for 1 h before sieving to reduce the adherence of smaller particles to bigger particles based on observations in a pre-study (see Chapter 2A). Samples were weighed before and after oven-drying to determine their DM concentrations at sieving. Each subsample was subjected to sieving using a Penn State Particle Separator (PSPS, Nasco, Fort Atkinson, United States). The operation of the PSPS device was conducted according to Jones and Heinrichs (2016) to separate the samples into the following particle fractions: long (> 19 mm), medium (≤ 19 mm, > 8 mm), small (≤ 8 mm, > 4 mm), and fine (≤ 4 mm) particles. Sample material on each sieve and the bottom pan was weighed and the proportion of material retained on each sieve of the total sample weight (wt/wt) was determined.

3.3.7 Calculations

The DM intake (kg/d) of each group of five cows was calculated as the difference between the total DM offered to and refused by each group. Daily DM intake of individual animals (kg/d) was then calculated by multiplying the DM intake (kg/d) of each group by the ratio between FM intake (kg/d) of individual cows and total FM intake (kg/d) of the respective group as registered by the feeding trough system. Similarly, the intakes of OM, N, NDF, ME, and of each particle fraction were calculated by multiplying the total intake of the respective nutrient (kg/d), ME (MJ/d), or particle fraction of each group (kg/d) with the ratio between the FM intake (kg/d) of individual cows and total FM intake (kg/d) of the respective group. Individual intake of starch was calculated by multiplying starch concentration in offered diet with individual DM intake (kg/d).

The feeding rate (g DM intake/min eating time) was calculated as the quotient of daily DM intake (g/d) and the daily eating time (min/d) recorded by the pressure sensors of each animal. Daily number of trough visits (visits/d) and duration (min/visit) of each visit per animal were registered automatically by the trough system. Daily number of visits (visits/d) multiplied by the duration of each visit (min/visit) equaled the daily duration of total trough visits (min/d).

The average fecal DM excretion was estimated from the daily TiO_2 dosage and the concentration of TiO_2 in fecal DM (from pooled fecal sample) assuming a 100% recovery rate of the marker in the feces according to Glindemann et al. (2009). The digestible organic matter (DOM) intake was estimated from the animals' daily OM intake and their fecal OM excretion. The apparent total tract digestibilities of DM (aDMd), OM (aOMd), CP (aCPd), and NDF (aNDFd) of ingested diets were calculated using the average nutrient intake (kg/d) across each sampling period and the fecal nutrient excretion (kg/d) of individual cows.

Energy-corrected milk (ECM) yields were calculated according to Spiekers et al. (2009). The feed efficiency was calculated as the daily ECM yield (kg/d) divided by the daily DM intake (kg/d) of each animal.

The urinary N loss (g/d) of each animal was defined as the difference between daily N intake and the N losses via feces, skin, and hair (g/d), and the milk N secretion (g/d). The secretion of milk N was calculated by dividing the milk protein yield by 6.38 (McDonald et al., 2011), with the milk protein yield calculated as the product between milk yield (kg/d) and milk protein content (g/kg). Skin and hair N losses (g/d) were calculated by multiplying the daily metabolic BW of the animals ($kg^{0.75}$) by the factor 0.018 (g $N/kg^{0.75}$

BW) (GfE, 2001). The daily urine volume (l/d) of each cow was calculated by dividing the urinary N excretion (g/d) by the urine N concentration (g/l). The product between the cows' urine volume (l/d) and their urinary PD concentration (mmol/l) was defined as the urinary PD excretion (mmol/d). Thereafter, the duodenal absorption of microbial PD (mmol/d) was calculated following Verbic et al. (1990) and the intestinal flow of microbial N (g/d) calculated from absorbed PD according to Chen and Gomes (1992; equation 5). The efficiency of MPS was expressed as the intestinal flow of microbial N (g/d) per kilogram DM, DOM, and CP intakes.

The physical effectiveness factors (pef) and the peNDF concentrations were determined from the PS distribution of the diet samples averaged across the four replicates per day. For this, the sum of the proportions of material retained on two (19.0 and 8.0 mm) or three sieves (19.0, 8.0, and 4.0 mm) was defined as $pef_{>8.0}$ and $pef_{>4.0}$, respectively. The $peNDF_{>8.0}$ and $peNDF_{>4.0}$ concentrations were calculated by multiplying the dietary NDF concentration by $pef_{>8.0}$ and $pef_{>4.0}$, respectively. The geometric mean (X_{gm}) of the PS was determined using the spreadsheet by Jones and Heinrichs (2016) from the average PS distribution across the four replicates of each day. The pef, peNDF, and X_{gm} data were later averaged per cow and week.

The sorting index for each PSPS particle fraction was calculated according to Leonardi and Armentano (2003) by dividing the amount consumed of each particle fraction by the predicted intake of the respective fraction multiplied by 100%. The predicted intake was calculated by multiplying the concentration of the particle fraction in the offered diet by the DM intake of individual cows. A sorting value equal to 100% indicated no sorting, a value of < 100% indicated sorting against, and a value of > 100% indicated sorting for a

particular particle fraction. The sorting index was calculated per group (n = 4), as animals of each treatment group shared the same feed troughs throughout the study.

3.3.8 Statistical Analyses

All data were analyzed using the MIXED procedure of SAS (V9.4, SAS Institute Inc., Cary, United States). The model used for analyzing PSPS data (i.e. physical characteristics of diet) included peNDF concentration, RNB, the interaction between peNDF concentration and RNB, and period as fixed effects and cow within group as a random effect. For the sorting index data, the model included peNDF concentration, RNB, the interaction between peNDF concentration and RNB, and period as fixed effects and group as a random effect. The model for the rest of the data included peNDF concentration, RNB, the interaction between peNDF concentration and RNB, period, and DIM (covariable) as fixed effects and cow within group as a random effect. The DIM was included to correct for the effects of changing milk yield with advancing lactation and for individual animal differences.

The interactions between peNDF concentration and period, RNB and period, and the three-way interaction were originally included in all models but were removed, because they were not significant ($P \geq 0.10$). Effects were declared significant at $P < 0.05$ and tendencies were declared for P between ≥ 0.05 and < 0.10. Data were also tested for linear contrasts within RNB and within peNDF concentration using the ESTIMATE statement when the ANOVA showed a significant or a tendency for a significant interaction effect between peNDF concentration and RNB. The *P*-values of these pairwise comparisons are presented in the running text of the results section.

3.4 RESULTS

3.4.1 Chemical and Physical Properties of Diets

As intended, ingested diets were isoenergetic and isofibrous, but the CP concentration was greater in RNB0 than in RNB– diets (Table 3.2). At similar uCP supply across offered diets, the RNB were 0.1 g (RNB0) and – 1.5 g/kg DM (RNB–). There was an interaction effect between peNDF concentration and RNB for the proportion of material retained on each of the sieves, but not the one left in the pan. A greater proportion of material was retained on the top and lower sieves for RNB– than RNB0 diets at high peNDF, whereas it was similar for both RNB diets with low peNDF concentration (Table 3.3; $P < 0.01$ for all variables).

For the middle sieve, a greater proportion of material was retained at high than at low peNDF for RNB– diets ($P < 0.01$), whereas a similar proportion of material was retained irrespective of the peNDF concentrations for RNB0 diets ($P < 0.12$).

There was no interaction effect on $pef_{>8.0}$ and $pef_{>4.0}$ as well as concentrations of $peNDF_{>8.0}$ and $peNDF_{>4.0}$, but these variables were greater in high peNDF than in low peNDF diets and in RNB– than in RNB0 diets. There was a tendency for an interaction effect on $X_{gm.}$ The X_{gm} was greater for high than for low dietary peNDF concentrations independent of RNB ($P < 0.01$) and greater for RNB– than RNB0 diets with both peNDF concentrations ($P < 0.01$); however, the differences between RNB diets were more pronounced at high peNDF than low peNDF concentration and between peNDF concentrations for RNB– than RNB0 diets.

Table 3.2 Chemical composition of the total mixed rations differing in peNDF[1] and RNB[1] fed to lactating dairy cows (n = 4).

Chemical composition, g/kg DM[1]	Low peNDF[2]		High peNDF[2]	
	RNB0[3]	RNB–[3]	RNB0	RNB–
DM, g/kg fresh matter	420 ± 7.5	414 ± 4.1	416 ± 2.5	412 ± 6.0
OM[1]	918 ± 5.0	921 ± 2.7	919 ± 3.7	921 ± 1.3
Crude protein	147 ± 1.1	137 ± 3.4	148 ± 1.1	137 ± 2.9
Crude lipid	35.4 ± 0.61	33.3 ± 0.55	35.8 ± 1.12	33.5 ± 0.90
Neutral detergent fiber	377 ± 1.2	377 ± 0.4	378 ± 1.2	378 ± 1.7
Acid detergent fiber	201 ± 2.8	200 ± 2.1	195 ± 1.3	198 ± 4.4
Starch	112 ± 8.0	110 ± 10.9	115 ± 7.4	106 ± 10.2
Digestible OM,[4] g/100 g OM	73.7 ± 0.47	73.4 ± 0.60	73.2 ± 0.73	73.2 ± 0.48
Metabolizable energy,[5] MJ/kg DM	10.8 ± 0.07	10.7 ± 0.08	10.7 ± 0.15	10.7 ± 0.09
Net energy for lactation,[5] MJ/kg DM	6.6 ± 0.05	6.5 ± 0.06	6.5 ± 0.10	6.5 ± 0.06
Utilizable crude protein,[6] g/kg DM	147 ± 0.93	146 ± 2.24	147 ± 0.60	146 ± 2.02
RNB	0.1 ± 0.28	-1.5 ± 0.15	0.1 ± 0.18	-1.5 ± 0.38

[1]DM = dry matter; OM = organic matter; peNDF = physically effective neutral detergent fiber; RNB = rumen nitrogen balance.
[2]peNDF concentrations: low $peNDF_{>8.0}$ = 207 g/kg DM and high $peNDF_{>8.0}$ = 217 g/kg DM.
[3]RNB: RNB0 = 0.1 g/kg DM and RNB– = – 1.5 g/kg DM.
[4]Estimated by the *in vitro* Hohenheim gas test method (Menke and Steingass, 1988; equation 43f).
[5]Estimated by the *in vitro* Hohenheim gas test method (Menke and Steingass, 1988; equation 14f).
[6]Estimated by the modified *in vitro* Hohenheim gas test method (Steingass et al., 2001) to further calculate RNB as RNB (g/kg DM) = (crude protein (g/kg DM) – utilizable crude protein (g/kg DM))/6.25 (GfE, 2001).

Table 3.3 Physical characteristics of the total mixed rations differing in peNDF[1] and RNB[1] determined using the Penn State Particle Separator (n = 4).

Variable	Low peNDF[2]		High peNDF[2]		SEM[1]	*P*-value			
	RNB0[3]	RNB–[3]	RNB0	RNB–		Period	RNB	peNDF	RNB x peNDF
Particle size distribution,[4] g/100 g pre-dried material									
19.0 mm	38.9^{Aa}	39.3^{Aa}	41.9^{Ba}	43.7^{Bb}	0.43	<0.01	<0.01	<0.01	0.03
8.0 mm	14.7^{Aa}	17.1^{Ab}	14.2^{Aa}	15.0^{Bb}	0.21	<0.01	<0.01	<0.01	<0.01
4.0 mm	13.5^{Aa}	13.4^{Aa}	12.7^{Ba}	13.0^{Bb}	0.09	<0.01	<0.01	<0.01	<0.01
Pan	32.9	30.2	31.3	28.3	0.25	<0.01	<0.01	<0.01	0.36
$pef_{>8.0}$[5]	0.54	0.56	0.56	0.59	0.003	<0.01	<0.01	<0.01	1.00
$pef_{>4.0}$[5]	0.67	0.70	0.69	0.72	0.003	<0.01	<0.01	<0.01	0.32
$peNDF_{>8.0}$,[6] g/kg DM[1]	202	213	212	221	0.53	<0.01	<0.01	<0.01	0.45
$peNDF_{>4.0}$,[6] g/kg DM	253	263	259	271	0.73	<0.01	<0.01	<0.01	0.52
X_{gm},[7] mm	8.6^{Aa}	9.1^{Ab}	9.2^{Ba}	9.8^{Bb}	0.07	<0.01	<0.01	<0.01	0.08

[A, B]Means with different uppercase superscripts in the same row within an RNB differed significantly at $P < 0.05$.
[a, b]Means with different lowercase superscripts in the same row within a peNDF concentration differed significantly at $P < 0.05$.
[1]DM = dry matter; peNDF = physically effective neutral detergent fiber; RNB = rumen nitrogen balance; SEM = standard error of means.
[2]peNDF concentrations: low $peNDF_{>8.0}$ = 207 g/kg DM and high $peNDF_{>8.0}$ = 217 g/kg DM.
[3]RNB: RNB0 = 0.1 g/kg DM and RNB– = – 1.5 g/kg DM).
[4]Sample DM concentrations of low and high peNDF diets at sieving were 484 and 488 g/kg fresh matter for RNB0 and 488 and 487 g/kg fresh matter for RNB–, respectively.
[5]$pef_{>8.0}$ and $pef_{>4.0}$ = physical effectiveness factor determined as the proportion of material retained on sieves 19.0- and 8.0-mm, and 19.0-, 8.0-, and 4.0-mm, respectively.
[6]$peNDF_{>8.0}$ and $peNDF_{>4.0}$ = peNDF determined as the dietary neutral detergent fiber concentration (g/kg DM) of feed multiplied by $pef_{>8.0}$ and $pef_{>4.0}$, respectively.
[7]X_{gm} = geometric mean of particle size calculated according to Jones and Heinrichs (2016).

3.4.2 Intake and Digestibility of Nutrients

There were no effects of RNB, peNDF concentration, and the interaction thereof on BW of individual cows (Table 3.4). Also, there were no interactions between RNB and peNDF concentration for any of the intake variables. The DM intake was similar between RNB; however, it was greater in low peNDF than in high peNDF diets. A similar pattern was observed for intakes of OM, DOM, NDF, and ME. Starch and N intakes were, however, greater for low peNDF than high peNDF diets and for RNB0 than RNB– diets.

There was a tendency for an interaction effect for aDMd and aOMd, and a significant effect for aCPd, where aDMd and aOMD followed the same pattern as aCPd. The aCPd was lower at high than at low peNDF concentration in RNB– diets ($P = 0.03$), but similar ($P = 0.22$) between peNDF concentrations in RNB0 diets. The RNB had also no effect in diets with low peNDF concentration ($P = 0.16$), but aCPd was lower in RNB– than RNB0 diets with high peNDF concentration ($P < 0.01$). Instead, aNDFd was similar across treatments.

Table 3.4 Intake and nutrient digestibility of lactating dairy cows fed a total mixed ration differing in peNDF[1] and RNB[1] (n = 20).

Variable	Low peNDF[2]		High peNDF[2]		SEM[1]	*P*-value			
	RNB0[3]	RNB–[3]	RNB0	RNB–		Period	RNB	peNDF	RNB x peNDF
Body weight, kg	700	699	695	698	6.9	<0.01	0.60	0.11	0.13
Intake, kg/d									
DM[1]	26.2	26.3	25.4	25.7	0.25	<0.01	0.31	<0.01	0.43
OM[1]	24.1	24.2	23.3	23.7	0.23	<0.01	0.20	<0.01	0.50
Digestible OM[2]	16.4	16.7	16.2	16.1	0.18	<0.01	0.64	0.04	0.37
NDF[1]	9.8	9.9	9.5	9.7	0.10	<0.01	0.15	<0.01	0.28
ME,[1] MJ/d	283	282	273	276	2.7	<0.01	0.75	<0.01	0.36
Starch	3.0	2.9	2.9	2.7	0.04	<0.01	<0.01	0.01	0.12
N,[1] g/d	620	576	604	565	6.4	<0.01	<0.01	<0.01	0.69
Apparent total tract digestibility, g/100 g									
DM[5]	66.0	67.0	67.2	65.6	0.37	0.23	0.68	0.90	0.09
OM[5]	68.3	69.2	69.4	67.9	0.36	0.29	0.69	0.90	0.08
CP[1]	64.9^{Aa}	63.3^{Aa}	66.2^{Aa}	60.9^{Bb}	0.46	0.26	<0.01	0.47	0.02
NDF	50.5	51.8	52.9	50.7	0.69	0.02	0.71	0.57	0.15

[A, B]Means with different uppercase superscripts in the same row within an RNB differed significantly at $P < 0.05$.
[a, b]Means with different lowercase superscripts in the same row within a peNDF concentration differed significantly at $P < 0.05$.
[1]DM = dry matter; ME = metabolizable energy; N = nitrogen; NDF = neutral detergent fiber; OM = organic matter; peNDF = physically effective neutral detergent fiber; RNB = rumen nitrogen balance; SEM = standard error of means.
[2]peNDF concentrations: low $peNDF_{>8.0}$ = 207 g/kg DM and high $peNDF_{>8.0}$ = 217 g/kg DM.
[3]RNB: RNB0 = 0.1 g/kg DM and RNB– = – 1.5 g/kg DM).
[4]Digestible OM intake estimated by multiplying apparent total tract digestibility of OM by the OM intake (kg/d) and dividing it by 100.
[5]Test for orthogonal polynomial contrast did not show any significant and tendency for significant differences ($P \geq 0.10$).

3.4.3 Chewing and Feeding Behavior

There were interactions between dietary peNDF concentration and RNB for daily eating time, number of eating chews, and feed intake rate (g DM/min eating time), which were greater with high peNDF than with low peNDF concentration within each RNB (Table 3.5; $P \leq 0.04$). Yet, these variables were greater in RNB0 than RNB– diets with low peNDF concentration ($P \leq 0.02$), but similar between RNB when diets contained high concentrations of peNDF ($P \geq 0.39$). When related to the DM and NDF intakes of cows, there were no interaction effects for eating time and number of eating chews, but all variables were greater with high peNDF than with low peNDF concentration ($P < 0.01$) and with RNB0 than RNB– ($P \leq 0.04$).

No effect of RNB was observed on the time spent in the feed troughs per day (min/d) and per visit (min/visit), nor on the number of trough visits (n/d). In concordance with the daily eating time measured by the chewing sensors, the daily time spent in the feed troughs was greater in high peNDF than in low peNDF diets, which was due to a greater number of trough visits and a similar time spent in the trough per visit at high peNDF than at low dietary peNDF concentrations.

There was a tendency for an interaction effect on daily rumination time and the number of rumination chews (Table 3.5). Daily rumination time ($P \leq 0.03$) and the number of rumination chews ($P \leq 0.09$) were greater for low than for high peNDF concentration irrespective of the RNB. Furthermore, rumination time was greater ($P = 0.04$) in RNB– than RNB0 diets with high peNDF concentration, but similar ($P = 0.84$) between RNB for diets with low peNDF concentrations. Rumination time and the number of rumination

chews expressed per kilogram DM and NDF intakes were similar for all dietary treatments.

There was an interaction between peNDF concentration and RNB for the total number of chews per day and daily chewing time, which were greater with high than with low peNDF concentration for RNB– diets (Table 3.5; $P \leq 0.01$), but similar between peNDF concentrations for RNB0 diets ($P \geq 0.41$). Also, total chewing time and the number of chews were lower ($P < 0.01$) for RNB– than RNB0 diets with low peNDF concentration, but similar between RNB diets with high peNDF concentration ($P \geq 0.26$).

When expressed per kilogram of DM and NDF intakes, there was no interaction between peNDF concentration and RNB for total chewing time and number of chews, but all variables were greater with high than with low peNDF concentration. Total chewing time per kilogram DM intake was similar across RNB, but total chews per kilogram DM intake tended to be lower for RNB– than RNB0 diets. Finally, total chewing time and total chews per kilogram NDF intake were greater with RNB0 than RNB– diets.

There was an interaction effect between peNDF concentration and RNB related to the selective intake of long, small, and fine particles (Table 3.5). Cows sorted more against long particles and for small and fine particles when offered the RNB0 than the RNB– diet at high peNDF concentrations ($P < 0.01$ for all variables). At low peNDF concentration, cows fed the RNB0 diet also sorted more against long particles ($P < 0.01$) and for fine particles ($P < 0.01$), but sorting for small particles did not differ between RNB ($P = 0.58$). Also, cows sorted more against long particles and for small particles when fed high peNDF than low peNDF for RNB0 ($P \leq 0.04$ for both variables), whereas no difference

was observed between peNDF concentrations for RNB– diets ($P \geq 0.14$ for both variables). Similarly, cows sorted more for fine particles at high peNDF diets ($P \leq 0.02$), the difference being more pronounced in RNB0 than RNB– diets.

Table 3.5 Chewing and feeding behavior parameters of lactating dairy cows fed a total mixed ration differing in peNDF[1] and RNB[1].

Variable[4]	Low peNDF[2]		High peNDF[2]		SEM[1]	*P*-value			
	RNB0[3]	RNB–[3]	RNB0	RNB–		Period	RNB	peNDF	RNB x peNDF
Eating									
min/d	409^{Aa}	386^{Ab}	424^{Ba}	424^{Ba}	6.1	0.39	0.03	<0.01	0.04
min/kg DM[1] intake	16	15	17	17	0.3	0.31	0.04	<0.01	0.25
min/kg NDF[1] intake	42	40	45	44	0.8	0.19	0.02	<0.01	0.36
g DM/min	66.2	70.2	61.1	62.5	2.31	0.91	0.02	<0.01	0.25
n/d	30663^{Aa}	28606^{Ab}	32181^{Ba}	31906^{Ba}	637.1	0.08	0.01	<0.01	0.04
n/kg DM intake	1169	1094	1280	1255	26.6	0.13	0.02	<0.01	0.21
n/kg NDF intake	3118	2917	3427	3337	72.1	0.15	<0.01	<0.01	0.30
Rumination									
min/d	591^{Aa}	590^{Aa}	570^{Ba}	580^{Bb}	3.6	0.09	0.17	<0.01	0.09
min/kg DM intake	23	23	23	23	0.2	0.01	0.76	0.67	0.88
min/kg NDF intake	60	60	61	61	0.7	<0.01	0.80	0.51	0.58
n/d	40596^{Aa}	40342^{Xa}	39131^{Ba}	39730^{Ya}	442.3	<0.01	0.50	<0.01	0.09
n/kg DM intake	1554	1553	1564	1563	23.2	0.23	0.96	0.43	1.00
n/kg NDF intake	4142	4138	4192	4157	64.0	0.02	0.60	0.34	0.67
Total chewing									
min/d	1000^{Aa}	977^{Ab}	994^{Aa}	1003^{Ba}	6.0	0.07	0.22	0.09	<0.01
min/kg DM intake	38	38	40	39	0.4	0.05	0.21	<0.01	0.48

min/kg NDF intake	102	100	106	105	1.2	<0.01	0.09	<0.01	0.74
n/d	71261^{Aa}	68959^{Ab}	71313^{Aa}	71622^{Ba}	890.9	0.03	0.06	0.01	0.01
n/kg DM intake	2722	2648	2844	2818	43.4	0.18	0.08	<0.01	0.38
n/kg NDF intake	7260	7055	7619	7493	119.5	0.07	0.04	<0.01	0.61
Trough visits[5]									
min/visit	6.4	6.6	6.1	6.2	0.23	0.04	0.57	0.15	0.86
min/d	291	285	308	309	8.8	<0.01	0.53	<0.01	0.33
visits/d	52	49	55	55	1.9	0.30	0.38	0.04	0.43
Sorting index for particle fractions,[6] % predicted intake									
> 19.0 mm	98.5^{Aa}	99.2^{Ab}	98.2^{Ba}	99.5^{Ab}	0.11	<0.01	<0.01	0.66	0.01
> 8.0 mm to ≤ 19.0 mm	98.8	98.6	98.1	97.6	0.12	<0.01	0.11	<0.01	0.37
> 4.0 mm to ≤ 8.0 mm,	100.7^{Aa}	100.6^{Aa}	101.0^{Ba}	100.5^{Ab}	0.06	<0.01	<0.01	0.36	<0.01
≤ 4.0 mm	102.0^{Aa}	101.5^{Ab}	102.7^{Ba}	101.8^{Bb}	0.09	<0.01	<0.01	<0.01	0.04

$^{A, B}$Means with different uppercase superscripts in the same row within an RNB differed significantly at $P < 0.05$.
$^{a, b}$Means with different lowercase superscripts in the same row within a peNDF concentration differed significantly at $P < 0.05$.
^{X,Y}Means with different uppercase superscripts in the same row within an RNB tended to differ at $P \geq 0.05$ to < 0.10.
[1]DM = dry matter; NDF = neutral detergent fiber; peNDF = physically effective neutral detergent fiber; rumen nitrogen balance = RNB; SEM = standard error of means.
[2]peNDF concentrations: low $peNDF_{>8.0}$ = 207 g/kg DM and high $peNDF_{>8.0}$ = 217 g/kg DM.
[3]RNB: RNB0 = 0.1 g/kg DM and RNB– = – 1.5 g/kg DM.
[4]Chewing parameters: n = 18 for RNB– with high peNDF concentration, n = 19 for the rest.
[5]Trough data: n = 20.
[6]Sorting index: n = 4.

3.4.4 Rumen Microbial Protein Synthesis and Nitrogen Balance

The intestinal flow of microbial N (g/d) tended to be greater in cows fed diets with low peNDF than with high peNDF concentrations, with no effects of RNB and the interaction between RNB and peNDF concentration (Table 3.6). There was a tendency for an interaction effect for the efficiency of MPS expressed per kilogram DM and CP intakes. The efficiency of MPS (g N/kg DM and CP intakes) was greater for low than high peNDF concentration in RNB– diets ($P \leq 0.04$), but similar between peNDF concentrations in RNB0 diets ($P \geq 0.61$). Also, the efficiency of MPS expressed per kilogram CP intake was greater in RNB– than RNB0 diets with low peNDF concentration ($P < 0.01$), but similar between RNB diets with high peNDF concentration ($P = 0.86$). There was no effect of RNB, peNDF concentration, or the interaction thereof on the efficiency of rumen MPS expressed in g N/kg DOM intake.

There was no interaction effect on milk N secretion and milk N use efficiency (g milk N/100 g N intake). Milk N secretion tended to be greater with low peNDF compared to high peNDF diets and greater for RNB0 than RNB– diets. The milk N use efficiency was lower in RNB0 than in RNB– diets, but similar between peNDF concentrations. There was a tendency for an interaction effect between peNDF concentration and RNB for urinary N excretion. Urinary N excretion was greater in RNB0 than RNB– diets for both peNDF concentrations ($P \leq 0.01$). However, while urinary N excretion was greater for low peNDF than high peNDF concentration in RNB– diets ($P = 0.02$), it was similar between peNDF concentrations in RNB0 diets ($P = 0.91$). There was also an interaction effect on fecal N excretion, whereas fecal N excretion was greater in RNB– than RNB0

diets at high peNDF concentration ($P = 0.02$), but similar between RNB diets at low peNDF concentration ($P = 0.39$).

An interaction effect between dietary treatments was also observed for the partitioning of N excretion between urine and feces. A greater proportion of N was excreted via feces and a lower proportion via urine in RNB– diets with high rather than with low peNDF concentration ($P \leq 0.03$), while the variables did not differ between peNDF concentrations in the RNB0 diet ($P \geq 0.22$). Also, a greater proportion of N was excreted via feces and a lower proportion via urine in RNB– than RNB0 diets with high peNDF concentration ($P \leq 0.02$). While the proportion of ingested N excreted via feces was similar between RNB at low peNDF concentration ($P = 0.16$), urine N excretion as a proportion of N intake was greater in RNB0 than RNB– diets with low peNDF concentration ($P \leq 0.01$).

Table 3.6 Metabolism of N[1] in lactating dairy cows fed a total mixed ration differing in peNDF[1] and RNB[1] (n = 20).

Variable	Low peNDF[2]		High peNDF[2]		SEM[1]	*P*-value			
	RNB0[3]	RNB–[3]	RNB0	RNB–		Period	RNB	peNDF	RNB x peNDF
N[1] intake, g/d	620	576	604	565	6.4	<0.01	<0.01	<0.01	0.69
Microbial N									
g N/d	451	482	446	423	11.6	<0.01	0.81	0.07	0.13
g N/kg DM[1] intake	17.0^{Aa}	18.2^{Aa}	17.6^{Aa}	16.4^{Ba}	0.67	<0.01	0.93	0.27	0.07
g N/kg DOM[1] intake	27.3	28.6	27.6	26.1	0.97	<0.01	0.91	0.20	0.10
g N/kg CP[1] intake	116^{Aa}	133^{Ab}	118^{Aa}	119^{Ba}	4.7	<0.01	0.04	0.20	0.08
Milk N secretion, g/d	191	186	188	182	3.2	0.18	<0.01	0.06	0.96
N losses, g/d									
Urinary N	209^{Aa}	177^{Ab}	210^{Aa}	159^{Bb}	4.9	<0.01	<0.01	0.10	0.08
Fecal N	217^{Aa}	211^{Aa}	204^{Aa}	220^{Ab}	3.0	0.40	0.29	0.68	0.03
Skin and hair N	2.50	2.40	2.40	2.40	0.02	<0.01	0.57	0.11	0.15
Milk N, g/100 g N intake	30.9	32.4	31.3	32.5	0.46	<0.01	<0.01	0.55	0.58
Urinary N, g/100 g N intake	33.6^{Aa}	30.6^{Ab}	34.7^{Aa}	28.1^{Bb}	0.01	0.02	<0.01	0.38	0.03
Fecal N, g/100 g N intake	35.1^{Aa}	36.7^{Aa}	33.8^{Aa}	39.1^{Bb}	0.46	0.26	<0.01	0.47	0.02

[A, B]Means with different uppercase superscripts in the same row within an RNB differed significantly at $P < 0.05$.
[a, b]Means with different lowercase superscripts in the same row within a peNDF concentration differed significantly at $P < 0.05$.
[1]CP = crude protein; DOM = digestible organic matter; DM = dry matter; N = nitrogen; peNDF = physically effective neutral detergent fiber; RNB = rumen nitrogen balance; SEM = standard error of means.
[2]peNDF concentrations: low $peNDF_{>8.0}$ = 207 g/kg DM and high $peNDF_{>8.0}$ = 217 g/kg DM.
[3]RNB: RNB0 = 0.1 g/kg DM and RNB– = – 1.5 g/kg DM.

3.4.5 Milk Production and Composition

No interaction effects between dietary peNDF concentration and RNB were observed for any milk variables (Table 3.7). Milk yield was not affected by peNDF concentration; however, ECM, fat, and protein yield tended to be greater in low peNDF compared to high peNDF diets. Also, cows had greater milk, ECM, fat (tendency) and protein yields when fed RNB0 than RNB– diets. Milk fat and protein contents were similar across all dietary treatments. There were no effects of peNDF concentration on milk lactose content and yield; however, both variables were lower at RNB– than at RNB0. Concentrations of milk urea-N were similar between peNDF concentrations, but lower in RNB– compared to RNB0 diets. The feed efficiency (ECM yield (kg/d)/DM intake (kg/d)) was similar between peNDF concentrations, but lower in RNB– than in RNB0 diets.

Table 3.7 Milk yield and composition of lactating dairy cows fed a total mixed ration differing in peNDF[1] and RNB[1] (n = 20).

Variable	Low peNDF[2]		High peNDF[2]		SEM[1]	*P*-value			
	RNB0[3]	RNB–[3]	RNB0	RNB–		Period	RNB	peNDF	RNB x peNDF
Milk yield, kg/d	36.6	35.7	36.2	35.2	0.77	0.28	<0.01	0.15	0.96
ECM yield, [4] kg/d	35.1	34.2	34.5	33.7	0.61	0.48	0.01	0.06	0.93
Fat yield, kg/d	1.34	1.31	1.31	1.29	0.023	0.45	0.08	0.06	0.87
Protein yield, kg/d	1.22	1.19	1.20	1.16	0.020	0.16	<0.01	0.07	0.96
Lactose yield, kg/d	1.78	1.73	1.76	1.70	0.039	0.14	<0.01	0.16	0.68
Milk fat, g/kg milk	37.1	37.3	36.8	37.1	0.05	0.03	0.43	0.44	0.98
Milk protein, g/kg milk	33.6	33.6	33.5	33.3	0.03	0.44	0.47	0.22	0.69
Milk lactose, g/kg milk	48.4	48.3	48.5	48.0	0.02	0.23	0.04	0.77	0.17
Milk urea-nitrogen, mg/dl	24.3	17.9	24.3	18.3	0.56	<0.01	<0.01	0.56	0.59
Feed efficiency[5]	1.34	1.30	1.37	1.32	0.032	<0.01	<0.01	0.18	0.42

[1]DM = dry matter; peNDF = physically effective neutral detergent fiber; RNB = rumen nitrogen balance; SEM = standard error of means.
[2]peNDF concentrations: low $peNDF_{>8.0}$ = 207 g/kg DM and high $peNDF_{>8.0}$ = 217 g/kg DM.
[3]RNB: RNB0 = 0.1 g/kg DM and RNB– = – 1.5 g/kg DM).
[4]Energy-corrected milk yield (ECM) calculated as milk yield (kg) x ((0.38 x milk fat (g/100 g) + 0.21 x milk protein (g/100 g) + 1.05)/3.28).
[5]Calculated as ECM yield (kg/d) divided by DM intake (kg/d).

3.5 DISCUSSION

This study hypothesized that increasing dietary peNDF concentrations would lead to greater chewing activity, saliva production, and N recycling in dairy cows and hence, may partly compensate for possible negative effects of negative RNB on rumen MPS, diet digestibility, and milk production, while reducing urinary N excretion of dairy cows. To adjust the different peNDF concentrations of the diets, two different feed mixing times were chosen. The RNB exhibited a significant effect on PS distribution, with more material retained on the sieves of the RNB– than the RNB0 diets, resulting in greater $peNDF_{>8.0}$, $peNDF_{>4.0}$, and X_{gm} for RNB– than RNB0 diets. As a result, values of RNB– diet with low peNDF concentration were comparable to the RNB0 diet with high peNDF concentration rather than to the RNB0 diet with low peNDF concentration, even though a mixing protocol was established for the study to ensure similar peNDF concentrations between RNB diets. Presumably, the addition of sugar in RNB– diets resulted in small particles adhering to longer particles, leading to a greater proportion of sample being retained in the sieves after sieving for the RNB– than RNB0 diets. Hence, due to the identical forage amount and ingredients in the diets as well as compliance with the mixing protocol, it can be assumed that the peNDF concentrations were similar between the two RNB. Thus, RNB exhibited no effect on DM intake, but only on intake of nutrients, due to the different composition of the diets.

3.5.1 Intake and Digestibility

The sorting index was calculated as the actual intake divided by the predicted intake, with 100% indicating no occurrence of sorting. In the present study, sorting occurred in

all four dietary treatments with cows sorting against long and for small and fine particles to a small degree. The difference in sorting between treatments was less than 1% and is thus likely of no biological relevance. Thus, although sorting may have occurred, treatments were not altered, and ingested diets remained similar to the formulated diets. Regardless of the sorting activity of cows, the animals' uCP and energy requirements were met for all diets.

Studies in the literature reported greater (Krause and Combs, 2003, X_{gm} = 2.5 – 4.2 mm), similar (Krause et al., 2002, X_{gm} = 2.8 – 6.3 mm; Maulfair et al., 2011, X_{gm} = 4.5 – 5.8 mm), or lower DM intakes (Kononoff et al., 2003, X_{gm} = 7.4 – 8.8 mm; Soita et al., 2002, X_{gm} = 4.7 – 18.8 mm) with increasing dietary PS. Amongst other factors, these contradictory responses to dietary PS may be related to the proportion of forage in the animals' diets and its PS as indicated by X_{gm}. Feeding diets containing long dietary PS is generally associated with slower solid digesta passage rate as well as slower rates and extents of ruminal feed nutrient degradation, leading to distension of the reticulorumen and other compartments of the gastrointestinal tract and therefore limiting voluntary DM intake (Allen, 2000). For both RNB in the present study, DM intake of cows decreased when they were fed the diets with high peNDF concentration, which is in line with the results obtained by Kononoff and Heinrichs (2003, X_{gm} = 4.1 – 6.8 mm) and Kononoff et al. (2003) who also offered diets with long PS to lactating Holstein cows or by Soita et al. (2002) who fed diets with high proportions of forage (100% barley silage) of long PS to steers.

As stated above, the reduction in intake was, however, not only observed for RNB–, but also RNB0 with high peNDF concentration. Presumably, the greater PS was the major

determinant for intake, which increased the filling effect of the diet, and thus triggered satiety in cows. The intake of nutrients, except N and starch, followed the same pattern as that of the DM intake, as their concentrations were formulated to be similar between diets. Additionally, the lactation stage may alter the effect of PS on DM intake, with cows in early lactation being more sensitive to changes in dietary PS. Therefore cows in an advanced lactation stage can more easily meet their energy requirements, so that reducing dietary PS may not encourage them to increase their DM intake (Kononoff and Heinrichs, 2003). Hence, the negative effect of NDF is more pronounced in dairy cows with high energy requirements fed a low energy diet, so a high energy diet may counteract the negative effects of NDF on DM intake of cows. Accordingly, no difference in the DM intake of lactating dairy cows was found in the first *in vivo* study (see Chapter 2B) when the PS of their TMR was varied by the feed mixing time with a similar forage to concentrate ratio (54:46) and even longer PS (X_{gm} = 11.1 – 14.6 mm) than in the present study. Cows in the first *in vivo* study were more advanced in their lactation stage (197 ± 67.7 DIM) compared to those in the present study (103 ± 59.4 DIM).

Irrespective of the dietary peNDF concentration, the low rumen N supply at a dietary RNB of – 1.5 g/kg DM (~ – 39 g/d) did not reduce the DM intake of cows when compared to those fed the RNB0 diets 0.1 g/kg DM (~ 2.6 g/d). This supports the results of König et al. (2005) who also found no effect of RNB on feed intake of lactating dairy cows offered maize- and grass silage-based diets with an RNB of either 0.5 or – 1.9 g/kg DM (9.5 or – 37 g/d) at a mean DM intake of around 20 kg/d. Similarly, Lebzien et al. (2006) did not observe any differences in DM intake or OM-flow at the duodenum (g/100 g OM intake) of lactating dairy cows when RNB was reduced from 1.6 to – 7.5 g/kg DM. The

lack of effects of negative dietary RNB on feed intake of cows observed by König et al. (2005) and in the present study was likely due to the fact that endogenous N supply to the rumen system was sufficient to maintain rumen OM fermentation, and thus aOMd even at negative RNB.

There was a tendency for an interaction effect between peNDF concentration and RNB on aDMd and aOMd. Although pairwise comparisons did not show any differences between individual treatments, both, aDMd and aOMd followed numerically the same pattern as aCPd with lowest aDMd, aOMd, and aCPd for RNB– diets with high peNDF concentration. The peNDF concentration and X_{gm} were greatest in the RNB– diet with a high peNDF concentration. Despite a presumably prolonged retention time of solid feed in the rumen, greater dietary PS reduces rumen degradation and digestibility of nutrients due to decreased surface area and access of microbial enzymes to feed substrate (Zebeli et al., 2012). Additionally, the lower dietary CP concentration and a reduced ruminal CP degradability in the RNB– diets, that did not include any urea, might have resulted in a lack of RDP for rumen microbes, which was possibly amplified by the larger dietary PS and thus even lower ruminal CP degradation (Kononoff and Heinrichs, 2003) in the diet with high peNDF concentration. Hence, the low RDP supply to rumen microbes in RNB– diets with high peNDF concentration may thus have impaired rumen microbial growth and activity and thus apparent total tract nutrient digestibility. Nevertheless, the efficiency of MCP synthesis per unit of CP intake was similar between RNB for diets with high peNDF concentration and even greater for RNB– than RNB0 diet with low peNDF concentration. Moreover, the efficiency of MCP synthesis per unit of DOMI did not differ

between treatments, suggesting that rather than RDP, nutrient digestibility, and with this fermentable carbohydrate supply, limited rumen microbial growth and activity.

Although apparent total tract nutrient digestibility was not affected by RNB0 compared to the RNB– diet, DM intake of cows was reduced for both RNB diets when offered at high peNDF concentration. Hence, presumably, the lower solid passage rate was the determinant for the lower DM intake of cows in the present study. The aNDFd was similar across diets in the present study. The lower DM intake of cows offered the diets with high peNDF concentrations suggests a slower ruminal NDF degradation, which might have been compensated, however, by an increased post-ruminal fermentation of NDF, explaining the similar aNDFd across diets (Yang and Beauchemin, 2006a). Milk fat content, however, was not affected by the peNDF concentration, RNB, nor the interaction thereof in the present study, which might be due to the high NDF and peNDF concentrations of the offered diets, also discerned by the high total chewing time of cows (38 – 40 min/kg DM intake), which indicates a reduced risk of digestive disorders in cows (Sudweeks et al., 1981).

3.5.2 Chewing and Feeding Behavior

It was hypothesized that greater peNDF concentration would promote the chewing activity (i.e. total number of chews and chewing time (min) expressed per day and per kilogram DM intake) of cows irrespective of the RNB. The following discussion will only focus on the eating, rumination, and total chewing time, because the number of eating, rumination, and total chews followed the same pattern as the duration of time spent for the respective behavior.

It was discerned in the present study that cows needed a longer time to chew (total chewing time in min/kg DM intake) with increasing peNDF concentration, with no difference between RNB. As DM intake was greater for low than high peNDF diets, total chewing time (min/d) was thus longer with high than low peNDF concentration for RNB– diets, but similar for both RNB0 diets. Also, the effect of RNB was more pronounced at low dietary peNDF concentration, with shorter total chewing time of cows in RNB– than RNB0 diets. When related to DM or NDF intake, several studies observed prolonged total chewing time of lactating dairy cows in response to increasing peNDF concentration of their TMR, which was either due to an increased rumination time (Beauchemin et al., 2003), a prolonged eating time (Yang et al., 2001; Kahyani et al., 2013), or both increased rumination and eating times (Kononoff and Heinrichs, 2003; Jiang et al., 2017). In general, increasing the dietary PS is more effective in stimulating eating than rumination time, because additional mastication during eating reduces the differences in the PS of the swallowed bolus (Beauchemin, 2018). Indeed, change in total chewing activity was more likely to be caused by a change in eating activity while rumination activity was similar across peNDF concentrations.

As opposed to the situation with total chewing time, eating time was greater for high peNDF concentration in both RNB. Moreover, feeding cows an RNB– diet with low peNDF concentration reduced eating time compared to when they were offered the RNB0 diet with low peNDF concentration. Similarly, the number of eating chews per kilogram DM and NDF intakes, as well as the daily number of trough visits and the time cows spent in the feeding troughs each day as recorded by the weighing system were greater in high than in low peNDF diets, which is in line with the observations during the first *in vivo*

study (see Chapter 2B) in which high-performing dairy cows were offered four total mixed rations varying solely in their PS. Total chewing time (16.2 – 16.7 h/d) and eating time (6.4 – 7.1 h/d) of cows were high and close to their maximum capacity, which, in the case of total chewing time, was estimated to be 16 h/d (Beauchemin, 2018). Hence, as cows were chewing to near their physiological maximum capacity, increasing peNDF concentration resulted in a minor increase in total chewing time and consequently possibly in saliva production and N recycling.

For both peNDF concentrations, the RNB was changed by altering the concentrate mixture without changing the forage ingredients and proportions. Thus, the addition of sugar in RNB– diets in combination with the small PS could have increased the palatability of the RNB– diet with low peNDF concentration, resulting in a greater feed intake rate of cows given this diet. This was, however, not the case for the RNB– diet with high peNDF concentration, for which the feed intake rate was reduced. Despite the palatability added by the sugar, the reduction in feed intake rate was likely due to the physical constraint in feed apprehension and mastication induced by the greatest dietary PS amongst all treatments. In combination with a possibly lower ruminal NDF degradation, the greater need for feed apprehension and mastication of long feed particles and thus reduced feed intake rate (g DM/min eating time) in the diets with high peNDF concentration probably reduced the DM intake of cows. Moreover, the high peNDF concentrations probably also increased rumen fill and thus satiety, reducing numerically the duration of trough visits, while increasing the number of trough visits each day.

Daily rumination time (min/d) was shorter for high than for low peNDF concentration, irrespective of the RNB, which is likely related to the longer eating time of cows offered the high peNDF diets and thus the reduced need for further mastication of feed particles. There were no differences in rumination time between RNB in diets with low peNDF concentrations, whereas daily rumination time was slightly longer for RNB– than RNB0 diets with a high peNDF concentration. An increase in rumination activity may be a behavioral response of the animals to compensate for a potential reduction in the digestibility of feed (Schiavon et al., 2015; Kand and Dickhoefer, 2021). The longer rumination time for the RNB– diet with high peNDF concentration may suggest that the extent and rate of rumen degradation were reduced by a lack of RDP supply to rumen microbes in RNB– diets with high peNDF concentration. When expressed per kilogram of DM or NDF intakes, cows spent a similar time ruminating regardless of the dietary peNDF concentration and RNB. Similarly, rumination time (5161 – 562 min/d) of high-yielding dairy cows offered a TMR increased with increasing dietary PS (X_{gm} = 11.1 – 14.6 mm) in the first *in vivo* study in Chapter 2B, but was similar across treatments when expressed per kilogram of DM (22.7 min) or NDF intake (62.2 min). Hence, DM or NDF intakes rather than peNDF concentration or RNB are the main determinants of rumination activity.

The longer total chewing time and the greater number of total chews, when expressed relative to DM and NDF intakes, in the diets with high rather than with low peNDF concentration might have stimulated saliva secretion (Mertens, 1997) and consequently N recycling. Thus, it was initially postulated that increasing peNDF promotes chewing activity and saliva secretion. However, the absolute differences in total chewing time

between treatments observed in the present study were small and probably had only a minor impact on total saliva production and thus, on N recycling. For comparison, Maekawa et al. (2002) found that the daily total chewing time of dairy cows increased by 107 min/d (14.4%) from the lowest to highest recorded total chewing time in response to increasing the forage proportion of their TMR from 40 to 60% (on DM basis) while their saliva secretion increased by 25 L/d (11%). Alternatively, raising the peNDF concentration in the present study prolonged the total chewing time of cows by only 26 min/d (2.7% from the lowest to highest recorded total chewing time) with increasing peNDF concentration in RNB– diets, which was likely too small to substantially increase the daily saliva production of cows.

3.5.3 Nitrogen Metabolism and Performance

It was hypothesized that increasing dietary peNDF concentration may at least partially compensate for possible negative effects of RNB– on rumen MPS (g N/d), diet digestibility, and thus, milk production of dairy cows while reducing urinary N excretion due to an increase in saliva secretion and N recycling. However, contrary to these expectations, rumen MPS tended to decrease with greater peNDF concentration in the diet, with no effects on RNB or the interaction between RNB and peNDF concentration. Nevertheless, there was a tendency for there to be an interaction between dietary peNDF concentration and RNB with the efficiency of MPS (g N/kg DM intake and g N/kg CP intake), with lower efficiency of MPS (g N/kg CP intake) for the RNB– diet, and with high than with low peNDF concentration, which is in accordance to the observed differences in aDMd, aOMd, and aCPd (see above). Thus the efficiency of MCP synthesis expressed per kilogram of digestible OM intake was similar across treatments. Hence,

contrary to the initial postulate, prolonged chewing time (min/d) for both RNB with high peNDF concentration presumably did not result in a significant increase in rumen N re-entry via saliva as the increase in total chewing time was small.

Other studies such as Yang and Beauchemin (2006b; X_{gm} = 7.4 – 10.3 mm) and Yang and Beauchemin (2007; X_{gm} = 7.0 – 9.1 mm) reported no effect of dietary peNDF concentration modified by PS on MPS (g N/d and g N/kg OM truly digested in the rumen). These studies, although they tested diets with greater X_{gm} of particles than in the present study, had much lower dietary $peNDF_{>8.0}$ concentrations (202 – 221 g/kg DM) due to the lower NDF concentration in the diets of Yang and Beauchemin (2006b, 321 – 333 g/kg DM; 2007, 303 –345 g/kg DM) and also the offered diets were high in CP (Yang and Beauchemin, 2006b, 165 – 168 g/kg DM; 2007, 199 – 218 g/kg DM). Thus, the greater N supply and lower peNDF concentration in the other studies may have offset the potential effects of high peNDF concentration on rumen MPS, which was observed for the RNB0 diet in the present study and confirmed the lack of N supply for the RNB– diet with high peNDF concentration.

Despite the tendencies or significant interactions observed for nutrient digestibility, there were no interactions between RNB and peNDF concentration for any of the variables related to milk yield and composition. Milk yield was similar, whereas ECM tended to be lower for the high peNDF than for the low peNDF diets, in line with the lower DM, starch, and ME intakes of cows offered the TMR with high peNDF concentrations. In a review by Zebeli et al. (2012), it was concluded the milk yield was generally unaffected by a change in dietary peNDF concentration probably due to the

relatively short experimental periods of 21 days. Milk and ECM yields were also lower for RNB– than RNB0, which confirms the results of a study by Riemeier (2004) with dairy cows fed corn silage. He observed a lower milk yield with an RNB of – 1.2 g/kg DM (18 g/d) compared to an RNB of 1.2 g/kg DM (– 18 g/d). However, in the present study, neither DM intake nor aOMd (and thus digestible OM) intake differed between RNB so that feed conversion efficiency (kg ECM/kg DM intake) was lower for RNB– than RNB0 diets. Observed decline in milk and ECM yields may be at least partly attributable to lower starch intake of cows fed RNB– than RNB0 diets, resulting in lower energy available for milk synthesis of cows fed RNB– diets. Hence, milk yield declined for RNB– diets compared to RNB0 diets. Starch digestion may be shifted from the rumen to the small intestine with increasing dietary PS (Yang and Beauchemin, 2006a). Such a shift in starch digestion to the intestine may have also occurred in the present study for both RNB diets with high peNDF concentration, resulting in a more efficient energetic use and uptake of glucose by the mammary gland for milk synthesis (Reynolds, 2006), with consequently attenuating negative effects of high dietary PS on milk yield. The lack of effect on milk fat content was not surprising, because all diets contained great amounts of peNDF, which was also indicated by the high total chewing time observed across diets as discussed above. However, milk fat yield was slightly lower for high than low peNDF and for RNB– than RNB0 diets, which was due to the numerical lower milk yield observed for low RNB and high peNDF diets.

Increasing the peNDF concentration did not improve the milk N use efficiency for either RNB diet; however, the conversion of ingested N into milk N was greater in cows fed RNB– than RNB0 diets. Similar to the results related to milk yield, there was also no

interaction between the main treatments for milk N secretion (g N/d), but it was greater in RNB0 than RNB– diets. Moreover, urinary N excretion was lower in cows fed the RNB– than those offered the RNB0 diets irrespective of the peNDF concentration. Similarly, Kand and Dickhoefer (2021) observed an increase in the proportion of ingested N secreted via milk and a lower proportion of ingested N excreted via urine in high-performing dairy cows fed diets with a negative RNB of – 3.2/kg DM (~65 g/d) than those offered a TMR with an RNB close to 0 g/kg DM. Yet, the proportion of ingested N excreted via urine in the present study was even lower in the RNB– diet with high than with low peNDF concentration. Accordingly, the proportion of ingested N excreted via feces was greater in RNB– than RNB0 diets with high peNDF concentration, due to the lower N intake and aCPd observed in cows fed RNB– diet with high peNDF concentration. Assuming similar peNDF concentrations and small changes in concentrate composition between RNB, these results indicate that lowering the RNB in combination with increasing peNDF concentration of a diet can result in greater partitioning of N excretion towards feces than urine, which confirms the present study's hypothesis. However, this shift in N partitioning was more likely due to lower digestibility and shift in starch digestion rather than due to greater N recycling from the rumino-hepatic cycle.

3.6 CONCLUSIONS

Increasing peNDF concentration decreases the DM intake of lactating dairy cows in early lactation. Dietary peNDF concentration interacts with dietary RNB, whereas the potential negative effects of increased peNDF concentration on total chewing time and chews, apparent total tract digestibility of nutrients, MPS, and N balance are more pronounced when cows are offered a diet with a low RNB. Although high peNDF

concentrations prolong total chewing time (min/kg DM and NDF intakes) and may stimulate saliva secretion and N recycling, the effect is likely too small to compensate for the negative effect of low RNB on milk yield. More research with regards to the interaction between N supply and peNDF concentration may be beneficial for a better understanding of the peNDF concept. Moreover, the long-term effects on nutrient intake and digestibility, performance, and reproduction of dairy cows fed diets with low RNB and high peNDF concentration need to be evaluated.

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CHAPTER 4

EFFECT OF PHYSICALLY EFFECTIVE FIBER CONCENTRATION ON CHEWING BEHAVIOR, RUMEN FERMENTATION, DIGESTA PASSAGE, AND PROTEIN METABOLISM OF LACTATING DAIRY COWS

4.1 ABSTRACT

The aim of the study was to evaluate the effects of physically effective neutral detergent fiber (peNDF) concentration on feed intake, chewing behavior, rumen fermentation, passage rate, nitrogen (N) metabolism, and performance in lactating dairy cows. Four lactating rumen-cannulated Holstein cows with (mean ± one standard deviation) 31.9 ± 2.69 kg/d of milk yield and 75 ± 8.4 days in milk were assigned to a 4 x 4 Latin square consisting of 21-day-periods with 13 days of adaptation and 8 days of sample collection. Cows were offered one of four total mixed rations which were identical in their chemical composition and varied solely in their peNDF concentration adjusted by reducing their mixing time in the feed mixer wagon: 60, 45, 30, and 15 min which corresponded to low (L), medium-low (ML), medium-high (MH), and high (H) peNDF concentration, respectively. The concentrations of $peNDF_{>8.0}$ (202, 208, 221, and 238 g/kg dry matter (DM); particles > 8.0 mm) increased linearly from L to H diet, respectively. Diets were formulated to have a negative rumen N balance (RNB; – 2.1 g/kg DM). The PROC MIXED in SAS 9.4 (SAS Institute Inc., Cary, United States) was used to analyze all data.

Nutrient intakes, apparent total tract digestibility of organic matter, total chewing time (min/d), and number of total chews (n/d) responded quadratically to increasing dietary peNDF concentrations with greater values observed for MH and ML, with no differences in milk yield and composition across diets. Liquid and solid digesta passage rates as well as rumen pH and concentrations of ammonium-N and volatile fatty acids in rumen fluid were not affected by peNDF concentration. However, molar proportions of acetate increased linearly and of propionate decreased linearly with increasing peNDF

concentration. Rumen microbial protein synthesis was lower for H diet than L, ML, and MH diets. Excess in utilizable crude protein supply for all diets and greater post-ruminal starch digestion resulted in greater efficiency of milk synthesis and hence, a lower proportion of ingested N was excreted via urine and a greater proportion was secreted via milk for H compared to MH, ML, and L diets. Results indicate that feeding dairy cows a negative RNB diet with varying peNDF concentrations affects their intake, digestibility, and N metabolism which suggests a need for a better understanding of the effect of negative RNB as affected by peNDF concentration.

4.2 INTRODUCTION

The physically effective neutral detergent fiber (peNDF) concept was first introduced by Mertens (1997) and combines both, the physical and the chemical characteristics of fiber, namely dietary particle size (PS) and neutral detergent fiber (NDF) concentration. Besides affecting ruminal mat formation, dietary peNDF concentration reflects the ability of a feed to promote chewing and saliva secretion, which in turn affect rumen fermentation and digesta passage (Mertens, 1997). Increasing dietary peNDF concentration of total mixed rations (TMR) for high-yielding dairy cows increased their chewing activity and rumen pH (Krause et al., 2002), passage rate of liquid digesta, total tract nutrient digestibility (Kononoff and Heinrichs, 2003a; Yang and Beauchemin, 2005), and milk fat content (Yang et al., 2001; Kononoff et al., 2003). Also, increasing dietary PS enhanced the efficiency of microbial protein synthesis (MPS) in the rumen of lactating dairy cows fed 80% alfalfa hay and 20% corn-based concentrate (Rode et al., 1985).

As dietary peNDF concentration may affect feed intake, nutrient digestibility, and rumen MPS, it may also affect the animals' nitrogen (N) excretion and N use efficiency. In this line, Heering et al. (2020; Chapter 3) observed pronounced effects of marginal increases in dietary peNDF concentration on chewing activity, nutrient digestibility, and rumen MPS as well as N partitioning when cows were offered diets with reduced rumen-degradable crude protein (CP) supply, but not ones with adequate rumen-degradable CP supply. The study of Heering et al. (2020) did not evaluate variables such as rumen fermentation and digesta passage rates to explain the observed effects. Moreover, only two peNDF concentrations were tested. However the effects of increasing peNDF concentration on nutrient intake, chewing behavior, and milk performance differ depending on its level in the diet (Heering et al., 2020) and may be linear or quadratic. As increased chewing activity enhances saliva secretion (Mertens, 1997), and therefore possibly also endogenous N supply to the rumen, there may also be linear or quadratic effects of increasing peNDF concentration on rumen MPS, N balance, and milk N use efficiency.

Hence, the aim of the present study was to determine the effects of gradually increasing dietary peNDF concentration – varied solely in dietary PS – on chewing behavior, rumen fermentation, fractional passage rate, rumen MPS, and partitioning of N excretion in dairy cows at reduced rumen-degradable CP supply. A decrease in solid digesta passage rate with increasing peNDF concentration may increase fiber digestibility, while a greater liquid passage rate may enhance the post-ruminal flow of non-structural carbohydrates that can then be used directly by the animal for milk production. Nutrient intake and digestibility might, however, decline at low and high dietary peNDF concentrations.

Additionally, enhanced N recycling via the rumino-hepatic pathway due to more intensive chewing with increasing peNDF concentration may promote MPS, and consequently increase milk N use efficiency (i.e. g milk N/100 g N intake) by the animal and lower urinary N excretion. Hence, a quadratic effect of increasing dietary peNDF concentration on feed nutrient intake and digestibility, digesta passage rates, total chewing activity (i.e. total chewing time in min/d and total number of chews in n/d), MPS (g N/d), and milk yield was expected.

4.3 MATERIALS AND METHODS

4.3.1 Animals and Housing

A study was conducted at "Les Cedres" experimental farm belonging to "Herbipôle" experimental unit (https://doi.org/10.15454/1.5572318050509348E12) of the French National Institute for Agriculture, Food, and Environment (INRAE) in Saint-Genes Champanelle, France. The study comprised four periods of 21 days (13 days adaptation to the experimental diets and 8 days of data and sample collection) and lasted from January to July 2020. Since the study had to be interrupted after the third period end of March due to Covid-19 restrictions, the fourth period was conducted only from June to July 2020. The Auvergne Rhône-Alpes Ethics Committee for Studys on Animals approved all experimental procedures (DGRI's agreement APAFIS15401-2017062616304407, France), which were compliant with the guidelines established by the European Union Directive 2010/63/EU.

Initially, four multiparous, rumen-cannulated, lactating Holstein cows were selected. Nevertheless, in the second period, one animal fell sick and was replaced by a primiparous rumen-cannulated, lactating Holstein cow. All data from the sick animal were omitted

from the final dataset and no data were available for the replacement cow for the first period. At the beginning of the study, animals had (arithmetic mean ± one standard deviation) a milk yield of 31.9 ± 2.69 kg/d, body weight (BW) of 678 ± 51.8 kg, days in milk (DIM) of 75 ± 8.4. Cows were housed in individual tie stalls bedded with sawdust and were milked twice daily at 07:30 and 15:00 h in an auto-tandem milking parlor (C100E Basic SA, Delaval, Élancourt, France) with daily milk yield being recorded by in-parlor milk meters (MM27BC, Delaval, Élancourt, France) from day 15 to 21 of each experimental period). The BW was recorded daily after each milking using an automated walk-over-weighing system (AWS100, Delaval, Élancourt, France). Cows had *ad libitum* access to fresh drinking water.

4.3.2 Study Design and Diets

The study followed a 4 x 4 Latin Square. Cows were offered a TMR with a forage to concentrate ratio of 57:43 (on a dry matter (DM) basis). The TMR was formulated according to the German Feeding Recommendation System (GfE, 2001) to supply sufficient NE_L and utilizable CP (uCP) for a 720 kg-cow to produce 30 kg/d of milk containing 40 g fat and 35 g protein per kilogram milk with a DM intake of 25 kg/d. The uCP is defined as the sum of undegraded dietary CP and microbial CP and is further used to estimate the rumen N balance (RNB) by dividing the difference between dietary CP intake and the uCP supply by 6.25 (GfE, 2001).

The RNB was a parameter used in the present study to indicate the excess of rumen N supply not used by the microbes. The TMR was formulated to have a negative rumen N balance (RNB; – 2.1 g/kg DM) so that more pronounced effects of peNDF could be investigated (Heering et al., 2020). From the one TMR, four different experimental diets

were created that had the same ingredient composition and similar nutrient and energy concentrations (Table 4.1) but varied in their PS and thus peNDF concentration.

Table 4.1 Ingredient composition of the total mixed ration fed to lactating dairy cows.

Variable	Concentration
Ingredient, g/kg dry matter	
Corn silage	240
Grass haylage	135
Grass hay	170
Barley straw	25
Concentrate mixture[1]	340
Soybean grain meal	50
Corn grain meal	40
Mineral mixture,[2] g fresh matter/d and cow	250

[1]Concentrate composition (per kg dry matter) according to manufacturer information: 300 g sugar beet pulp, 227 g corn grain, 200 g barley grain, 150 g rapeseed, 79 g soybean grain, 15 g sugarcane molasses, 10 g $Ca_3(PO_4)_2$, 9 g trace elements, 5 g Mg, and 5 g NaCl (Centraliment).
[2]Mineral composition (per kg dry matter) according to manufacturer information: 35 g P, 200 g Ca, 45 g Mg, and 10 g Na in the form of $Mg_3(PO_4)_2$, $CaHPO_4$, MgO, and NaCl, 400,000 UI Vitamin A, 120,000 UI Vitamin D3, 1,600 UI Vitamin E, 6,000 mg ZnO, 3,500 mg MnO, 1,300 mg $CuSO_4$, 90 mg $Ca(IO_3)_2$, 36 mg $CoCO_3$, and 20 mg Na_2SeO_3.

The dietary PS and thus peNDF concentration were adjusted by increasing the mixing time of the TMR in the feed mixer wagon (Unifeed Dessilmix 80, Jeulin SA, Évreux, France): 60, 45, 30, and 15 min corresponding to low (L), medium-low (ML), medium-high (MH), and high (H) peNDF concentrations, respectively. The ingredients were loaded in the following order into the mixer wagon which was equipped with horizontal stir wing mixers fitted with blades: barley straw, grass silage, grass haylage, concentrate and mineral mixtures, corn grain and soybean grain meals, and corn silage. The grass silage and haylage originated from a meadow containing a mixture of herb and grass species. The mixing protocol is given in the Appendix (Table A.4). The diets were prepared in the mornings of days 1, 3, 5, 8, 10, 12, 15, 17, and 19 of every period. Every period, each cow was fed one of the four experimental diets. From day 1 to 13, diets were

offered twice daily in two equal meals for *ad libitum* consumption at 09:00 and 16:00 h with amounts offered being adjusted daily to allow for refusals of 10% of the offered diet (on DM basis). The amount of mineral mixture (Table 1) added to the TMR was adjusted prior to TMR mixing to achieve a daily intake from the mineral mixture of 250 g fresh matter (FM)/cow. Cows were subjected to restrictive feeding from day 14 to 21 by reducing the amounts of the offered diets to 95% of the average *ad libitum* DM intake (10% refusals) of each animal recorded during the adaptation phase to ensure a stable nutrient and energy intake of cows during the sampling and data collection phase.

4.3.3 Feed Intake, Milk Performance, and Feces and Urine Excretion

Offered and Refused Diet.

Samples of offered diets were taken on days 15, 17, and 19 of each period directly after TMR mixing for analyses of chemical composition and PS distribution. For this, one sample of about 400 g FM of each diet was collected, weighed (Spider SW, Mettler, Viroflay, France), frozen at – 20°C, and lyophilized (Pilote LPCCPLS15, Cryotec, Saint-Gély-du-Fesc, France) for 96 h. Additionally, one sample of 1.2 kg FM each was taken for immediate determination of PS distribution (see below). Refusals of each animal were collected daily from the troughs and weighed in the morning before feeding and samples (400 g FM) for analysis of chemical composition were taken only when the amount of refused diet of an individual cow was ≥ 1 kg FM. Samples of refusals were also weighed, frozen at – 20°C, and lyophilized for 96 h. Lyophilized samples of offered and refused diets were weighed, ground to pass a 2-mm sieve (SM 1, Retsch GmbH, Hahn, Germany), and pooled per diet and period by taking equal amounts from each day.

Milk

Milk samples were taken daily from each cow from day 15 until day 1 of the subsequent period (before the new diet was offered), alternating between afternoon and morning milking. Samples (30 ml) were collected directly in bottles, preserved with bronopol (2-bromo-2-nitropropane-1,3-diol; final concentration 0.03%, wt/vol; UNITED CAPS, Messia-sur-Sorne, France), and stored at 4°C for later analyses of milk fat, protein, lactose, and milk urea-N (MUN).

Urine

Urine spot samples (≥ 300 ml) were collected by perineal massage once daily from each cow from day 14 to 19, alternating between afternoon and morning feeding, resulting in a total of six samples per cow and period. Immediately after collection, urine samples were homogenized and filtered through a gauze with a pore size of 0.5 mm to remove impurities. A subsample of 250 ml was then acidified using an aqueous solution of H_2SO_4 (20%; vol/vol*;* Roth GmbH, Karlsruhe, Germany) to reduce urine pH to below 3. Four aliquots of the acidified urine (40 ml each) were transferred into 50-ml falcon tubes of which two were used for the analysis of N and the other two for the analysis of purine derivatives (PD). All urine samples were stored at – 20°C until the end of each period. The two aliquots per cow and day for N analysis were then thawed and pooled by period by taking the same amount from each aliquot. From this pooled sample, two aliquots of 12 ml each were transferred into 15-ml centrifugation tubes and frozen at – 20°C until analysis of N. The two aliquots per cow and day for PD analysis collected on two consecutive days were also thawed and pooled by taking the same amount from each

aliquot, resulting in a total of three pooled samples per cow and period. Each pooled sample was then sieved through filter paper (DP 400 185, Ø 185 mm, pore size 7 – 12 µm, Hahnemühle FineArt GmbH, Dassel, Germany); subsequently, 20 ml of the filtrate were diluted with distilled water (1:5; vol/vol) and hand shaken for homogenization. Two aliquots of 12 ml each were then transferred into 15-ml centrifugation tubes and frozen again at – 20°C until analysis of PD.

Feces

Daily fecal excretion of cows and consequently, the apparent total tract nutrient digestibility were determined using the external fecal marker titanium dioxide (TiO_2, 60797, Kronos® 1171, Kronos Worldwide Inc., Dallas, United States). For this, 17.5 g (± 0.02) of TiO_2 were weighed into gelatin capsules (Size XL, 10 ml volume, Kapselwelt, Hude, Germany), which were inserted directly into the rumen through the fistula twice daily during morning and afternoon feeding (i.e. a daily dosage of 35 g/cow) from day 10 to 18. Fecal spot samples (400 g FM) were collected once daily on days 14 (afternoon) and 19 (morning) and twice daily (morning and afternoon) from day 15 to 18. Samples were taken either from boxes placed directly behind the animals to avoid discomfort to the animals or via manual grab from the rectum if cows did not voluntarily excrete any feces until 1 h after feeding. Whenever possible, sampling was conducted simultaneously with fecal sampling for passage rate determination (see the section below) to minimize the discomfort of the animals. Immediately after collection, samples were frozen at – 20°C. At the end of each sampling period, samples were thawed again and pooled by cow and period by taking the same amount from each sample. Pooled samples were then homogenized and, a subsample (180 g) taken, its weight recorded, and then frozen at

– 20°C. After lyophilization (Pilote LPCCPLS15, Cryotec, Saint-Gély-du-Fesc, France), the subsample was weighed back and ground through a 1-mm screen (SM 1, Retsch GmbH, Hahn, Germany).

The passage rates of liquid and solid digesta through the gastrointestinal tract were determined using cobalt (Co) - ethylenediaminetetraacetate (EDTA) and ytterbium (Yb)-marked fiber particles, respectively. For the preparation of Yb-marked fiber, barley straw was manually cut to 1 – 2 cm length, boiled for 1 h in a neutral-detergent-solution that was free of EDTA and then rinsed repeatedly with tap water. Thereafter, the fiber particles were dried at 70°C for 48 h, before being soaked in 12.4 m*M* aqueous Yb (III) acetate tetrahydrate (Sigma-Aldrich, St. Louis, MO; Roth GmbH, Karlsruhe, Germany) for 48 h and rinsed again with tap water. Afterward, the marked fiber was soaked in 100 m*M* solution of acetic acid for 6 h to eliminate unabsorbed Yb (Teeter et al., 1984), rinsed once more with tap water, and then dried at 70°C for 48 h. The Yb concentration of the obtained marked fiber was 66.87 mg/g DM (see chemical analysis).

The Co-EDTA powder was prepared according to the procedure described by Udén et al. (1980). For this, 249.08 g Co (II) acetate tetrahydrate (Roth GmbH, Karlsruhe, Germany), 292.24 g EDTA (PanReac AppliChem, Barcelona, Spain), and 43 g lithium hydroxide monohydrate (Alfa Aesar, Kandel, Germany) were dissolved in a 10-ml-beaker with 2 l of distilled water. Then, 200 ml of hydrogen peroxide (PanReac AppliChem, Barcelona, Spain; 30%, vol/vol) was added and the solution was left to stand at room temperature overnight. The next morning, 3 l of ethanol (Merck KGaA, Darmstadt, Germany; 95%, vol/vol) was added and the solution was refrigerated at 4°C for 24 h. The

solution was then filtered (Whatman No. 2 filter paper, Sigma-Aldrich, St. Louis, United States) and the precipitate thoroughly rinsed several times with ethanol (80%, vol/vol) until the supernatant was clear, dried at 65°C overnight, and stored in an airtight jar. Immediately before dosing, the Co-EDTA precipitate was solubilized in 250 ml of tap water.

Single doses of Yb-marked fiber (5.6 mg of Yb kg^{-1} BW; Richter and Schlecht (2006) and Co-EDTA (23.56 mg kg^{-1} of BW; Ali et al. (2019)) solution were directly administered to the rumen via the fistula on day 15 of each experimental period during morning feeding (09:00). No attempt was made to manually mix the markers with the ruminal contents. The time of marker application (t_0) was recorded individually for each cow as the time when the Co-EDTA solution was inserted into the rumen.

For Yb and Co analysis, fecal samples were taken at 0, 4, 6, 8, 10, 12, 14, 16, 22, 24, 28, 32, 36, 40, 46, 52, 58, 64, 70, 76, 82, 88, 96, 104, 112, 120, 128, 136, and 148 h after marker application (t_0; Ali et al. (2019)). Sampling was conducted as explained for fecal sampling for TiO_2 analysis. Collected samples (400 g FM) were directly homogenized and a subsample of 60 g FM was weighed into a plastic box and dried at 60°C for 2 d. Then, dried subsamples were weighed and ground to pass a 1-mm sieve (SM 1, Retsch GmbH, Hahn, Germany).

4.3.4 Chewing Behavior

The chewing behavior (i.e. eating and rumination) of animals was recorded from day 15 to 21 using automatic jaw movement recorders at 10-Hz-frequency (RumiWatch System, Itin & Hoch GmbH, Liestal, Switzerland). The 24-h-resolution option of the

RumiWatch conversion software V0.7.3.2 was used to convert recorded data to daily eating and rumination time (min/d) and daily number of eating and rumination chews (n/d) with their sum being defined as total chewing time (min/d) and total number of chews (n/d), respectively. Eating, rumination, and total chewing time as well as number of eating, rumination, and total chews were also expressed per kilogram of DM and NDF intakes. The RumiWatch conversion software V0.7.3.36 was used to convert recorded data to daily number of eating meals (meals/d) and rumination events (events/d). The converter considered a meal as occurring when eating lasted for a minimum of 7 min with an intra-meal interval of less than 7 min; a rumination event was considered as occurring when rumination lasted for a minimum of 3 min with an intra-event interval of less than 1 min. The eating time spent per meal (min/meal) was calculated by dividing the eating time (min/d) by the number of meals per day. The rumination time spent per event (min/event) was calculated by dividing the rumination time (min/d) by the number of events per day. All data were averaged per cow and period prior to statistical analysis.

4.3.5 Rumen pH and Fermentation

Ruminal fluid (about 300 ml) was collected from the ventral sack of the rumen using a probe (manufactured by INRAE) at 0.0, 1.5, 3.5, 5.5, 6.5, 8.5, 11.5, 14.5, 21.5, and 24.5 h starting after morning feeding at 9:00 on day 19 of each period. Ruminal pH was measured directly using a portable pH meter (Multi 340i, WTW GmbH, Weilheim, Germany) and a pH electrode (InLabR Easy, Mettler-Toledo, Greifensee, Switzerland), which was calibrated daily using pH 4.0 and 7.0 standards. Thereafter, samples were filtered through a nylon mesh (100 µm pore size) and two subsamples of the filtrate (10 ml each) transferred into 15-ml centrifugation tubes filled with 2 ml each of HPO_3

(25%; wt/vol; Roth GmbH, Karlsruhe, Germany). All samples were stored at – 20°C until analysis of volatile fatty acids (VFA) and ammonium-N (NH_4-N) (see below). The maximum and minimum recorded rumen pH of each animal and period was noted.

4.3.6 Sample Analyses

Proximate analysis of offered diets, refused diets, urine, and feces were performed in duplicate according to the official analytical methods in Germany (VDLUFA, 2007). The DM and crude ash (methods 3.1 and 8.1, respectively) were analyzed in offered diets, refused diets, and fecal samples to further estimate the organic matter (OM) concentration (g/kg DM). The crude lipid concentrations in offered and refused diets were determined according to method 5.1.1. Analyses of N concentrations in samples of the offered and refused diets, feces, and urine were performed using Kjeldahl digestion (KT20 KJELDAHLTHERM®, C. Gerhardt GmbH & Co. KG, Königswinter, Germany), distillation (B324, Büchi Labortechnik GmbH, Essen, Germany), and titration (719 S Titrino, Metrohm AG, Herisau, Switzerland) to further calculate the CP concentrations as the product of N concentration and 6.25 (method 4.1.1).

The NDF and ADF concentrations in offered and refused diets were determined sequentially inclusive of residual ash using an Ankom200 Fiber Analyzer (Ankom Technology, Fairport, United States) (methods 6.5.1 and 6.5.2). Additionally, fecal samples were analyzed for NDF (method 6.5.1). All NDF analyses were conducted with heat-stable α-amylase (ANKOM Technology, Macedon, United States) and sodium sulfite (Merck KGaA, Darmstadt, Germany). Starch in samples of offered diets was analyzed in duplicate using an enzymatic kit (Test-Combination Nr. 10 207 748 035, R-Biopharm AG, Darmstadt, Germany).

For samples of the offered and refused diets, gas production during 24 h of *in vitro* incubations was determined in triplicate on two days and used to estimate concentrations of metabolizable energy (ME) and net energy of lactation (NE_L) (equation 14f) and digestible OM (equation 43f) according to Menke and Steingass (1988). For uCP determination, pooled samples of offered diets were additionally pooled across treatments per period by taking the same amount of each treatment, generating one pooled diet sample per period. The uCP concentrations in pooled diet samples were estimated from changes in the NH_4-N concentrations in rumen inoculum during 24 h of *in vitro* incubation, which was performed in triplicate on two different days (Steingass et al., 2001).

The PD (allantoin and uric acid) concentrations of urine spot samples were determined in duplicate according to the procedures described by Balcells et al. (1992) and George et al. (2006) with minor modifications using HPLC (Varian 920-LC, Palo Alto, CA).

Fecal samples were analyzed in duplicate for TiO_2 using a spectrophotometer (Varian Cary 50 Bio UV-Visible Spectrophotometer, Varian Australia Pty Ltd, Australia) following the procedure of Boguhn et al. (2009) with the slight modification that the Kjeldahl digestion was carried out for 4 h instead of only 40 min.

The Yb and Co in fecal samples were extracted by sealed chamber digestion following Anderson and Henderson (1986). After a 1:10 dilution with distilled water, Yb and Co were analyzed with single determinations using atomic absorption spectrophotometry (Spectra AA, 220 FS, Varian Australia Pty Ltd, Australia).

The concentration of NH_4-N in rumen fluid was determined following the method described by Weatherburn (1967) using a spectrophotometer (Varian Cary 50 Bio, UV–

vis, Palo Alto, United States). Rumen fluid was also analyzed for VFA using a GC (GC14-A Shimadzu Corp., Kyoto, Japan) equipped with an auto-injector (AOC–20i, Shimadzu Corp., Kyoto, Japan).

Milk fat, protein, lactose, and MUN concentrations were determined in duplicate by Agrolabs in Aurillac, France, using a Fourier Transform Infrared Spectrometer (MilkoScan™ FT+, Foss, Hillerød, Denmark).

All chemical analyses were repeated when the coefficient of variation between duplicate or triplicate analyses exceeded 5%.

The PS distribution of fresh samples of offered diets was determined using the Penn State Particle Separator (Nasco, Fort Atkinson, United States) with three sieves (19.0, 8.0, and 4.0 mm) and a bottom pan (Jones and Heinrichs, 2016). Collected samples were divided into four equal parts and individually sieved, resulting in four repetitions per sample. After sample sieving, the material on each sieve and the bottom pan was weighed and the weight of material retained on each sieve was recorded and averaged across the four replicates.

4.3.7 Calculations

The physical effectiveness factor (pef) of the experimental diets is the ratio between the sum of the amount of material retained on two ($pef_{>8.0}$; 19.0 and 8.0 mm) or three sieves ($pef_{>4.0}$; 19.0, 8.0, and 4.0 mm) and the total weight of sieved material (all in g). The $peNDF_{>8.0}$ and $peNDF_{>4.0}$ concentrations of the experimental diets were calculated by multiplying the dietary NDF concentration by the $pef_{>8.0}$ and $pef_{>4.0}$, respectively (Jones and Heinrichs, 2016). The geometric mean (X_{gm}) of the PS was estimated

according to Jones and Heinrichs (2016). The $pef_{>8.0}$, $pef_{>4.0}$, $peNDF_{>8.0}$, $peNDF_{>4.0}$, and X_{gm} were then averaged per diet and period.

Daily DM intake of individual animals (kg/d) was calculated by multiplying the offered FM (kg/d) by the DM concentration (g/kg FM) in the respective diet minus the refused amount of DM calculated in the same way. The offered and refused amounts of OM, N, NDF, and ME were calculated by multiplying individual DM offered and refused amounts (kg/d) with the concentrations (g or MJ/kg DM) of the respective nutrients or ME in the diets. Finally, the nutrient and ME intakes were calculated as the difference between offered and refused amounts of the respective nutrients or ME in the diet. Starch intakes were calculated by multiplying the starch concentration in the diet (g/kg DM) with the DM intake (kg/d) of individual cows.

Daily fecal DM excretion was estimated from the daily TiO_2 dosage and the concentration of TiO_2 in fecal DM assuming a recovery rate of the marker in feces of 100% according to Glindemann et al. (2009). The apparent total tract digestibility of DM (aDMd), OM (aOMd), CP (aCPd), and NDF (aNDFd) of the ingested diets were estimated for each cow from its average nutrient intake (kg/d) across each sampling period and its fecal nutrient excretion (kg/d) estimated from the pooled sample of each sampling period. The digestible OM intake was derived from the daily OM intake of cows multiplied by the measured aOMd.

Milk protein, fat, and lactose yields were calculated by multiplying milk yield (kg/d) by the respective component concentration (g/kg milk) in milk. The energy-corrected

milk (ECM) yields were calculated according to Spiekers et al. (2009). The feed efficiency was calculated as ECM yield (kg/d) divided by DM intake (kg/d) of animals.

The urinary N loss of each animal was defined as the difference between its daily N intake and the sum of its N losses via feces, skin, and hair, and its milk N secretion (all in g/d). Milk N secretion was calculated by dividing the milk protein yield by 6.38 (McDonald et al., 2011). Skin and hair N losses (g/d) were estimated by multiplying the metabolic BW of the animals ($kg^{0.75}$) by 0.018 (g $N/kg^{0.75}$ BW) (GfE, 2001). No significant BW change was observed for any of the animals throughout the study and thus N mobilization or retention in BW was not considered in estimating the urinary N excretion. Finally, urine volume (l/d) of individual cows was calculated by dividing the estimated urinary N excretion (g/d) by the urine N concentration (g/l).

Urinary PD excretion (mmol/d) of individual animals was calculated as the product of their urine volume (l/d) and the urinary PD concentration (mmol/l). The duodenal absorption of microbial PD (mmol/d) was then estimated according to Verbic et al. (1990) and used to calculate MPS (g N/d) according to equation 5 of Chen and Gomes (1992). The efficiency of MPS was expressed in grams of N per kilogram of DM, digestible OM, and CP intakes.

The NLIN procedure (PROC NLIN method = dud) in SAS (V9.4, SAS Institute Inc., Cary, United States) was used to compute the first-time appearance of the marker in feces (TT; equivalent to post-ruminal laminar flow), the ruminal passage rate, the retention time in the mixing compartment (CMRT:2 x passage $rate^{-1}$), and the retention time in the total gastrointestinal tract (TMRT: CMRT+TT) for solid and liquid digesta. For this, the

double-compartment Gamma-2 model of Richter and Schlecht (2006) was used for the Yb-marker (i.e. solid digesta), whereas the double-compartment, age-dependent G1G1 model of Moore et al. (1992) was used for the Co-EDTA (i.e. liquid digesta). Data of passage rate and MPS parameters were averaged per animal and period.

4.3.8 Statistical Analyses

All data were analyzed using the MIXED procedure of SAS (V9.4, SAS Institute Inc., Cary, United States). For rumen fermentation parameters, the model accounted for the effects of peNDF concentration, sampling time, period, and the interaction between sampling time and peNDF concentration as fixed effects and cow per period included as repeated measurements. For the remaining variables, the model accounted for the effects of peNDF concentration, period, and DIM (covariable) as fixed effects and cow as random effect. The included DIM corrected for the effects of changing milk yield with advancing lactation as well as individual animal differences. The interaction between peNDF concentration and period was originally included in both models as well as the interaction between sampling time and period in the first model, but were removed due to insignificancy ($P \geq 0.10$). All variables were tested for linear and quadratic orthogonal contrasts using the CONTRAST statement. All means are presented as least squares means. Effects were declared significant at $P < 0.05$ and trends were recognized at $0.05 \leq P < 0.10$.

4.4 RESULTS

4.4.1 Chemical and Physical Properties of Diets

As intended, diets were isoenergetic and isonitrogenous (Table 4.2), with an RNB of

– 2.1 g/kg DM. Increasing the peNDF concentration from L to H by reducing the mixing time, increased linearly the proportion of material retained on the 19.0-mm sieve ($P < 0.01$; Table 4.3) while decreasing linearly the proportion of material in the bottom pan ($P < 0.01$). No differences in the proportions of material on the 8.0-mm and 4.0-mm sieves were found ($P \geq 0.27$). Accordingly, reducing the mixing time increased linearly $pef_{>8.0}$, $pef_{>4.0}$, and X_{gm} as well as the concentrations of $peNDF_{>8.0}$ and $peNDF_{>4.0}$ from diet L to H ($P < 0.01$ for all variables).

Table 4.2 Chemical composition of offered experimental diets differing in physically effective neutral detergent fiber (peNDF) concentration fed to lactating dairy cows (n = 4).

Variable	peNDF[1]			
	L	ML	MH	H
Chemical composition, g/kg DM				
DM,[2] g/kg fresh matter	534 ± 14.8	537 ± 22.0	535 ± 24.8	524 ± 18.8
OM	908 ± 3.0	912 ± 2.7	907 ± 7.5	908 ± 4.5
CP[2]	138 ± 0.9	138 ± 2.3	138 ± 1.6	137 ± 1.7
Crude lipid	17.4 ± 1.37	17.1 ± 2.16	17.2 ± 2.16	17.2 ± 1.34
Neutral detergent fiber	396 ± 2.3	400 ± 12.2	405 ± 17.8	406 ± 16.2
Acid detergent fiber	216 ± 4.3	219 ± 8.8	224 ± 13.9	227 ± 11.2
Starch	95.1 ± 1.26	96.8 ± 7.37	102 ± 5.3	88.0 ± 6.59
Digestible OM,[3] g/100 g OM	70.7 ± 1.85	71.1 ± 1.68	70.7 ± 2.07	70.5 ± 0.34
ME,[2,4] MJ/kg DM	10.4 ± 0.28	10.5 ± 0.24	10.4 ± 0.32	10.4 ± 0.04
NE_L,[2,4] MJ/kg DM	6.3 ± 0.20	6.3 ± 0.17	6.3 ± 0.22	6.3 ± 0.03
Utilizable CP,[5] g/kg DM	150 ± 4.0	150 ± 4.8	150 ± 4.7	150 ± 1.7
RNB,[2] g/kg DM	-2.1	-2.1	-2.1	-2.1

[1]peNDF concentration: low (L), medium-low (ML), medium-high (MH), and high (H).
[2]CP = crude protein; DM = dry matter; ME = metabolizable energy; net energy of lactation = NE_L; OM = organic matter; RNB = rumen nitrogen balance.
[3]Estimated by the *in vitro* Hohenheim gas test method (Menke and Steingass, 1988; equation 43f).
[4]Estimated by the *in vitro* Hohenheim gas test method (Menke and Steingass, 1988; equation 14f).
[5]Estimated by the modified *in vitro* Hohenheim gas test method (Steingass et al., 2001) to further calculate RNB as RNB (g/kg DM) = (CP (g/kg DM) – utilizable CP (g/kg DM))/6.25 (GfE, 2001) with n = 1.

Table 4.3 Physical characteristics of offered experimental diets differing in physically effective neutral detergent fiber (peNDF) concentration fed to lactating dairy cows (n = 4).

Variable	peNDF[1]				SEM[2]	Contrast	
	L	ML	MH	H		Linear	Quadratic
Particle size distribution, g/100 g fresh matter retained							
19.0 mm	17.4	18.5	21.1	25.0	0.84	<0.01	0.02
8.0 mm	33.5	33.5	33.4	33.6	0.68	1.00	0.84
4.0 mm	20.5	21.0	21.7	21.0	0.41	0.40	0.27
Pan	28.6	27.0	23.8	20.5	0.93	<0.01	0.13
$pef_{>8.0}$[3]	0.51	0.52	0.55	0.59	0.009	<0.01	0.03
$pef_{>4.0}$[3]	0.71	0.73	0.76	0.80	0.009	<0.01	0.14
$peNDF_{>8.0}$,[4] g/kg DM[2]	202	208	221	238	4.8	<0.01	0.14
$peNDF_{>4.0}$,[4] g/kg DM	283	292	309	323	5.1	<0.01	0.56
X_{gm}[5] of particle size, mm	7.3	7.6	8.2	9.0	0.18	<0.01	<0.01

[1]peNDF concentration: low (L), medium-low (ML), medium-high (MH), and high (H).
[2]DM = dry matter; SEM = standard error of means.
[3]$pef_{>8.0}$ and $pef_{>4.0}$ = physical effectiveness factor determined as the proportion of DM retained on 19.0- and 8.0-mm sieves, and 19.0-, 8.0-, and 4.0-mm sieves, respectively.
[4]$peNDF_{>8.0}$ and $peNDF_{>4.0}$ = peNDF determined as the dietary neutral detergent fiber concentration multiplied by $pef_{>8.0}$ and $pef_{>4.0}$, respectively (Jones and Heinrichs, 2016).
[5]X_{gm} = geometric mean of particle size determined according to Jones and Heinrichs (2016).

4.4.2 Body weight, Feed Intake, and Nutrient Digestibility

The BW was similar across dietary peNDF concentration ($P \geq 0.40$; Table 4.4). Intakes of DM, OM, digestible OM, ME, CP, and starch responded quadratically to increasing dietary peNDF concentrations with greater intakes for both, ML and MH, than for the H and L diets ($P \leq 0.03$). Increasing dietary peNDF concentration tended to quadratically affect aOMd ($P = 0.06$) with the greatest aOMd observed for ML and MH diets. However, no linear ($P \geq 0.19$) or quadratic ($P \geq 0.11$) responses to increasing peNDF concentration were observed for aDMd, aCPd, and aNDFd.

4.4.3 Milk Production and Composition

There were no linear or quadratic relationships between dietary peNDF concentration and any of the variables related to milk yield and composition ($P \geq 0.10$; Table 4.5). Feed efficiency (kg ECM yield/kg DM intake) responded quadratically to increasing peNDF concentration, with greater values observed for H and L diets, where also the lowest DM intakes were observed ($P < 0.01$).

Table 4.4 Nutrient and energy intakes and apparent total tract nutrient digestibility (ATTD) in lactating Holstein cows fed diets differing in physically effective neutral detergent fiber (peNDF) concentration.

Variable	peNDF[1]				SEM[2]	Contrast	
	L n = 4	ML n = 4	MH n = 3[3]	H n = 4		Linear	Quadratic
Body weight, kg	687	692	692	685	46.5	0.80	0.40
Intake, kg/d							
Dry matter	21.9	22.1	22.5	20.2	1.45	0.02	0.01
OM[2]	19.9	20.1	20.3	18.2	1.31	0.02	0.01
Digestible OM[4]	13.1	13.5	13.7	11.8	0.89	0.03	0.01
NDF[2]	8.7	8.8	8.9	8.1	0.58	0.08	0.03
ME,[2,5] MJ/d	228	231	234	209	15.1	<0.01	<0.01
Starch	2.1	2.1	2.3	1.8	0.15	0.03	<0.01
ATTD, g/100 g							
Dry matter	63.0	64.1	63.7	61.5	4.18	0.27	0.16
OM	65.8	67.4	66.9	64.2	4.37	0.22	0.06
Crude protein	57.2	59.8	57.6	56.0	3.89	0.28	0.11
NDF	51.3	52.9	52.3	50.7	3.82	0.76	0.41

[1]peNDF concentration: low (L), medium-low (ML), medium-high (MH), and high (H).
[2]ME = metabolizable energy; NDF = neutral detergent fiber; OM = organic matter; SEM = standard error of means.
[3]No data available in period 1.
[4]Digestible OM intake estimated as the difference between total OM intake and fecal OM excretion.
[5]Dietary ME concentration (MJ/kg DM) estimated by the *in vitro* Hohenheim gas test method (Menke and Steingass, 1988; equation 14f) multiplied by dry matter intake (kg/d).

Table 4.5 Milk yield and composition of lactating dairy cows fed diets differing in physically effective neutral detergent fiber (peNDF) concentration.

Variable	peNDF[1]				SEM[2]	Contrast	
	L	ML	MH	H		Linear	Quadratic
	n = 4	n = 4	n = 3[3]	n = 4			
Milk yield, kg/d	27.2	26.7	25.8	27.2	1.90	0.70	0.20
ECM yield,[2,4] kg/d	27.3	26.7	26.0	27.0	1.88	0.39	0.13
Milk fat, g/kg	40.6	40.4	40.4	39.7	2.79	0.58	0.82
Milk protein, g/kg	33.3	33.4	34.1	33.2	2.22	0.69	0.13
Milk lactose, g/kg	51.4	51.6	51.3	51.7	3.33	0.60	0.82
Milk fat yield, kg/d	1.10	1.07	1.03	1.08	0.077	0.18	0.10
Milk protein yield, kg/d	0.91	0.89	0.88	0.90	0.063	0.76	0.36
Milk lactose yield, kg/d	1.41	1.38	1.34	1.41	0.099	0.70	0.14
MUN,[2] mg/dl	22.5	21.8	24.0	23.0	1.60	0.16	0.79
Feed efficiency[5]	1.24	1.21	1.15	1.33	0.085	0.12	<0.01

[1]peNDF concentration: low (L), medium-low (ML), medium-high (MH), and high (H).
[2]ECM = energy-corrected milk; MUN = milk urea-nitrogen; SEM = standard error of means.
[3]No data available for period 1.
[4]ECM yield calculated as milk yield (kg) x ((0.38 x milk fat (g/100 g) + 0.21 x milk protein (g/100 g) + 1.05)/3.28) according to Spiekers et al. (2009).
[5]Calculated as ECM yield (kg/d) divided by DM intake (kg/d).

4.4.4 Chewing Behavior and Rate of Passage

A quadratic response of eating time (min/d), number of eating chews (n/d), and number of meals (n/d) to increasing peNDF concentration was observed with greater eating time, number of eating chews, and meals for ML and MH diets ($P \leq 0.04$; Table 4.6). Eating time and number of eating chews per kilogram of DM or NDF intakes as well as feeding rate (g DM intake/min eating time) were not affected by peNDF concentration ($P \geq 0.31$). Rumination time (min/d), number of rumination chews (n/d), and number of rumination events (n/d) were not affected by dietary peNDF concentration ($P \geq 0.13$), but rumination time and number of rumination chews per kilogram of DM or NDF intakes increased linearly with increasing peNDF concentration ($P \leq 0.04$).

As a result, total chewing time (min/d) responded quadratically to increasing dietary peNDF concentration, whereas a greater total chewing time was observed for ML and MH diets than for L and H diets ($P = 0.02$; Table 4.6). Similarly, a tendency for a quadratic response of total number of chews to increasing peNDF concentration was observed following the same pattern as total chewing time ($P = 0.05$). Total chewing time per kilogram DM intake tended to increase linearly with increasing peNDF concentration ($P = 0.09$). Nonetheless, total chewing time per kilogram NDF intake and total number of chews per kilogram DM or NDF intakes were similar across peNDF concentrations ($P \geq 0.12$).

There were neither differences in rumen nor in total digestive tract liquid and solid digesta passage rates and retention times across the four diets ($P \geq 0.37$; Table 4.7). Liquid passage rate averaged 10.6, 10.6, 10.4, and 10.4 %/h and solid passage rate averaged 3.5, 3.7, 3.4, and 3.4 %/h for L, ML, MH, and H diets, respectively.

Table 4.6 Chewing behavior of lactating dairy cows fed diets differing in physically effective neutral detergent fiber (peNDF) concentration.

Variable	peNDF[1]				SEM[2]	Contrast	
	L	ML	MH	H		Linear	Quadratic
	n = 4	n = 4	n = 3[3]	n = 4			
Eating							
min/d	369	382	410	347	9.5	0.46	0.01
min/kg DM[2] intake	17	17	18	18	0.4	0.37	0.36
min/kg NDF[2] intake	43	43	46	44	0.9	0.48	0.38
n/d	28,014	29,105	31,838	25,460	954	0.48	0.04
n/kg DM intake	1,277	1,321	1,424	1,290	36.6	0.71	0.31
n/kg NDF intake	3,229	3,314	3,591	3,226	97.3	0.79	0.33
meals/d	7.7	8.7	8.8	7.1	0.47	0.54	0.04
min/meal	52	48	47	52	5.3	0.88	0.17
Rumination							
min/d	551	565	568	557	9.1	0.71	0.28
min/kg DM intake	25	26	25	28	0.5	0.01	0.08
min/kg NDF intake	63	64	65	70	1.4	0.01	0.09
n/d	37,127	38,258	38,087	36,600	1,026	0.62	0.13
n/kg DM intake	1,694	1,738	1,720	1,838	58	0.04	0.27
n/kg NDF intake	4,283	4,357	4,378	4,619	163	0.04	0.30
events/d	14.5	14.0	15.1	15.3	0.36	0.27	0.59
min/event	39	41	38	37	3.6	0.34	0.28

Total chewing							
min/d	920	947	973	899	13.8	0.63	0.02
min/kg DM intake	42	43	44	45	0.6	0.09	0.85
min/kg NDF intake	106	108	111	113	1.8	0.12	0.91
n/d	65,185	67,407	70,570	62,705	1,411	0.66	0.05
n/kg DM intake	2,973	3,062	3,177	3,162	71.7	0.29	0.67
n/kg NDF intake	7,518	7,676	8,050	7,926	211	0.32	0.65

[1]peNDF concentration: low (L), medium-low (ML), medium-high (MH), and high (H).
[2]DM = dry matter; NDF = neutral detergent fiber; SEM = standard error of means.
[3]No data available in period 1.

Table 4.7 Liquid and solid digesta passage in lactating dairy cows fed diets differing in physically effective neutral detergent fiber (peNDF) concentration.

Variable	peNDF[1]				SEM[2]	Contrast	
	L n = 4	ML n = 4	MH n = 3[2]	H n = 4		Linear	Quadratic
Liquid digesta passage							
λ,[2] %/h	10.6	10.6	10.4	10.4	0.74	0.70	0.96
TT,[2] h	6.5	6.8	6.7	6.8	0.21	0.45	0.80
CMRT,[2] h	19.0	19.3	19.4	19.5	0.54	0.72	0.94
TMRT,[2] h	25.6	26.0	26.3	26.4	0.61	0.48	0.82
Solid digesta passage							
λ, %/h	3.5	3.7	3.4	3.4	0.24	0.37	0.74
TT, h	7.8	6.6	7.5	7.3	0.57	0.95	0.55
CMRT, h	57.5	55.2	60.3	58.5	1.89	0.54	0.93
TMRT, h	65.3	61.7	67.8	65.8	2.01	0.63	0.82

[1]peNDF concentration: low (L), medium-low (ML), medium-high (MH), and high (H).
[2]CMRT = retention time in the rumen; λ = ruminal passage rate; SEM = standard error of means; TMRT = retention time in total gastrointestinal tract; TT = post-ruminal transit time.
[3]No data available for period 1.

4.4.5 Rumen pH and Fermentation

Ruminal NH_4-N concentration and average rumen pH were similar between peNDF concentrations ($P \geq 0.35$; Table 4.8); however, the maximum rumen pH increased linearly and the minimum rumen pH decreased linearly with increasing peNDF concentration of the diets ($P < 0.01$ for both variables). Total VFA concentration and the molar proportions of butyrate and valerate in rumen fluid were similar across peNDF concentrations ($P \geq 0.12$). However, the molar proportion of propionate decreased linearly while those of acetate, as well as the acetate to propionate ratio, increased linearly with increasing peNDF concentration ($P \leq 0.03$).

Table 4.8 Ruminal pH and fermentation parameters of lactating dairy cows fed diets differing in physically effective neutral detergent fiber (peNDF) concentration.

Variable	peNDF[1]				SEM[2]	Contrast	
	L	ML	MH	H		Linear	Quadratic
pH							
Mean[3]	6.41	6.45	6.39	6.43	0.06	0.96	0.88
Minimum[4]	5.86	5.85	5.78	5.76	0.039	<0.01	0.55
Maximum[4]	6.89	6.95	6.89	7.00	0.018	<0.01	0.08
Total VFA,[2,5] m*M*	84.2	85.2	81.4	83.8	3.79	0.72	0.82
VFA profile, mmol/100 mmol							
Acetate	66.7	66.9	68.3	68.0	0.30	0.03	0.66
Propionate	18.7	18.6	17.3	17.9	0.19	<0.01	0.25
Butyrate	11.3	11.4	11.1	10.7	0.16	0.14	0.57
Valerate	2.5	2.5	2.4	2.4	0.03	0.16	0.66
Acetate:Propionate	3.61	3.62	4.00	3.86	0.056	<0.01	0.44
NH_4-N,[2,5] m*M*	2.9	2.8	3.3	3.2	1.35	0.64	0.98

[1]peNDF concentration: low (L), medium-low (ML), medium-high (MH), and high (H).
[2]NH_4-N = ammonium-nitrogen, SEM = standard error of means, VFA = volatile fatty acids.
[3]Mean rumen pH: n = 29 for MH, n = 39 for ML and L, and n = 40 for H.
[4]Minimum and maximum rumen pH: n = 3 for MH and n = 4 for H, ML, and L.
[5]VFA and NH_4-N parameters: n = 18 for MH and n = 24 for H, ML, and L.

4.4.6 Nitrogen Metabolism and Turnover

The amount (g N/d) and efficiency of rumen MPS (g N/kg DM intake) responded quadratically to increasing peNDF concentration with greater amount and efficiency of MPS observed for ML and MH diets ($P \leq 0.04$; Table 4.9). Efficiency of rumen MPS expressed per kilogram digestible OM and CP intakes decreased linearly with increasing peNDF concentration ($P \leq 0.04$).

Milk N secretion was similar between peNDF concentrations ($P \geq 0.37$). A quadratic relationship was observed between peNDF concentration and daily urinary N loss ($P < 0.01$), where urinary N loss was greater for ML and MH diets. The proportion of ingested N excreted via feces was similar between peNDF concentrations ($P \geq 0.11$); however, the proportion of ingested N excreted via urine and secreted via milk responded quadratically to increasing peNDF concentration with greater proportion of ingested N excreted via urine and less secreted via milk for ML and MH diets ($P < 0.01$ for all variables).

Table 4.9 Nitrogen (N) balance and rumen microbial protein synthesis in lactating dairy cows fed diets differing in physically effective neutral detergent fiber (peNDF) concentration.

Variable	peNDF[1]				SEM[2]	Contrast	
	L	ML	MH	H		Linear	Quadratic
	n = 4	n = 4	n = 3[3]	n = 4			
N intake, g/d	484	487	499	445	32	0.01	<0.01
Microbial protein synthesis[4]							
g N/d	373	428	388	279	35.5	0.08	0.02
g N/kg dry matter intake	17	19	17	14	1.2	0.03	0.03
g N/kg digestible organic matter intake[5]	28	31	28	24	1.6	0.03	0.04
g N/kg crude protein intake	123	141	124	100	8.2	0.03	0.04
Milk N secretion, g/d	142	139	138	141	9.8	0.70	0.37
N excretion, g/d							
Urinary N	132	149	141	106	11.6	0.02	<0.01
Fecal N	207	196	211	195	14.2	0.45	0.68
Skin and hair N losses, g/d	2.4	2.4	2.4	2.4	0.16	0.59	0.38
Milk N, g/100 g N intake	29.3	28.6	27.5	31.3	2.01	0.11	<0.01
Urinary N, g/100 g N intake	27.3	30.8	29.4	23.8	2.31	0.04	<0.01
Fecal N, g/100 g N intake	42.9	40.2	42.4	44.0	2.94	0.28	0.11

[1]peNDF concentration: low (L), medium-low (ML), medium-high (MH), and high (H).
[2]SEM = standard error of means.
[3]No data available for period 1.
[4]Estimated from the duodenal absorption of microbial purine derivatives (estimated according to Verbic et al. (1990)) according to equation 5 of Chen and Gomes (1992).
[5]Digestible organic matter (OM) intake estimated as the difference between total OM intake and fecal OM excretion.

4.5 DISCUSSION

4.5.1 Relationships between Physically Effective Fiber, Feed Intake, Nutrient Digestibility, and Performance

Feeding dairy cows diets containing long dietary PS reduces solid passage rate and distends the reticulorumen, therefore limiting their DM intake (Allen, 2000). In the present study, a quadratic response of intake, digestibility, and performance in cows to increasing peNDF concentration was expected. Reducing the mixing time of the TMR resulted in a linear increase in $peNDF_{>8.0}$ concentration from 202 to 238 g/kg DM and in X_{gm} of PS from 7.3 to 9.0 mm. Indeed, increasing dietary peNDF concentration had a quadratic effect on nutrient intakes, with greater intakes observed for ML and MH diets. Positive effects on feed intake of smaller dietary PS were observed mostly in studies with a great proportion of forage (Soita et al., 2002, 100% barley silage fed to steers) or those including long forage PS in the diet (Kononoff et al., 2003, X_{gm} = 7.4 – 8.8 mm; Kononoff and Heinrichs, 2003, X_{gm} = 4.1 – 6.8 mm, fed to lactating Holstein cows). With X_{gm} = 7.3 – 9.0 mm, such as in the present study, extended the range of previous studies but confirmed their results. Surprisingly, passage rate, CMRT, and TMRT of liquid and solid digesta were similar across all peNDF concentrations. Moreover, nutrient intakes were lower for L than for ML and MH diets. Nonetheless, the differences in DM intake between L and both, ML and MH, were small (≤ 0.6 kg DM/d) and might not be of biological relevance. The quadratic effect observed was rather caused by the pronounced decrease in DM intake of cows fed the H diet, which was likely related to a greater rumen fill due to the long PS, and the lower aOMd of the diet (see below).

In line with the effects on DM intake, a tendency for a quadratic response of aOMd to varying peNDF concentrations was observed where aOMd was greater for ML and MH diets. Although mean aDMd, aNDFd, and aCPd were statistically similar across peNDF concentrations, they followed numerically the same pattern as aOMd. Similar to DM intake, differences in mean aOMd between ML and MH, and L diets were small (≤ 1.6 g/100 g OM), which suggested that the biological significance of these differences was also small. Yet, the lower aOMd for H was likely due to reduced surface area of the dietary particles for microbial adherence and attack due to the long particles (Zebeli et al., 2012). As the passage rate of solids was similar across dietary peNDF concentrations, prolonged CMRT did not compensate for supposedly reduced microbial fermentation at high peNDF concentration. Thus, the longer daily rumination time (per kilogram of DM and NDF intakes) for H than for L, ML, and MH diets supports the assumption of reduced ruminal OM and NDF degradation (Schiavon et al., 2015; Kand and Dickhoefer, 2021). Additionally, the observed decline in aOMd for H diets may have been due to the lower starch and higher NDF and ADF concentrations as compared to the other diets, although these differences were rather small.

Although OM intake and aOMd were greater for both, ML and MH diets, milk yield was not affected by dietary peNDF concentration in the present study. Milk fat, protein, and lactose contents and yields were also not affected by dietary peNDF concentration despite the differences in nutrient intakes and digestibility. There were no differences in the BW and thus ME requirements for maintenance of the animals between treatments. Similarly, increased chewing intensity due to increasing dietary PS would have even increased the energy expenditures for chewing (Susenbeth et al., 1998). The lack of

effects on milk yield and composition despite the pronounced decline in digestible OM and ME intakes cannot be fully explained. However, cows fed the L, ML, and MH diets might have deposited body fat and/or those offered the H diet might have mobilized body tissue to sustain milk yield. Due to the short duration of the experimental periods, such changes in BW might not have been detected, although animals were weighed frequently before morning feeding. Additionally, it has been shown that increasing dietary peNDF concentration by varying the PS of a barley silage-based TMR shifts starch digestion from the rumen to the small intestine in high-yielding dairy cows (Yang and Beauchemin, 2006a). In the present study, the molar proportion of propionate linearly decreased and that of acetate increased linearly with increasing dietary peNDF concentration, which may indicate a shift in starch digestion to the duodenum.

Also, although animals were fed a diet with negative RNB, and hence, low N supply to rumen microbes, mean uCP supply exceeded the requirements of animals (2,688 g/d; GfE, 2001) in all diets with 3,308, 3,329, 3,436, and 3,145 g/d for L, ML, MH, and H diets, respectively. This excess of uCP supply may explain the similar milk protein content and yield across diets. Moreover, increasing CP flow to the small intestine may improve starch digestion through greater pancreatic amylase capacity (Harmon and Swanson, 2020). The energy in form of glucose arising from postruminal starch digestion can be used energetically more efficiently by the animal for milk synthesis as well as for omental and mesenteric fat as compared to the energy derived from starch fermented in the rumen (Reynolds, 2006). Hence, the excess in uCP supply may have improved intestinal starch digestion in the present study, and thus may have enabled cows fed the H diet to maintain their milk yield and BW. However, the magnitude of the positive effect

of the shift in starch digestion in combination with excess uCP was likely to be small, because dietary starch concentration was lower in the H diet than in the other diets.

The lack of effects on milk fat content and yield was presumably due to the overall high peNDF concentrations of offered diets, with the lowest dietary $peNDF_{>8.0}$ concentration in the present study of 202 g/kg DM, and thus, was much greater than the recommendation for $peNDF_{>8.0}$ by Zebeli et al. (2008) of 149 – 185 g/kg DM for dairy cows. Consequently, high milk fat content of, on average, 40.3 g/kg milk was observed in the present study.

4.5.2 Relationships between Physically Effective Fiber, Chewing Behavior, and Passage Rate

The present study hypothesized that increasing dietary peNDF concentration also increases the intensity of total chewing activity (minutes and chews per kilogram DM intake). Due to a quadratic response of nutrient intake and aOMd to increasing peNDF concentration, total chewing activity (in minutes and chews per day), and liquid and solid digesta passage rates were also expected to be quadratically affected by peNDF concentration. Similarly, total chewing time (min/kg DM intake) tended to increase linearly with increasing peNDF concentration, although total number of chews (chews/kg DM intake) did not differ between diets. The increase in total chewing time (min/kg DM intake) in response to increasing peNDF concentration resulted from a prolonged rumination time (min/kg DM intake) rather than a longer eating time (min/kg DM intake). Nonetheless, due to the quadratic response of DM intake to increasing peNDF concentration, daily rumination time (min/d) was similar between diets, whereas daily eating time (min/d) responded quadratically with greater eating time for both, ML and

MH diets, than for L and H diets.

Compared to eating time (min/kg DM intake), rumination time (min/kg DM intake) is generally less affected by dietary PS or peNDF concentration, because additional mastication during eating reduces the need for additional PS break-down during rumination (Beauchemin, 2018). Similarly, Heering et al. (2019, Chapter 2B) observed a linear increase in eating time (min/kg DM intake) but not in rumination time (min/kg DM intake) as $peNDF_{>8.0}$ concentration increased from 237 to 283 g/kg DM. In comparison to the present study (X_{gm} = 7.3 – 9.0 mm), the X_{gm} of PS was greater in Heering et al. (2019; X_{gm} = 11.1 – 14.6 mm). Allen (1997) proposed that only small or limited increases in rumination time can be expected when X_{gm} is above a threshold of 10 mm, which confirms observations in the present study. Moreover, diets in the present study contained more NDF and had a greater proportion of forage, so that cows needed more time to chew per kilogram NDF intake (43 – 44 min) compared to those in the study of Heering et al. (2019; 31 – 38 min). However, intensive mastication during eating might still not have been sufficient to reduce the need for additional mastication during rumination, as rumination time per kilogram NDF intake was still longer in the present study (63 – 70 min) than that reported by Heering et al. (2019; 60 – 65 min).

Passage rate of solid digesta was similar across diets in the present study and was not affected by the peNDF concentration of the diet. This is in line with observations by Yang et al. (2001) who were feeding high-yielding dairy cows TMR with increasing forage PS, but is in contrast to the results of Rode et al. (1985) where passage rates of solid material declined when the forage PS of diets fed to dairy cows was increased. Moreover, previous studies (Rode et al., 1985; Kononoff and Heinrichs, 2003a) recorded a faster liquid

passage rate in dairy cows with greater dietary PS, which was probably due to a rise in rumination activity (minutes and chews per kilogram DM intake) and salivary flow as forage PS in the diets fed to them increased. In contrast, passage rate of liquids was not affected by peNDF in the present study. However, compared to the situation in the present study, Rode et al. (1985) tested a broader range of dietary PS (ground to long alfalfa hay) with a greater proportion of forage (80% on DM basis) in their diets. Moreover, the lack of effect of peNDF on solid digesta passage rate in the present study and that of Yang et al. (2001) might be due to the increase in total chewing time (min/kg DM intake) and mastication with increasing peNDF concentration, suggesting that PS reduction is not a rate-determining step in ruminal particulate passage. Furthermore, PS reduction due to greater chewing intensity would also explain the lack of effect observed on liquid passage rate. Besides the narrow range in peNDF concentrations in the present study, the greater chewing intensity with increasing peNDF concentration may not have induced change in ruminal mat formation and hence in liquid passage rate although, an increase in chewing and rumination intensity (min/kg DM intake) for MH and ML diets probably enhanced saliva secretion, and hence N recycling, as speculated by Kand and Dickhoefer (2021).

Nevertheless, the increments in total saliva secretion and thus in N recycling were probably very small. For instance, Maekawa et al. (2002) determined an increase in saliva production of only 25 L/d (11%) when total chewing time of dairy cows increased by 107 min/d (14.4%) as the proportion of forage in their diet was raised from 40 to 60%. This difference in daily chewing time was considerably greater than the one observed in the present study, in which daily chewing time increased by only 74 min (8.2%) from H diet (with the shortest daily chewing time) to MH diet (with the longest daily chewing time).

The lack of effect of peNDF concentration on rumen pH contradicts the commonly accepted principle that greater peNDF concentration promotes chewing and salivation and hence increases buffering capacity within the rumen (Mertens, 1997). The average rumen pH across all peNDF concentrations was 6.43 and similar to that in other studies with diets of comparable forage to concentrate ratios than in the present study (Kononoff et al., 2003; Yang and Beauchemin, 2009). Moreover, the observed mean rumen pH was greater than the recommended minimum mean rumen pH of 6.16 that is needed to lower the risk of acidosis in high-yielding dairy cows (Zebeli et al., 2008). The satisfactory pH and the similar pH across diets are attributable to the overall high NDF concentration (396 – 406 g/kg DM) and range of X_{gm} in experimental diets (X_{gm} = 7.3 – 9.0 mm) offered to cows in the present study as well as their low starch concentrations (88 – 102 g/kg DM). In addition, total chewing time of cows was ≥ 42 min/kg DM intake, which is greater than the threshold of ≥ 30 min/kg DM intake of chewing proposed by Sudweeks et al. (1981) to reduce the risk of digestive disorders.

As was the case with mean rumen pH, there was no difference in total VFA concentrations in rumen fluid between diets. The present study, however, only analyzed VFA concentrations and not the actual amount produced within the rumen, which may have been different depending on the liquid turn-over rate and the rate of VFA absorption via the rumen wall (Storm and Kristensen, 2010). Importantly, the mixing of ruminal contents through ruminal motility is decisive for an effective VFA absorption (Storm and Kristensen, 2010) and in this regard the process of eating and rumination (Beauchemin, 2018). Thus, greater chewing time (in min/kg DM intake) with increasing peNDF concentration probably stimulated VFA absorption via the rumen wall, leading to similar

VFA concentrations across the experimental diets.

4.5.3 Relationships between Physically Effective Fiber, Nitrogen Metabolism and Turnover

It was proposed that increasing peNDF concentration promotes rumen MPS (g N/d) via enhanced N recycling due to increased total chewing time (min/d) and number of chews (n/d) of cows, when N is limiting in the rumen. However, after a certain peNDF concentration, both MPS and chewing activity, is expected to decrease. As expected, increasing peNDF concentration elevated MPS (g N/d) only up to a certain point, after which the dietary PS was too large and negatively affected feed intake and aOMd. The quadratic response in MPS (g N/d) with increasing peNDF concentration is in line with results of a study of Yang and Beauchemin (2006b) with lactating dairy cows offered diets with 47% barley silage and 53% concentrate on DM basis, as well as $peNDF_{>8.0}$ concentrations of 100 – 176 g/kg DM. However, while efficiency of rumen MPS (g N/kg digestible OM intake) also tended to respond quadratically to increasing peNDF concentrations in the study of Yang and Beauchemin (2006b), it declined linearly with increasing peNDF concentration in the present study. The efficiency of MPS is, among other factors, affected by type of feed, forage quality, synchronization of N and energy from the diet, the rumen pH and, outflow rate (Harun and Sali, 2019). The lower efficiency of rumen MPS for the H diet compared to the other diets was probably attributable to a lower supply of rumen-degradable CP and fermentable energy due to lower digestible OM, starch, and N intakes, the lower aCPd, and the reduced ruminal starch degradation in response to increasing dietary PS (see above), as well as the unexpectantly similar solid and liquid digesta passage rates.

Irrespective of the differences in rumen MPS, ruminal NH_4-N and MUN concentrations were similar across diets. Since only spot-measurements of the concentration of NH_4-N were made, possible effects of dietary peNDF concentration on ruminal NH_4-N production cannot be fully excluded. Nevertheless, the excess of uCP supply over the animals' requirements was greater for L, ML, and MH diets as compared to the H diet. Together with the prolonged chewing time and greater number of chews, this presumably resulted in greater endogenous N supply to the rumen microbes as indicated by Kand and Dickhoefer (2021). Additionally, CP intakes were greater for L, ML, and MH diets than for the H diet, suggesting that ruminal NH_4-N release was probably higher for these diets. At the same time, the greater MPS for the L, ML, and MH diets as compared to the H diet indicates an increased incorporation of NH_4-N by rumen microbes, thus explaining the similar ruminal NH_4-N and also MUN across diets. All four diets resulted in high MUN, which is most likely due to the excess of uCP supply to cows.

Feeding excess N to animals beyond their requirements is known to increase excretion of N, in particular via urine (Huhtanen et al., 2008), while reducing the proportion of ingested N being utilized by the animal for milk protein synthesis (Castillo et al., 2000). In the present study there was a quadratic response of N intake to increasing peNDF concentration. The proportion of N excreted via urine was greater, and the proportion of ingested N secreted via milk was lower for ML and MH diets while there was no effect on the proportion of ingested N excreted via feces. Although N intake was lower and the proportion of ingested N secreted via milk was greater in L compared to ML and MH diets, absolute differences in both parameters between these treatments were small, and may not be of relevance. Instead, the differences in the proportions of ingested N excreted

via urine or secreted via milk were greater for the H diet compared to the other diets, due to a much lower N intake of the animals receiving this diet. Moreover, the greater milk N use efficiency (g milk N/100g N intake) observed for the H diet could also be due to enhanced efficiency of milk synthesis as a result of greater intestinal starch digestion and uptake of glucose in the duodenum (see above).

Increasing the efficiency of N use in dairy production is currently an environmental challenge. Feeding diets with reduced N supply (i.e. a slightly negative RNB) and optimal dietary peNDF concentration with respect to feed intake and health to dairy cows may provide a promising opportunity to reduce potential negative effects of low RNB on apparent total tract digestibility of nutrients and rumen MPS, while at the same time maintaining animal performance and decreasing N emissions.

4.6 CONCLUSIONS

Under conditions similar to those in the present study, increasing peNDF concentration of diets with negative RNB and moderate to high NDF concentrations quadratically affects nutrient intake and digestibility, with no effects on ruminal fermentation, passage rates of solid and liquid digesta, as well as performance of high-yielding dairy cows. Increasing dietary peNDF concentration also increases total chewing time and affects the fate of ingested N in the animal, by enhancing amount and efficiency of MPS, increasing the percentage of ingested N being secreted via milk and lowering urine N excretion, suggesting an increase in saliva secretion and N recycling. Hence, offering dairy cows diets with a low RNB and high dietary peNDF concentration may reduce potential negative effects of low RNB, while maintaining the animals' performance and providing

environmental benefits. Yet, a better understanding is needed of the interaction between RNB and dietary peNDF concentration and its effect on protein metabolism of dairy cows.

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CHAPTER 5

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Chapter 5

A major challenge in feeding today's high-yielding dairy cows is to balance the energy- and protein-dense diets with adequate fiber supply to maximize the animal's milk production, while maintaining the animal's health (Zebeli et al., 2012). The concept of physically effective neutral detergent fiber (peNDF) has been widely adopted to measure the adequacy of fiber in dairy cows' diets to reduce the risk of rumen acidosis in dairy cows. The peNDF has been considered as the fraction of fiber that stimulates chewing activity, and consequently salivation in ruminants (Mertens, 1997).

A vast amount of research has been conducted to understand the physiological role of peNDF in dairy cattle (Zebeli et al., 2012). However, the effects of peNDF on the protein metabolism of animals has not been extensively evaluated. In particular, the effect of peNDF on the partitioning of nitrogen (N) excretion in animals has not been the focus of research so far despite the possible links between dietary peNDF and various aspects of rumen N turnover, such as its effect on microbial growth, rumen fermentation and pH, nutrient degradability and absorption, and hence, on rumen N recycling. Up to 20% of microbial crude protein (CP) may be derived from urea-N recycled via the rumino-hepatic pathway (GfE, 2001). Entry of circulating N into the rumen may occur either via saliva or by diffusion from the blood through the rumen wall (Reynolds and Kristensen, 2008). Importantly, stimulating the chewing activity of dairy cows by increasing the peNDF concentration of a diet may simultaneously promote N influx into the rumen via saliva.

This doctoral dissertation focuses on i) Penn State Particle Separator technique and methods of varying peNDF concentration (Chapter 2A and 2B) and ii), *in vivo* studies on the effects of peNDF concentration on feed intake, chewing behavior, rumen fermentation

and microbial protein synthesis (MPS), digesta passage, partitioning of N excretion, and performance of dairy cows (Chapters 2B, 3, and 4).

All studies were performed with the overall aim of testing the doctoral dissertation's hypotheses:

I. Increasing peNDF concentration of a diet stimulates chewing activity, and thus promotes salivation up to a certain peNDF concentration, after which chewing activity declines as a result of lower dry matter (DM) intake.

II. Greater chewing activity and salivation may stimulate rumen N recycling and MPS and compensate for the potential negative effects of reduced rumen degradable CP supply on feed intake, nutrient digestibility, and performance of dairy cows.

A meta-analysis of several recent studies found in literature was performed to get a better overview of the physiological effects of peNDF in dairy cows. The result of the meta-analysis is presented in section 5.1.1. The subsequent sections (5.1.2, 5.1.3, and 5.2) compare and discuss the results obtained from the studies performed within the scope of the doctoral project including the meta-analysis with the overall aim of answering the questions posed by the present thesis' hypotheses. The future perspectives are presented in section 5.3.

5.1 RELATION BETWEEN PHYSICALLY EFFECTIVE FIBER, INTAKE, CHEWING ACTIVITY, AND DIGESTA PASSAGE

5.1.1. Animal Responses in Physically Effective Fiber Studies – A Meta-Analysis

Data from several studies on the effects of peNDF were analyzed quantitatively to evaluate the physiological responses of dairy cows to intake of peNDF. Studies were included which were conducted in the last two decades, because dairy cows' diets have been adjusted during this time to meet their nutritional demands while maintaining high levels of milk production. The data set included 25 studies comprising 128 treatment means which were published in English as peer-reviewed articles from 2001 to 2020 (see Appendix Table A.5). Inclusion criteria for published articles were the availability of the chemical and the physical characteristics (i.e. peNDF concentration, particle size (PS) distribution, or the geometric mean (X_{gm}) of particles) of the diet. In cases where the peNDF concentration was not available in a study but the PS distribution was given, then the former was calculated from the latter.

The $peNDF_{>8.0}$ is estimated as the neutral detergent fiber (NDF) concentration multiplied by the proportion of DM retained on 19.0- and 8.0-mm sieves (Lammers et al., 1996). The $peNDF_{>4.0}$ is estimated as the NDF concentration multiplied by the proportion of DM retained on 19.0-, 8.0-, and 4.0-mm sieves (Jones and Heinrichs, 2016) and the $peNDF_{>1.18}$ is estimated as the NDF concentration multiplied by the proportion of DM retained on 19.0-, 8.0-, and 1.18-mm sieves (Kononoff et al., 2003a). The $peNDF_{>4.0}$ is only reported in four of the 25 identified studies in the present meta-analysis and hence had to be excluded from the statistical analysis, but the results of the descriptive analysis were still shown (Table 5.1).

Table 5.1 Description of animal characteristics, dietary factors, and response variables included in the present study.

Variable	n_{Treat}[2]	n_{Ref}[3]	Mean	SEM[1]	Minimum	Maximum	Median
Animal characteristics							
Body weight, kg	128	25	650	4.86	580	848	632
Days in milk	128	25	93	4.35	-13.4	195	104
Dietary factors							
Forage, %	128	25	49.6	0.95	35.0	80.0	47.0
Concentrate, %	128	25	50.4	0.95	20.0	65.0	53.0
NDF,[1] g/kg DM[1]	128	25	333	4.41	232	490	326
Forage NDF, g/kg DM	80	15	226	13.4	151	449	216
ADF,[1] g/kg DM	124	24	212	4.93	130	315	209
NDF:ADF	124	24	1.60	0.03	1.24	2.35	1.50
X_{gm},[1] mm	82	18	5.82	0.39	1.28	10.9	5.91
$peNDF_{>8.0}$,[1] g/kg DM	124	24	138	5.2	21.1	253	135
$peNDF_{>4.0}$,[1] g/kg DM	21	4	271	16.9	32.1	343	209
$peNDF_{>1.18}$,[1] g/kg DM	102	21	203	5.9	95.1	470	275
Crude protein, g/kg DM	128	25	175	1.93	130	218	171
Starch, g/kg DM	95	16	280	13.7	138	412	292
NFC,[1] g/kg DM	59	13	379	25.1	279	442	395
NE_L,[1] MJ/kg DM	110	21	6.81	0.23	5.84	7.53	6.84
Response variables							
DM intake, kg/d	120	25	22.5	0.59	15.5	31.6	22.1
ATTD,[1] g/100 g							
DM	60	15	65.5	4.25	58.7	75.5	65.0
NDF	74	16	46.0	2.80	30.6	61.3	45.9
Crude protein	56	12	63.8	4.42	51.8	86.2	62.9

Milk yield, kg/d	95	22	34.6	1.67	22.9	49.5	35.1
Milk fat, g/kg	95	22	35.5	16.3	24.3	54.0	35.1
Milk protein, g/kg	95	22	31.8	14.2	26.3	44.0	32.0
Eating time, min/d	79	20	264	15.5	163	412	255
Rumination time, min/d	83	20	433	23.6	236	596	444
Total chewing time							
min/d	79	20	693	39.3	446	949	697
min/kg DM	79	20	31.4	1.86	17.1	50.9	30.9
Passage rate, %/h							
Liquid	30	8	12.2	0.97	5.90	17.2	12.0
Solid	29	8	3.84	0.30	2.70	5.00	4.00
Microbial N[1]							
g N/d	31	8	283	22.6	155	406	291
g N/kg RFOM[1]	27	7	24.2	10.30	18.2	39.8	21.9
Ruminal pH	87	22	6.08	0.31	5.49	6.75	6.08
Total VFA, m*M*	73	19	124	7.40	68.0	162	121
Acetate-to-propionate ratio	77	20	2.68	0.16	1.60	3.95	2.61
Acetate, mol/100 mol VFA	77	20	63.7	32.3	49.4	90.4	60.8
Propionate, mol/100 mol VFA	77	20	24.9	13.0	14.6	43.8	24.1
Ammonium-nitrogen, m*M*	68	17	9.67	5.37	3.14	17.0	10.4

[1]ADF = acid detergent fiber; ATTD = apparent total tract digestibility; DM = dry matter; N = nitrogen; NDF = neutral detergent fiber; NE_L = net energy of lactation; NFC = non-fiber carbohydrates; $peNDF_{>8.0}$ = physically effective NDF concentration estimated as NDF concentration multiplied by proportion of DM retained on 19.0- and 8.0-mm sieves (Lammers et al., 1996); $peNDF_{>4.0}$ = physically effective NDF concentration estimated as NDF concentration multiplied by proportion of DM retained on 19.0-, 8.0-, and 4.0-mm sieves (Kononoff et al., 2003a); $peNDF_{>1.18}$ = physically effective NDF concentration estimated as the NDF concentration multiplied by the proportion of DM retained on 19.0-, 8.0-, and 1.18-mm sieves (Kononoff et al., 2003a); RFOM = organic matter that was truly fermented in the rumen; SEM = standard error or means; VFA = volatile fatty acids; X_{gm} = geometric mean of particle size. [2]Number of treatment means. [3]Number of included studies.

The identified studies were performed with lactating primi- and/or multiparous Holstein dairy cows averaging (arithmetic mean ± one standard deviation) 93 ± 49.2 days in milk (DIM), weighing 650 ± 55 kg, and producing 34.6 ± 16.3 kg/d of milk (Table 5.1). Animals in identified studies received their diet *ad libitum*. Animal response variables included feed intake, apparent total tract digestibility of nutrients, chewing behavior, passage rate, ruminal fermentation, MPS, and milk yield and composition. The dietary factors included forage and concentration proportions in the diet, concentrations of NDF, forage NDF, acid detergent fiber (ADF), $peNDF_{>8.0}$, $peNDF_{>4.0}$, $peNDF_{>1.18}$, CP, starch, non-fiber carbohydrate (NFC), NDF to ADF ratio, X_{gm}, and net energy of lactation (NE_L).

Data analysis was performed using the MIXED procedure of SAS (V9.4, SAS Institute Inc., Cary, United States). A simple linear regression was performed to test the relationship between various animal responses and all dietary factors listed in Table 5.1. Hence, the independent variables in the present study included all dietary factors and the dependent variable was the animal response variable.

The model accounted for dietary factor as a fixed effect and included the random effects of the study and the interaction between dietary factor and study. For simplicity, only the significant dietary factors are shown ($P < 0.05$, see Appendix Tables A.6 – A.9). A dietary factor or an animal response variable was only included in the statistical analysis, if more than six studies were available on the respective variable. To consider the unequal variance between the studies, all animal response variables were weighted by the reciprocal of their squared standard error of means.

All significant dietary factors were further tested using forward elimination multiple regression. In the case of collinearity between significant dietary factors, only the one with the highest *P*-value was included in the model. For simplicity, only the equations of multiple regression of those animal response variables which further improved the relationship obtained from the linear regression are reported (see Appendix Table A.10).

5.1.2 Adjustment and Concentration of Physically Effective Fiber in Diets

Overall, a wide range peNDF concentrations was covered across studies with $peNDF_{>8.0}$ concentration ranging from 21.1 to 352 g/kg DM, $peNDF_{>4.0}$ concentration ranging from 32.1 to 343 g/kg DM, and $peNDF_{>1.18}$ concentration ranging from 95.1 to 470 g/kg DM (Table 5.1). The great variation in peNDF concentrations was also reflected in the wide range of X_{gm} between studies of 1.28 to 10.9 mm. These variations in the physical structure of the tested diets within and across studies were achieved either by changing the forage proportion of the diet (Jiang et al., 2017), by varying the PS of the forage or whole diet (Kononoff and Heinrichs, 2003a; b; Bhandari et al., 2008), or by a combination of both (Yang and Beauchemin, 2007a; Li et al., 2020). Changing the PS of forages or diets involved cutting forage ingredients with a forage harvester to the desired theoretical chop length (Yang and Beauchemin, 2007b), re-chopping of forages using a threshing machine (Kahyani et al., 2013), or by varying the mixing time in the feed mixer wagon (Leonardi and Armentano, 2003).

According to Heinrichs et al. (1999), the theoretical PS of forages prior to feed preparation does not reflect the actual PS consumed by cows, because TMR mixing and distribution to cows affect the physical structure of the TMR. Indeed, the pre-study

conducted in Chapter 2A could demonstrate that a prolonged mixing time of the TMR (i.e. 35 and 55 min) reduced the $peNDF_{>8.0}$ concentration from 261 to 241 g/kg DM and the X_{gm} of PS from 12.7 to 11.7 mm. Hence, the TMR mixing affects the final X_{gm} and consequently the peNDF concentration of the TMR. This observation resulted in the decision to use the mixing time in the feed mixer wagon to vary the peNDF concentration in the three performed *in vivo* studies of the present thesis. As a result, the chemical composition of the TMR was identical and observed animal responses were solely related to the effect of the change in the physical structure of the TMR without any confounding effects of chemical constituents (e.g. NDF concentration or sources). In the present thesis, the $peNDF_{>8.0}$ concentration ranged from 202 to 284 g/kg DM (Chapters 2B, 3, and 4; Table 5.2) and the $peNDF_{>4.0}$ concentration from 253 to 323 g/kg DM (Chapters 2B, 3, and 4), and were thus within the upper range of peNDF concentrations in diets of dairy cows reported in the literature.

Although widely used, the peNDF concept has not been adopted in feed formulations systems (GfE, 2001; NRC, 2001) and thus no official recommendations on the peNDF concentration in dairy cows have been made. The requirements for peNDF reported in the literature are based on the principles of maintaining a certain rumen pH to reduce the risk of sub-acute ruminal acidosis (SARA) and sustaining milk fat content while avoiding a reduction in DM intake of cows at high peNDF concentrations. Defining the method of measuring peNDF is a pre-requisite for comparison between studies and also for making recommendations, because the requirement for peNDF varies with the methods used (i.e. $peNDF_{>8.0}$, $peNDF_{>4.0}$, and $peNDF_{>1.18}$).

Table 5.2 Overview of some measured variables of the three *in vivo* studies of the doctoral thesis.

Study	n[1]	Days in milk	peNDF$_{>8.0}$,[2] g/kg DM[2]	X_{gm},[2] mm	RNB[2]		DM intake, kg/d	ATTD,[2] g/100 g		Microbial N[2]		g N/100 g N intake			Milk yield, kg/d
					g/kg DM	g/d		OM[2]	CP[2]	g N/d	g N/kg CP intake	Urine	Feces	Milk	
1	8	197 ± 67.7	237	11.1	0.6	14.2	24.3	–	–	–	–	–	–	–	38.7
	8		249	12.0	0.5	11.3	24.9	–	–	–	–	–	–	–	39.2
	8		267	13.0	0.6	14.5	24.6	–	–	–	–	–	–	–	39.3
	8		284	14.6	0.5	11.2	24.1	–	–	–	–	–	–	–	38.7
2	20	103 ± 59.4	202	8.6	0.1	2.6	26.2‡	68.3	64.9	451	116	33.6	35.1	30.9	36.6
	20		212	9.2	0.1	2.5	25.4‡	69.4	66.2	446	118	34.7	33.8	31.3	36.2
	20		213	9.1	-1.5	-39.4	26.3‡	69.2	63.3‡	482	133‡	30.6‡	36.7‡	32.4	35.7
	20		221	9.8	-1.5	-38.6	25.7‡	67.9	60.9‡	423	119‡	28.1‡	39.1‡	32.5	35.2
3	4	75 ± 8.4	202	7.3	-2.1	-45.5	21.9^{T}	65.8	57.2	373^{T}	123*	27.3^{T}	42.9	29.3^{T}	27.2
	4		208	7.6	-2.1	-45.8	22.1^{T}	67.4	59.8	428^{T}	141*	30.8^{T}	40.2	28.6^{T}	26.7
	4		218	8.2	-2.1	-48.1	22.5^{T}	66.9	57.6	388^{T}	124*	29.4^{T}	42.4	27.5^{T}	25.8
	4		235	9.0	-2.1	-43.0	20.2^{T}	64.2	56.0	279^{T}	100*	23.8^{T}	44.0	31.3^{T}	27.2

*Significant linear effect ($P < 0.05$) of peNDF concentration on measured variable within a study.

TSignificant quadratic effect ($P < 0.05$) of peNDF concentration on measured variable within a study.

‡Means with different uppercase superscripts in the same column within a study and RNB, and between peNDF concentrations differed at $P < 0.05$.

-Not measured in the study.

[1]Number of animals.

[2]ATTD = apparent total tract digestibility; CP = crude protein; DM = dry matter; N = nitrogen; OM = organic matter; peNDF = physically effective neutral detergent fiber; peNDF$_{>8.0}$ = peNDF estimated as NDF concentration multiplied by proportion of DM retained on 19.0- and 8.0-mm sieves (Lammers et al., 1996); RNB = rumen N balance; X_{gm} = geometric mean of particle size.

However, the capabilities of peNDF$_{>8.0}$ and peNDF$_{>1.18}$ to predict animal responses are similar for some variables such as rumen pH as already observed by Zebeli et al. (2012) from their modeling studies (Zebeli et al., 2008, 2010). The meta-analysis performed in the present thesis revealed similar prediction capabilities of these two variables for DM intake (Table A.6), chewing behavior (Table A.7), milk constituents (Table A.8), and rumen pH (Table A.9). These findings would infer that these two variables, peNDF$_{>8.0}$ and peNDF$_{>1.18}$, can be used interchangeably to predict the mentioned animal responses.

Although a minimum peNDF$_{>1.18}$ concentration between 190 – 220 g/kg DM was reported to be sufficient to maintain an average rumen pH of 6.0 in Holstein cows (Mertens, 1997; Zebeli et al., 2006), Zebeli et al. (2008) using a modeling approach reported a greater risk of SARA when feeding Holstein cows less than 149 g/kg DM of peNDF$_{>8.0}$ or less than 312 g/kg DM of peNDF$_{>1.18}$. A peNDF concentration above 149 g/kg DM of peNDF$_{>8.0}$ or 312 g/kg DM of peNDF, however, may decrease DM intake of dairy cows (Zebeli et al., 2008). Hence, optimal concentrations of peNDF$_{>8.0}$ between 149 – 185 or of peNDF$_{>1.18}$ between 300 – 330 g/kg DM reduce the risk of SARA and of reduced feed intake and performance of high-yielding dairy cows (Zebeli et al., 2008). With this in mind, all diets offered to cows in the present thesis' *in vivo* studies would have been above this stated recommendation for peNDF$_{>8.0}$ (Chapters 2B, 3, and 4) and for peNDF$_{>1.18}$ (Chapter 2B). Hence, based on the stated recommendation of Zebeli et al. (2008), the peNDF concentrations offered in the present thesis studies would have result in lower DM intake of cows (see section 5.1.3). However, it was not possible to reduce further the peNDF concentrations of the tested diets, as the NDF concentration of the tested diets was high and ranged between 361 and 406 g/kg DM and prolonging the

mixing of the TMR would have resulted in a feed mush. Also, the NDF concentration of the available forages were high and hence, there was no wide scope for reducing the NDF concentration of the overall diet.

5.1.3 Physiological Effects of Physically Effective Fiber in Dairy Cows

Essentially, the animal response which is associated with peNDF is chewing activity, and thus, chewing has been proposed as an indicator to assure adequate peNDF concentrations in the diet of dairy cows to maintain rumen health and functioning (Mertens, 1997; Zebeli et al., 2012). As peNDF comprises both, NDF concentration and PS, it is also related to ruminal mat formation and consequently, to the selective entrapment of fiber in the rumen affecting the dynamics of ruminal fermentation and passage, and stimulating rumination (Mertens, 1997). Nevertheless, too large dietary PS may lower the passage of digesta, reduce surface area for microbial attack, and decrease rumen fiber degradation, and consequently, decrease feed nutrient intake (Zebeli et al., 2012).

The effect of peNDF concentration on DM intake in the three studies within the present thesis differed. There was no effect of peNDF concentration on DM intake in the first study (Chapter 2B), whereas peNDF concentration decreased DM intake in the second study (Chapter 3) and affected quadratically the DM intake in the third study (Chapter 4). Table A.6 shows the results of the linear response of DM intake to different dietary factors as determined from the meta-analysis. The dietary concentrations of $peNDF_{>8.0}$, forage NDF, X_{gm}, and $peNDF_{>1.18}$ negatively affected DM intake, whereas DM intake increased as a response to the increasing proportion of concentrate and concentrations of starch, NFC, and NE_L in diets. The analysis of forward elimination of multiple regression where

all significant dietary factors were included in the model showed that concentrations of dietary peNDF$_{>8.0}$ (quadratically) increased and forage NDF (linearly) decreased DM intake. Similarly, Zebeli et al. (2006) reported negative effects of peDNF$_{>1.18}$ and NDF concentrations and positive effects of NFC:NDF ratio on DM intake of cows fed a TMR in *ad libitum*. The response of DM intake to forage NDF, NDF, peNDF$_{>1.18,}$ and peNDF$_{>8.0}$ concentrations might be attributable to their effect on rumen fill.

Feeding dairy cows long PS is generally associated with reduced voluntary feed intake as a result of slower digesta passage rates and greater fill of the reticulorumen by distension (Allen, 2000). In all three studies of the present thesis, diets with high X_{gm} were offered ranging from 7.4 to 14.6 mm (Table 5.2) which, compared to identified studies listed in Table A.5 with a median of the X_{gm} at 5.9 mm (Table 5.1), are classified as long PS. Following the principles of Allen (2000), reducing the PS of a diet may potentially lead to greater voluntary feed intake by the animals when fed very long PS. This observation is substantiated by other studies, where an increase in feed intake by animals with decreasing dietary PS was observed when steers were offered a diet with high proportions of forage (Soita et al., 2002; 100% barley silage) or when lactating Holstein cows were offered a diet with forages of long PS (Kononoff and Heinrichs, 2003a, X_{gm} = 7.4 – 8.8 mm; Kononoff et al., 2003b, X_{gm} = 4.1 – 6.8 mm). Given the high inclusion levels of forages (53 – 57% of diet DM) in tested diets of the present thesis, a positive effect of reducing PS and thus peNDF concentration would have been expected. Nonetheless, although the peNDF$_{>8.0}$ concentration in the first study was greater compared to the other two studies, the feed intake of cows in the first study was not affected by peNDF concentration. The first study was a short study to test whether

marginal increases in peNDF concentration adjusted by the feed mixing time affects DM intake and chewing activity of dairy cows.

The effect of peNDF concentration on solid passage rate was estimated in the third study. While the effect on solid passage rate remained absent, the DM intake responded quadratically to increasing peNDF concentration. Therefore, the decline in DM intake of cows was not due to the slower digesta passage rate in the present study. The meta-analysis of the present thesis has shown that solid passage rate is negatively affected by the NDF concentration and positively related to the NFC and CP concentration in the diet (Table A.7). The lack of effect of PS on solid passage rate in the present thesis meta-analysis and in the third study was in line with several studies (Yang et al., 2001, 2002; Zebeli et al., 2007) and in contrast to another study (Rode et al., 1985) with high-yielding Holstein cows, who observed a quadratic response of solid digesta passage rate when reducing the PS from long hay to ground hay. Observed effects on the latter study were likely due to the wider range of tested dietary PS. The lack of relationship between chewing activity and passage rate of solid ruminal contents across studies suggested that PS reduction was not a rate-determining step in ruminal particulate passage, as indicated by Yang et al. (2001).

In a study by Allen (1996) the negative effect of rumen fill due to factors such as high NDF concentration and PS on DM intake was more pronounced in cows with high energy requirements who were fed a low energy diet, while a high energy diet counteracted the negative effect of dietary NDF concentration on DM intake. This could be one of the explanations for the discrepancy between the results of the three studies in the present

thesis. Cows in the first study were in a more advanced lactation stage (197 ± 67.7 DIM) compared with cows in the second (103 ± 59.4 DIM) and third (75 ± 8.4 DIM) study. Although X_{gm} of experimental diets were greater in the first compared to the second and third study, the energy requirement of cows in the first study was probably adequate compared to that of those in second and third study, which resulted in a less pronounced effect of rumen fill on feed intake in the first study. Moreover, when diets contain too much fiber and the energy density is low, feed intake and performance of dairy cows decrease (Mertens, 1997).

Chewing is a prerequisite for maintaining an optimal rumen environment, because saliva is secreted during chewing to lubricate the bolus and to enable swallowing, and it buffers the ruminal environment (Beauchemin, 2018). Therefore, optimal chewing time can lower the risk of SARA and elevates fiber digestion and feed intake in dairy cows. The present thesis postulated that a greater dietary peNDF concentration stimulates chewing activity (i.e. total chewing time and chews) and thus promotes salivation up to a certain peNDF concentration after which chewing activity declines due to lower DM intake. This would imply that a gradual increase in peNDF concentration would trigger a quadratic response to DM intake. Also, that the total chewing time and chews per kilogram of DM intake increase with increasing peNDF concentration.

In the first study, where DM intake of cows was not limited by high peNDF concentration, total chewing time and number of chews linearly increased with greater peNDF concentration. In the second study, in which DM intake decreased with greater peNDF concentration, total chewing time tended to increase, and total number of chews significantly increased with greater peNDF concentration. A quadratic response of total

chewing time and total number of chews to dietary peNDF concentration was observed in the third study of the present thesis.

The eating (259 – 424 min/d), rumination (516 – 591 min/d), and total chewing time (782 – 1003 min/d) varies across the three studies. Similarly, total chewing time of dairy cows varies across selected studies from the literature, which can be seen in the great range in eating (163 – 412 min/d), rumination (236 – 596 min/d), and total chewing time (446 – 949 min/d) of the identified studies (Table 5.1). Hence, chewing time of cows from all three studies of the present thesis were close to or above the upper range of the results reported in previous studies. The high total chewing times of cows observed across the three studies were attributed to the high peNDF concentrations of the tested diets.

The linear regression of eating time (min/d), rumination time (min/d), and total chewing time (min/d and min/kg DM intake) to different dietary factors are shown in Table A.7. Eating time was negatively affected by dietary NFC, NE_L, and starch concentrations and the concentrate proportion in the diet and positively affected by $peNDF_{>8.0}$, $peNDF_{>1.18}$, and X_{gm}. Total chewing time (per day and kilogram DM intake) and rumination time positively responded to $peNDF_{>8.0}$, $peNDF_{>1.18}$, $X_{gm,}$ and NDF. Additionally, the forage proportion in the diet positively affected total chewing time (min/d). Both, NFC and NE_L concentrations negatively affected total chewing time (min/d and min/kg DM intake) and concentrate proportion in the diet additionally negatively affected the total chewing time (min/d) in the present analysis. In contrast, Zebeli et al. (2006) in their meta-analysis with lactating Holstein cows reported positive effects of NFC on total chewing time (min/d) due to positive effect of dietary NFC on DM intake. Multiple regression using forward selection showed that $peNDF_{>8.0}$

(quadratic) significantly affected rumination time (Table A.10). Overall, this would imply that not only the chemical and physical characteristics of fiber affect the animal's chewing activity as postulated by Mertens (1997), but also the proportion of concentrate and the concentration of NFC and starch in the diet.

The concept of peNDF does not account for differences in the fermentability of feedstuffs. Moreover, several studies reported that the requirement for peNDF is strongly dependent on several dietary factors such as forage to concentrate ratio (Tafaj et al., 2004), grain processing (Yang and Beauchemin, 2004) and source (Beauchemin and Rode, 1997), and the amount and fermentability of starch present in the diet (Silveira et al., 2007). Dietary starch concentration was low for all three studies of the present thesis and ranged between 82.9 and 177 g/kg DM. In comparison, the results of the quantitative analysis showed a median concentration of starch of 292 g/kg DM across selected studies from the literature (Table 5.1). The three studies had similar proportions of forage in the diet and type of forage, which was mostly based on maize silage and grass silage or haylage; hence, the peNDF concentrations across studies were comparable. However, the concentrate composition varied across the studies and thus, the fermentability of concentrate may have differed. However, due to the low starch concentration in all three studies of the present thesis, the observed differences in chewing behavior between the three studies may not be governed by the dietary starch concentration. Considering the various factors which can affect the effect of peNDF concentration in the animal, recommendations on peNDF should also take into consideration the NFC and starch concentration of the diet.

When related to the DM intake, eating and total chewing time and the number of eating and total chews were greater at high peNDF concentration for the first and second study, which suggests that increasing peNDF concentration stimulates the eating and total chewing activity of cows. Whereas no effect of peNDF concentration on eating and total chewing time and the number of eating and total chews per kilogram DM intake was observed in the third study, which suggests the DM intake rather than the peNDF concentration affects eating and total chewing activity of cows.

In contrast, rumination time and number of chews per kilogram of DM intake were unaffected by peNDF concentration in the first and second study, while rumination time and number of rumination chews per kilogram of DM intake linearly increased with greater peNDF concentration in the third study. This indicates that rumination time and the number of rumination chews per kilogram of DM intake was influenced by DM intake in the first two studies, whereby both variables were affected by the peNDF concentration in the third study.

According to Beauchemin (2018), eating time (min/kg DM intake) is generally more affected by dietary PS than rumination time (min/kg DM intake), which can be explained by the increasing need to chew prior to swallowing ingested feed and consequently, reducing the need for additional PS break-down during rumination. Moreover, greater dietary PS prolongs rumination time with the effects diminishing as PS increases, with no further increase in rumination time after a certain PS (Beauchemin, 2018). Allen (1997) recommended a mean PS of 10 mm after which only moderate or no further increase in rumination time occurs with greater dietary PS. In agreement, the X_{gm} of PS in the first (11.1 – 14.6 mm) and second study (8.6 – 9.8 mm) were greater than in the

third study (7.3 – 9.0 mm) and hence, there was a greater scope in the third study to increase the rumination activity of cows. Moreover, the intensive mastication in the third study may have not been sufficient to reduce the need for additional mastication during rumination, as rumination time per kilogram NDF intake was longer in the third study (63 – 70 min) than in the first (60 – 65 min) and second (60 – 61 min) study.

As postulated, the results of the present thesis show that the dietary peNDF concentration stimulates total chewing time and number of chews when related to the DM intake of cow, which can either be due to increase in eating or in rumination activity depending on the PS of the diet. Additionally, the effect of peNDF concentration on the total chewing time and number of chews expressed per day depends on the impact of peNDF concentration on DM intake of cows. Overall, all cows in all three studies had an average total chewing time (min/kg DM intake) of 39 ± 3.8, which is higher than the recommendation by Sudweeks et al. (1981) who identified a total chewing time of ≥ 30 min/kg DM intake as desirable to minimize the risk of digestive disorders.

Saliva secretion can be measured during eating or resting by collecting boluses or saliva in a collection bag through the rumen fistula directly at the cardia (Beauchemin, 2018), by esophageal fistula, or by cannulating the right parotid gland (Meyer et al., 1964). As summarized in a review by Beauchemin (2018), the average saliva production across different studies averaged 133 ml/min (91 – 156 ml/min) for resting (when the animal is not eating or ruminating) and 206 ml/min (192 – 250 ml/min) for eating. Saliva secretion during rumination is often assumed to be equal to that during eating as measuring saliva production during rumination in dairy cows presents a challenge (Beauchemin, 2018). Several studies that quantified the effect of prolonged chewing time

on salivation reported only a small net increase in daily salivation between the lowest and highest recorded chewing time when increasing the proportion of forage in the TMR fed to lactating Holstein cows (Maekawa et al., 2002; Jiang et al., 2017). Although Maekawa et al. (2002) reported an increase of 107 min/d (14.4%) in total chewing time of cows when the forage proportion in a barley silage-based TMR increased from 40 to 60% on a DM basis, estimated saliva secretion only increased by 25 l/d (11%). Similarly, Jiang et al. (2017) reported an increase in total chewing time by 141 min (19.8%) and salivary secretion by 16.6 l/d (7.1%) when cows were fed a TMR based on corn silage and alfalfa hay with increased forage proportion from 40 to 70% on DM basis. The small increase in net saliva production was due to a decline in saliva secretion during resting, when salivation during eating and ruminating increases with increasing forage proportion in the diet (Jiang et al., 2017).

Cassida and Stokes (1986) reported a high correlation ($r = 0.80$, $P < 0.01$) between total saliva production (l/d) and liquid outflow rate (l/h), indicating that saliva secretion is a major determinant of liquid outflow rate from the rumen as investigated in lactating Holstein cows fed corn or hay crop silage. However, the quantitative analysis of the present thesis could not find any relationship between the tested dietary factors and liquid passage rate (%/h), possibly due to lack of information as only four of the selected studies comprised information on liquid passage rate. Hence, the linear regressions for liquid passage rate of the meta-analysis were not presented. Although chewing activity responded quadratically to increasing dietary PS in the third study of the present thesis, no response of liquid passage rate could be observed, which suggests that saliva production was not elevated with increasing peNDF concentration as postulated.

For the present thesis, an increase in chewing time from the lowest to greatest in each study resulted in an average increase of 102 min/d (13%; first study), 11.5 min/d (1.2%; second study), and 74 min/d (8.2%; third study). Compared to the studies by Maekawa et al. (2002) and Jiang et al. (2017), the small increase in chewing time in the present thesis would have not resulted in much increase in total saliva production. Additionally, ruminal pH was similar between the tested peNDF concentrations and thus, it can be assumed that an increase in chewing did not necessarily increase total saliva output. Nevertheless, the rumen pH of all cows in the third study averaged 6.43, which is greater than the median of the quantitative analysis (Table 5.1), indicating that all cows in the present thesis had a smaller risk of developing SARA.

Overall, the first hypothesis, in which it was postulated that increasing peNDF concentration promotes chewing activity and total saliva output can be partly accepted, as increasing peNDF concentration increases chewing time and total number of chews of dairy cows; however, the effect on total saliva output might be too small. Moreover, the effect of peNDF concentration on chewing activity and thus salivation is mainly governed by the effects of peNDF concentration on DM intake of cows.

5.2 RELATION BETWEEN PHYSICALLY EFFECTIVE FIBER AND PROTEIN METABOLISM IN DAIRY COWS

Despite various studies that have been conducted on the concept of peNDF and its physiological effects in dairy cows, the effect of peNDF on the N utilization in dairy cows and particularly the partitioning in N excretion has not been a focus of research so far. Importantly, with increasing concerns on environmental emission from dairy production systems, especially related to the inefficient use of N by dairy cows generally offered

diets with a considerable safety margin in CP concentrations, finding alternative means for improving their N use efficiency plays a greater role. As stated in Chapter 1, reducing the CP concentration of a dairy cow's diet has been generally regarded as one of the ideal approaches to tackle this problem. Consequently, this will take advantage of the ruminant's ability to use recycled N from the rumino-hepatic cycle, which can re-enter the rumen either via saliva or by diffusion from the blood through the rumen wall (Reynolds and Kristensen, 2008). Thus, the present thesis postulated that increasing peNDF concentration of the diet will promote the chewing activity of dairy cows and thereby stimulate N re-entry into the rumen via increased saliva flow. Also, at reduced rumen-degradable CP supply, a greater effect on N metabolism of dairy cows may be observed, because N recycling plays a greater role to meet the N requirements of rumen microbes. Nonetheless, providing diets with a negative rumen N balance (RNB) of – 1.4 to – 5.0 g/kg DM (– 28 to – 94 g/d) to dairy cows either had no effect on feed intake and milk yield in some studies (Holthausen et al., 2000; König et al., 2005; van de Sand et al., 2006), or reduced DM intake and milk yield at an RNB of – 0.5 to – 7.0 g/kg DM (– 11 to – 106 g/d) in other studies (Riemeier, 2004; Steinwidder et al., 2009). Hence, it was expected that elevating chewing activity and salivation in dairy cows by increasing peNDF concentration of the diet may counteract the potential negative effects of reduced RNB on feed intake and performance of dairy cows due to a greater salvage of urea-N.

As stated in Section 5.1.3, although increased peNDF concentration prolonged total chewing time and total number of chews (per kilogram of DM intake) in dairy cows, observed increases were small and may not have resulted in a significant effect on saliva flow and consequently, on N recycling as initially postulated. Interestingly, increasing

peNDF$_{>8.0}$ concentration from 237 to 283 g/kg DM did not affect feed intake in the first study with an RNB of around 0.5 g/kg DM. However, increasing peNDF$_{>8.0}$ concentration from 202 to 212 g/kg DM and from 213 to 221 g/kg DM reduced feed intake of cows when offered diets with an RNB of 0.1 g/kg DM and – 1.5 g/kg DM, respectively, in the second study, despite lower peNDF$_{>8.0}$ concentrations compared to the first study (Table 5.2). Nonetheless, no interaction effect between concentration and RNB was observed on feed intake in the second study, with similar feed intakes within RNB diets. Moreover, feeding cows a negative RNB of – 2.1 g/kg DM exhibited a quadratic response of feed intake to increasing peNDF concentration from 202 to 238 g/kg DM with lower feed intake for high and low peNDF concentration compared to medium peNDF concentration. Hence, not only the RNB affects the DM intake of cows but also the peNDF concentration in the diet. Only one study found in the literature reported the peNDF concentration in an RNB study (Kand et al., 2021), while other studies on the effects of RNB found in the literature do not consider the physical characteristics of experimental diets and vice versa.

The second study showed interactions between dietary peNDF and RNB on several response variables such as apparent total tract digestibility of DM (aDMd), organic matter (aOMd), CP (aCPd), eating and total chewing time (min/d and chews/d), MPS efficiency (g N/kg digestible organic matter intake), and the partitioning of N excretion via urine and feces (g N/100 g N intake). The pairwise comparison within each treatment indicated for example that aCPd was not affected by varying peNDF concentrations at balanced RNB (0.1 g/kg DM); however, aCPd was lower with high than with low peNDF concentration when cows were offered a diet with negative RNB (– 1.5 g/kg DM intake).

Similar effects were observed for the other stated response variables such as total chewing time (min/d) and chews (chews/d), MPS efficiency, and partitioning of N excretion, with more pronounced effects of peNDF concentration at low RNB diets. The peNDF concentrations of both negative RNB diets in the second study were slightly greater than those of balanced RNB diets, which should not be disregarded and hence, may partly explain the decline in intake, digestibility, and rumen MPS with high peNDF concentration in the negative RNB diet in the second study. Nonetheless, the third study had lower $peNDF_{>8.0}$ concentrations and RNB compared to the first and second study. Here, as already discussed in Section 5.1, a quadratic response on aOMd, eating and total chewing time (min/d), MPS (g N/kg digestible organic matter intake), and the partitioning of N excretion via urine and milk (g N/100 g N intake) was observed.

As a result of a physiological response of the cow to compensate at least in part for the lower digestibility of nutrients and feed intake which occurred when they were offered the low RNB diet with high peNDF concentration, cows increased their rumination time (min/d) as observed in the second and third study, which is in line with observations made by other studies (Adin et al., 2009; Schiavon et al., 2015). The reduction in apparent total tract digestibility may, in the first place, occur due to the large PS of the tested diets which resulted in reduced surface area and access of microbial enzymes to feed substrate at high peNDF concentration (Zebeli et al., 2012). Increasing dietary PS is generally associated with increased retention time in the rumen, reduced solid digesta passage rate, and thus increased rumination activity and NDF fermentation (Mertens, 1997). However, increasing the peNDF concentration did not result in increased retention time in the rumen and decreased solid digesta passage rate in the third study. Furthermore, the efficiency of

MPS (g N/kg DM intake) decreased when lower nutrient intake was coupled with decreased nutrient digestibility in both the second and third study. In line with the present thesis observations, Yang and Beauchemin (2006a) offering diets with 160 g CP/kg DM at graded $peNDF_{>8.0}$ concentrations (174, 315, and 352 g/kg DM), which varied solely in dietary PS, to lactating Holstein cows, observed a quadratic response of aCPd, MPS yield and efficiency (g/kg digestible organic matter) with greater values for medium peNDF concentration. The range of tested X_{gm} of particles within the third study was presumably too small to achieve a substantial effect on the digesta passage rate. Hence, when a low RNB diet was coupled with greater peNDF concentration (i.e. greater PS in this case), digestibility decreased as presumably energy was limiting due to reduced microbial activity.

As stated above, reducing the RNB amplifies the effect peNDF concentration in the animal. Hence, at balanced RNB, no differences were observed in the partitioning of N excretion in the second study. However, for a low RNB diet, a decline in aCPd due to increasing peNDF concentration resulted in a greater proportion of ingested N excreted via feces, a smaller proportion via urine, and a similar proportion of ingested N was secreted via milk across peNDF concentrations. Further along the line, Yang and Beauchemin (2004) reported increased dietary N flow to the duodenum with greater forage PS which was probably attributable to lower N degradation in the rumen of dairy cows. Hence, lower ruminal N degradation might result in lower ruminal ammonium absorption and conversion to urea in the liver, followed by a reduced N excretion via urine for the diet with high peNDF concentration in the second study.

In contrast, a quadratic effect was observed on the proportion of ingested N excreted via urine and milk in the third study, while the proportion of ingested N excreted via feces was similar across peNDF concentrations. The partitioning of N excretion towards urine was greater and towards milk was lower for the medium peNDF concentration in contrary to the high and low peNDF concentrations. As discussed in Chapter 4, the differences in the measured variables between medium and low peNDF concentrations were small and not of biological relevance. Hence, the quadratic effect observed on the partitioning of N excretion was rather due to a pronounced decline in the proportion of ingested N excreted via urine and a greater proportion secreted via milk at high peNDF concentration. Although diets were initially designed to meet the utilizable CP (uCP) requirements of animals, final diets supplied animals with an excess in uCP supply. As DM intake was higher for the medium and low peNDF concentrations, from then on, excess in uCP was greatest for cows fed these diets. The surplus in uCP supply and presumably a deficit in ruminal energy supply meant that excess ammonia could not be used by rumen microbes and thus absorbed via the rumen wall.

While increasing peNDF concentration resulted in a lower urinary N excretion (g/100 g N intake) in the second and third study, there was consequently a greater fecal N excretion (g/100 g N intake) in the second study and in contrast, a greater milk N secretion (g/100 g N intake) in the third study. When comparing the two studies, N intake was greater in the second (565 – 620 g N/d) than in the third study (445 – 499 g N/d). According to Castillo et al. (2000) using the data from lactating dairy cows in a variety of studies carried out in different countries with a range of feeding situations and an N intake ranging between 200 to 750 g/d, a N intake of 400 g/d seems to be the critical point

in relation to the amount and form in which N is excreted in lactating dairy cows. The excretions via feces and urine, and secretion via milk increased linearly up to an intake of 400 g N/d, with feces being the major route of N excretion, whereby urinary N is the major route of N excretion at N intakes above this level, with the excretion in urine increasing exponentially while the rate of increase in N excretion in feces and milk declines linearly with increasing N intake (Castillo et al., 2000). These observations confirm those of the present thesis. The proportion of ingested N excreted via feces was lower and the proportion excreted via urine was higher in the second than the third study.

In spite of a decline in DM intake of cows with high peNDF concentration in all three studies regardless of RNB of the diet and also a reduction in the digestibility of nutrients at RNB–, no negative effects were observed on performance variables. The lack of effect of peNDF concentration on milk performance of cows, in line with other studies (Yansari et al., 2004; Yang and Beauchemin, 2006b) on peNDF, has been explained by the short experimental period of these studies (21 days) which were all conducted in a Latin-Square design. As further stated in Chapter 4, a shift in starch digestion with increasing peNDF concentration in combination with the surplus in uCP supply to the animals may have attenuated the negative effects of high dietary PS. The glucose arising from postruminal starch digestion can be used energetically more efficiently for milk synthesis by the animal (Nocek and Tamminga, 1991; Reynolds, 2006). However, the magnitude of the positive effect of a shift in starch digestion and surplus in uCP supply can only be speculated and was likely small, as the starch concentration of the tested diets was low.

Overall, the second hypothesis needs to be partly rejected. The increment in chewing activity due to an increase in peNDF concentration unlikely resulted in greater salivation

and N recycling in ruminants. Moreover, in addition to reduced DM intake, offering a negative RNB diet to cows with increased peNDF concentration resulted in a reduction in digestibility of nutrients and rumen MPS efficiency compared to when the cows were offered a balanced RNB diet with high peNDF concentration. Nonetheless, as postulated, the performance of dairy cows was not affected regardless of the negative effect on intake, digestibility, and MPS at low RNB.

5.3 FUTURE RESEARCH PERSPECTIVES

As previously outlined, the principle behind the peNDF concept is the ability of the combined effect of the physical and chemical characteristics of fiber to stimulate the chewing activity and saliva flow in ruminants. Although a positive response to increasing peNDF concentration was observed, the increment in chewing activity (i.e. total chewing time and the total number of chews) due to increasing peNDF concentration was rather small and seemed to be dependent on the RNB of the diet.

The pre-studies in Chapter 2A and 2B showed that the peNDF concentration can be varied by solely varying the mixing time of the TMR in the feed mixer wagon, however, only marginally, and consequently, the differences in the chewing activity of cows between the different peNDF concentration were rather small. Nonetheless, as discussed in Chapter 2A, it was not possible to increase the range of the peNDF concentrations in each of the studies, as prolonged mixing times resulted in a slushy TMR for cows. Moreover, in all three studies, experimental diets contained high concentrations of NDF, which directly resulted in high peNDF concentrations even in diets formulated to be low in peNDF concentration (i.e. prolonged mixing time).

The high NDF concentration of the experimental diets caused cows to chew near or within their physiological maximum capacity, which may have additionally limited the effect of peNDF concentration on chewing time. Hence, for future research studies, varying the theoretical chop length of forage ingredients of the TMR prior to mixing presents a better option when a greater range in peNDF concentrations is desired. Also, to observe a greater effect on chewing time, the NDF supply to the animals should not exceed much of the NDF requirements of dairy cows, especially if the X_{gm} of particles were already very long as in the present thesis.

As previously mentioned, the small increase in the response of chewing time to increasing peNDF concentration may not have a significant effect on saliva secretion of dairy cows in the present thesis and thus, contrary to expectations, N recycling may have not been promoted. To be able to properly answer this hypothesis, studys should be conducted to quantify the fates of urea that enters the digestive tract and in particular to quantify urea transfers via saliva in dairy cows for example using the labeled urea approach (e.g. [15N15N]urea, Lapierre and Lobley, 2001) when fed varying peNDF concentrations dependent on the RNB.

It was discerned from the present thesis, that the ability of peNDF to stimulate the chewing activity of cows depends on the RNB, but also the effect of RNB depends on the peNDF concentration of the diet. The RNB was adjusted by varying the dietary CP concentration, a factor that has not been considered in the concept of peNDF so far. Besides the energy supply, the CP supply is an important factor determining the ruminal yield and efficiency of MPS in the rumen (Bach et al., 2005). When cows were offered the balanced RNB diet, there was a lack of effect of peNDF concentration on chewing

time and other animal responses such as total tract digestibility of nutrients and N metabolism. In contrast, reducing the RNB of the offered diet amplified the negative effects of increasing peNDF concentration on animal response variables. Also vice versa, the negative effect of a low RNB diet was amplified at high peNDF concentration of the diet.

It was discerned that at negative RNB, increasing the peNDF concentration or in this case, the PS of the diet resulted in a shift in the partitioning of N excretion either from urine to feces (second study) or from urine to milk (third study). Only one study (Kand et al., 2021) found in the literature on the effect of RNB reported the peNDF concentration of their diets. It would be worthwhile to investigate whether observed animal response variables for the different RNB in other performed studies would differ if, for example, the PS of the diet was changed to verify their findings. Also, this means that animal response variables for different peNDF concentrations in other performed studies may differ when the RNB of the diet was varied. This would additionally mean that RNB thresholds need to be adjusted according to the dietary PS of the offered diet and the recommendations for peNDF needs to be adjusted according to the RNB of the offered diet.

Besides the reported reduction in feed intake and digestibility when a low RNB diet is offered to cows at high peNDF concentration, none of the three conducted studies could show any negative effect on milk performance and composition. Ensuring sufficient nutrient intake in dairy cows is known to be imperative to maintain the health and reproduction of dairy cows (Erickson and Kalscheur, 2020). Maintaining milk production

may result in lower nutrient availability for the reproduction of dairy cows particularly, if nutrient intake is concomitantly reduced. A negative energy balance may detrimentally impact the reproduction of dairy cows which could consequently lead to infertility (Nigussie, 2018). Few data exist on the long-term effects of feeding reduced levels of dietary CP to dairy cows on health and fertility, whilst some studies have observed increased risks of retained placenta and metabolic disease (Curtis et al., 1985; Rode et al., 1994) and decrease body fat mobilization during early lactation (Cadórniga and López Díaz, 1995). Along this line, conducting long-term peNDF studies is another area of interest that warrants further research to explore the long-term effects on the milk performance of dairy cows, especially when feeding diets with high concentrations of NDF and at a reduced dietary CP concentration. This would help in improving the recommendations for dietary peNDF in dairy cows' diets.

With the results of the present study in mind, feeding dairy cows diets high in peNDF at reduced rumen-degradable CP supply seems to present an option to increase the N use efficiency while reducing the amount of urinary N excretion and maintaining the performance of dairy cows. Nevertheless, more research is needed for a better understanding of the interaction between dietary peNDF concentration and RNB and to determine the optimal peNDF concentration in dairy cows' diets with regards to its effects on nutrient intake and digestibility that may affect future milk production, health, and reproduction of dairy cows. If milk production, health, and reproduction of dairy cows could be maintained, while reducing expensive protein supplementation, this would present economic benefits for farmers while, at the same time, reducing the environmental N emissions.

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CHAPTER 6

GENERAL CONCLUSIONS

GENERAL CONCLUSIONS

Results of the present thesis support the generally-accepted principle that increasing the physically effective neutral detergent fiber (peNDF) concentration in diets of lactating dairy cows by simply increasing dietary particle sizes promotes the animals' chewing activity. Increasing the peNDF concentration of the diet increased the total chewing time of cows per kilogram of dry matter (DM) intake across all three studies performed. The levels of DM intake consequently affected the total chewing time of cows per day probably because the DM intake declined due to greater peNDF concentrations of the diets. Nonetheless, the increments in chewing time and the total number of chews were small, and hence are unlikely to result in a significant increase in total saliva secretion and consequently in nitrogen (N) recycling in lactating dairy cows fed a total mixed ration in *ad libitum* and while chewing within their maximum physiological capacity.

The apparent total tract digestibility (ATTD) of nutrients and the yield and efficiency of rumen microbial protein synthesis (MPS) decreased when a diet with low rumen N balance (RNB) was offered to dairy cows with a high peNDF concentration compared to when the same diet was offered at low and medium peNDF concentrations. Although intake and ATTD of nutrients and MPS were reduced at high peNDF concentration, particularly for low RNB compared to the balanced RNB diet, the milk production of dairy cows and its composition could be maintained.

The effect of dietary peNDF concentration on chewing time as well as several animal response variables such as ATTD of nutrients, the efficiency of MPS, and partitioning of N excretion in cows differ, depending on the RNB of the offered diets. Also, the effect of RNB on ATTD of nutrients, yield and the efficiency of MPS, and partitioning of N

excretion in cows differ, depending on the peNDF concentration of the offered diets. Increasing the peNDF concentration reduced the yield and efficiency of MPS, in contrast to initially postulated and reduced ATTD of nutrients at low (– 1.5 g/kg DM) but not at balanced RNB (0.1 g/kg DM). Hence, increasing peNDF concentration does not compensate for negative effects on MPS and ATTD of nutrients at low RNB, but rather amplifies the effect of peNDF concentration. However, lowering the RNB reduced the proportion of ingested N excreted via urine in cows, when simultaneously the peNDF concentration in the diet was increased.

The RNB is a dietary factor that has not been considered in the peNDF concept so far. Besides considering the RNB of the diet in future peNDF studies, future RNB studies need also to take into account the peNDF concentration in ration formulation. With regards to this matter, the RNB threshold needs to be adjusted according to the desired peNDF concentration, and the peNDF concentration also needs to be adjusted according to the RNB of the diet. Additionally, long-term studies on the effect of peNDF dependent on the RNB of a diet on health and reproduction of dairy cows are needed for a better understanding of the long-term effects of reduced nutrient intakes and ATTD which can potentially arise in cows when fed diets with high concentrations of peNDF, especially at low RNB.

Adjusting both, the peNDF concentration and the RNB in diets of dairy cattle provides a tool to increase the milk N use efficiency in dairy cows. Ultimately, assuring adequate peNDF and slightly lowering RNB in the diets of dairy cows can, in the long-term, also provide economic and environmental benefits.

APPENDIX

Table A.1 Mixing protocol of experimental total mixed ration (TMR) of Chapter 2A.

Steps	Activities	Mixing	Individual mixing/ loading duration	Total mixing duration
Loading of feed ingredients				
1	Grass silage, 1st cut	x	4 min 30 s	
2	Grass silage, 2nd cut	x	5 min	9 min 30 s
3	Concentrate and Mineral-vitamin mixtures		30 min	
4	Corn silage	x	6 min	15 min 30 s
5	Grass hay, 1st cut	x	4 min	19 min 30 s
6	Barley straw	x	2 min	21 min 30 s
7	Grass hay, 2nd cut	x	4 min 30 s	26 min
8	Water	x	9 min	35 min
Positioning of feed mixer wagon and unloading of TMR into feed troughs				
9	1st TMR			35 min
10	2nd TMR	x	10 min	55 min

Table A.2 Mixing protocol of experimental total mixed ration (TMR) of Chapter 2B.

Steps	Activities	Mixing	Individual mixing/ loading duration	Total mixing duration
Loading of feed ingredients				
1	Grass silage, 1st cut	x	1 min 40 s	
2	Grass silage, 2nd cut	x	3 min 40 s	5 min 20 s
3	Molasses, Concentrate and Mineral-vitamin mixtures		20 min	
4	Corn silage	x	3 min 10 s	8 min 30 s
5	Grass hay, 1st cut		1 min	10 min 30 s
		x	2 min	
6	Grass hay, 2nd cut	x	2 min 30 s	13 min
7	Barley straw	x	2 min	15 min
8	Water	x	12 min	27 min
Positioning of feed mixer wagon to feed troughs				
9	Solely mixing	x	1 min	28 min
Unloading of TMR into feed troughs				
10	1st TMR			28 min
11	2nd TMR	x	15 min	43 min
12	3rd TMR	x	15 min	58 min
13	4th TMR	x	15 min	73 min

Table A.3 Mixing protocol of experimental total mixed ration (TMR) of Chapter 3.

Steps	Activities	Mixing	Individual mixing/ loading duration	Total mixing duration	
				RNB0[1]	RNB–[1]
Loading of feed ingredients					
1	Grass silage, 1st cut	x	1 min		
2	Grass silage, 2nd cut	x	6 min	7 min	7 min
3	Corn silage	x	1 min 30 s	8 min 30 s	8 min 30 s
4	Concentrate and Mineral-vitamin mixtures		20 min		
5	Barley straw	x	1 min	9 min 30 s	9 min 30 s
6	Grass hay, 1st cut	x	2 min 30 s	12 min	12 min
7	Grass hay, 2nd cut	x	2 min 30 s	14 min 30 s	14 min 30 s
8	Water	x	12 min 30 s	27 min	27 min
Positioning of feed mixer wagon to feed troughs					
9	Solely mixing	x	1 min	28 min	28 min
Unloading of TMR into feed troughs					
10	1st TMR			28 min	28 min
11	2nd TMR	x	30 min	58 min	58 min

[1]Rumen nitrogen balance (RNB): RNB0 = 0 g/kg dry matter and RNB– = – 1.5 g/kg dry matter.

Table A.4 Mixing protocol of experimental total mixed ration (TMR) of Chapter 4.

Steps	Activities	Mixing	Individual mixing/ loading duration	Total mixing duration
Loading of feed ingredients				
1	Barley straw			
2	Grass silage			
3	Grass haylage			
4	Corn silage			
5	Concentrate and Mineral-vitamin mixtures			
6	Corn grain			
7	Soybean grain			
8	Corn silage			
Mixing of TMR				
9	Solely mixing	x	15 min	
10	1st TMR			15 min
11	2nd TMR	x	15 min	30 min
12	3rd TMR	x	15 min	45 min
13	4th TMR	x	15 min	60 min

Table A.5 List of references and their reported variables[1] included in the analyses.

Reference	NDF	peNDF$_{>8.0}$	peNDF$_{>4.0}$	peNDF$_{>1.18}$	X_{gm}	DM intake	ATTD	Chewing activity	Passage rate	Ruminal fermentation	Microbial N yield	Milk variables
Beauchemin et al. (2003); Yang et al. (2002)	*	*		*	*	*	*	*	*	*	*	*
Beauchemin and Yang (2005); Yang and Beauchemin (2005)	*	*				*	*	*		*	*	*
Bhandari et al. (2007)	*	*		*	*	*				*		*
Bhandari et al. (2008)	*	*		*	*	*		*		*		*
Coon et al. (2018, 2019)	*	*	*			*		*		*		*
Jiang et al. (2017)	*	*	*			*		*		*		*
Kahyani et al. (2013)	*	*		*	*	*	*	*				*
Kononoff and Heinrichs (2003a)	*	*		*	*	*	*	*	*	*		*
Kononoff and Heinrichs (2003b)	*	*		*	*	*	*	*	*	*		*
Kononoff et al. (2003b)	*	*		*	*	*		*		*		*
Krause et al. (2002a; b)	*	*		*	*	*	*	*	*	*	*	*
Krause and Combs (2003)	*	*		*	*	*	*	*		*	*	*
Li et al. (2020); Zhao et al. (2020)	*	*		*	*	*	*			*	*	*
Maulfair et al. (2010)	*	*		*	*	*		*				*
Maulfair et al. (2011)	*	*		*	*	*	*			*		
Maulfair and Heinrichs (2013)	*	*		*		*		*		*		*
Rustomo et al. (2006)	*	*		*	*	*				*		*
Yang et al. (2001)	*	*		*	*	*	*	*	*	*		*
Yang and Beauchemin (2006a)	*	*		*		*	*	*		*	*	*

Yang and Beauchemin (2006b; c)	*	*			*	*	*	*	*	*	*	*
Yang and Beauchemin (2007a; b)	*	*		*	*	*	*	*		*	*	*
Yang and Beauchemin (2009)	*	*		*		*		*		*		*
Yansari et al. (2004)	*	*	*	*	*	*	*	*	*	*		*
Zebeli et al. (2007)	*		*	*	*	*	*	*	*			
Zebeli et al. (2008)	*	*		*		*	*			*		

[1]ATTD = apparent total tract digestibility; DM = dry matter; N = nitrogen; NDF = neutral detergent fiber; $peNDF_{>8.0}$ = physically effective NDF concentration estimated as NDF concentration multiplied by proportion of DM retained on 19.0- and 8.0-mm sieves (Lammers et al., 1996); $peNDF_{>4.0}$ = physically effective NDF concentration estimated as NDF concentration multiplied by proportion of DM retained on 19.0-, 8.0-, and 4.0-mm sieves (Kononoff et al., 2003a); $peNDF_{>1.18}$ = physically effective NDF concentration estimated as the NDF concentration multiplied by the proportion of DM retained on 19.0-, 8.0-, and 1.18-mm sieves (Kononoff et al., 2003a); X_{gm} = geometric mean of particle size.
‡List of references in Chapter 5.4.

Table A.6 Equations* for linear regression of response of feed intake and digestibility variables to different dietary factors in dairy cows.

Variable (Y)	Dietary factor (X)	Variable estimates				*P*-values
		Intercept	$SE_{Intercept}$[1]	Slope	SE_{Slope}[1]	
DM[1] intake, kg/d	$peNDF_{>8.0}$,[1] g/kg DM	25.8	0.89	-0.02	0.005	0.0002
	Concentrate, %	16.1	0.79	0.13	0.023	0.0046
	Starch, g/kg DM	17.1	1.71	0.20	0.069	0.0078
	NFC, [1] g/kg DM	14.6	2.54	0.20	0.073	0.0162
	Forage NDF[1] g/kg DM	26.3	1.58	-0.15	0.050	0.0166
	X_{gm},[1] mm	25.3	1.20	-0.41	0.157	0.0211
	$peNDF_{>1.18}$, g/kg DM	26.3	1.55	-0.01	0.035	0.0351
	NE_L,[1] MJ/kg DM	10.8	4.87	1.70	0.730	0.0424
ATDD,[1] g/100 g						
DM	NE_L, MJ/kg DM	9.75	8.44	8.33	1.250	0.0003
	Forage NDF g/kg DM	53.2	2.52	0.50	0.069	0.0019
	NFC, g/kg DM	35.1	4.96	0.84	0.118	0.0021
	$peNDF_{>1.18}$,[1] g/kg DM	73.7	2.76	-0.03	0.011	0.0266
	NDF:ADF	79.3	5.20	-8.21	3.430	0.0436
NDF	Starch, g/kg DM	57.0	4.08	-0.39	0.145	0.0356
Crude protein	NDF, g/kg DM	18.8	9.51	1.45	0.310	0.0015
	Forage NDF g/kg DM	45.3	3.47	0.51	0.145	0.0385

*Only significant relationships are shown ($P < 0.05$).

[1]ADF = acid detergent fiber; ATTD = apparent total tract digestibility; DM = dry matter; NDF = neutral detergent fiber; NE_L = net energy of lactation; NFC = non-fiber carbohydrate; $peNDF_{>8.0}$ = physically effective NDF concentration estimated as NDF concentration multiplied by proportion of DM retained on 19.0- and 8.0-mm sieves (Lammers et al., 1996); $peNDF_{>1.18}$ = physically effective NDF concentration estimated as the NDF concentration multiplied by the proportion of DM retained on 19.0-, 8.0-, and 1.18-mm sieves (Kononoff et al., 2003a); $SE_{Intercept}$ = standard error of intercept; SE_{Slope} = standard error of slope; X_{gm} = geometric mean of particle size.

‡List of references in Chapter 5.4.

Table A.7 Equations* for linear regression of response of solid passage rate and chewing variables to different dietary factors in dairy cows.

Variable (Y)	Dietary factor (X)	Variable estimates				*P*-values
		Intercept	$SE_{Intercept}$[1]	Slope	SE_{Slope}[1]	
Eating time, min/d	NFC,[1] g/kg DM	654	57.6	-10.2	1.43	<0.0001
	NE_L,[1] MJ/kg DM	985	124	-105	18.1	0.0007
	$peNDF_{>8.0}$,[1] g/kg DM[1]	199	12.0	0.51	0.13	0.0015
	NDF,[1] g/kg DM	93.6	42.6	5.22	1.32	0.0017
	Starch, g/kg DM	462	47.7	-6.81	1.50	0.0026
	$peNDF_{>1.18}$, [1] g/kg DM	166	29.1	0.41	0.12	0.0059
	X_{gm},[1] mm	199	16.8	19.2	5.53	0.0069
	Concentrate, %	406	31.2	-2.82	0.52	0.0319
Rumination time,	$peNDF_{>8.0}$, g/kg DM	338	21.2	0.81	0.16	0.0001
min/d	X_{gm}, mm	281	30.8	30.2	5.29	0.0003
	$peNDF_{>1.18}$, g/kg DM	292	47.3	0.52	0.16	0.0071
	NDF, g/kg DM	293	56.6	4.41	1.79	0.0273
Total chewing time						
min/d	X_{gm}, mm	489	54.8	53.5	11.1	0.0010
	NFC, g/kg DM	1267	126	-14.9	3.13	0.0015
	$peNDF_{>1.18}$, g/kg DM	437	67.0	1.0	0.24	0.0016

	peNDF$_{>8.0}$, g/kg DM	564	521	1.3	0.33	0.0023
	NDF, g/kg DM	408	80.2	8.94	2.58	0.0042
	NE_L, MJ/kg DM	1720	237	-150	35.1	0.0370
	Forage, %	455	1639	4.86	1.01	0.0406
min/kg DM intake	peNDF$_{>8.0}$, g/kg DM	20.6	1.52	0.08	0.01	<0.0001
	NFC, g/kg DM	79.5	8.90	-1.24	0.22	0.0005
	X_{gm}, mm	16.7	2.23	3.55	0.69	0.0006
	NE_L, MJ/kg DM	110	14.4	-11.4	2.11	0.0010
	peNDF$_{>1.18}$, g/kg DM	13.5	4.33	0.07	0.02	0.0011
	NDF, g/kg DM	16.6	5.02	0.45	0.16	0.0157
	Crude protein, g/kg DM	64.8	14.1	-2.02	0.85	0.0330
	Concentrate, %	49.0	3.80	-0.35	0.07	0.0325
Solid Passage, %/h	NDF, g/kg DM	5.27	0.40	-0.04	0.01	0.0189
	NFC, g/kg DM	0.32	0.53	0.09	0.01	0.0233
	Crude protein, g/kg DM	-1.06	1.60	0.29	0.09	0.0376

*Only significant relationships are shown ($P < 0.05$).
[1]DM = dry matter; NDF = neutral detergent fiber; NE_L = net energy of lactation; NFC = non-fiber carbohydrate; peNDF$_{>8.0}$ = physically effective NDF concentration estimated as NDF concentration multiplied by proportion of DM retained on 19.0- and 8.0-mm sieves (Lammers et al., 1996); peNDF$_{>1.18}$ = physically effective NDF concentration estimated as the NDF concentration multiplied by the proportion of DM retained on 19.0-, 8.0-, and 1.18-mm sieves (Kononoff et al., 2003a); $SE_{Intercept}$ = standard error of intercept; SE_{Slope} = standard error of slope; X_{gm} = geometric mean of particle size.
‡List of references in Chapter 5.4.

Table A.8 Equations* for linear regression of response of milk variables to different dietary factors in dairy cows.

Variable (Y)	Dietary factor (X)	Variable estimates				*P*-values
		Intercept	$SE_{Intercept}$[1]	Slope	SE_{Slope1}	
Milk yield, kg/d	NFC,[1] g/kg DM	24.0	4.50	0.340	0.109	0.0108
	$peNDF_{>8.0}$,[1] g/kg DM	37.8	1.69	-0.013	0.005	0.0235
Milk fat, g/kg	$peNDF_{>1.18}$,[1] g/kg DM	24.45	2.25	0.033	0.008	0.0011
	Forage NDF,[1] g/kg DM	54.60	4.72	-12.1	3.09	0.0014
	NDF:ADF[1]	27.86	1.73	0.285	0.057	0.0015
	$peNDF_{>8.0}$, g/kg DM	32.82	1.69	0.021	0.006	0.0022
	X_{gm},[1] mm	28.87	1.34	0.751	0.260	0.0138
	Crude protein, g/kg DM	16.61	7.39	1.13	0.421	0.0160
	Starch, g/kg DM	46.53	2.32	-0.290	0.105	0.0185
	Concentrate, %	43.72	2.41	-0.159	0.041	0.0315
	$peNDF_{>4.0}$, g/kg DM	36.53	5.83	0.029	0.008	0.0340
Milk protein, g/kg	Forage NDF g/kg DM	35.5	1.15	-0.015	0.003	0.0005
	$peNDF_{>1.18}$, g/kg DM	35.5	1.15	-0.015	0.003	0.0005
	NDF, g/kg DM	39.2	1.93	-0.211	0.052	0.0008
	NDF:ADF	26.6	1.62	3.60	1.08	0.0045

	Starch, g/kg DM	29.2	1.07	0.142	0.043	0.0067
	Forage, %	35.6	1.01	-0.066	0.010	0.0078
	$peNDF_{>8.0}$, g/kg DM	33.4	0.98	-0.008	0.003	0.0150
	NE_L,[1] MJ/kg DM	21.5	4.48	1.59	0.678	0.0475
MNUE,[1] g/g	Crude protein, g/kg DM	0.52	0.08	-0.013	0.005	0.0119
	Forage, %	0.21	0.02	1.48×10^{-3}	0.43×10^{-3}	0.0421

*Only significant relationships are shown ($P < 0.05$).
[1]ADF = acid detergent fiber; DM = dry matter; MNUE = milk N use efficiency (g milk N/g N intake); N = nitrogen; NDF = neutral detergent fiber; NE_L = net energy of lactation; NFC = non-fiber carbohydrate; $peNDF_{>8.0}$ = physically effective NDF concentration estimated as NDF concentration multiplied by proportion of DM retained on 19.0- and 8.0-mm sieves (Lammers et al., 1996); $peNDF_{>1.18}$ = physically effective NDF concentration estimated as the NDF concentration multiplied by the proportion of DM retained on 19.0-, 8.0-, and 1.18-mm sieves (Kononoff et al., 2003a); $SE_{Intercept}$ = standard error of intercept; SE_{Slope} = standard error of slope; X_{gm} = geometric mean of particle size.
‡List of references in Chapter 5.4.

Table A.9 Equations* for linear regression of response of rumen fermentation and microbial nitrogen variables to different dietary factors in dairy cows.

Variable (Y)	Dietary factor (X)	Variable estimates				
		Intercept	$SE_{Intercept}$[1]	Slope	SE_{Slope}[1]	*P*-values
Microbial N[1], g/d	NDF,[1] g/kg DM	628	121	-11.9	4.18	0.0462
Rumen pH	$peNDF_{>1.18}$,[1] g/kg DM	5.47	0.14	2.26×10^{-3}	0.00054	0.0013
	$peNDF_{>8.0}$,[1] g/kg DM	5.90	0.07	0.00135	0.00046	0.0104
	NDF, g/kg DM	5.47	0.26	0.01854	0.00782	0.0316
VFA,[1] m*M*	X_{gm},[1] mm	145	8.32	-3.89	0.817	0.0015
	$peNDF_{>8.0}$, g/kg DM	139	6.10	-0.11	0.030	0.0042
C2[1]:C3[1]	NE_L,[1] MJ/kg DM	8.37	1.51	-0.857	0.217	0.0075
	NDF, g/kg DM	1.38	0.51	0.036	0.016	0.0451
C2, mol/100 mol	NDF, g/kg DM	31.8	7.22	0.986	0.2006	0.0002
	Forage NDF g/kg DM	52.7	5.71	0.591	0.0761	0.0002
	NE_L, MJ/kg DM	132.5	16.62	-9.99	2.50	0.0040
	NFC,[1] g/kg DM	99.8	7.59	-0.904	0.2311	0.0045
	NDF:ADF[1]	84.9	6.85	-12.6	4.04	0.0091
	Starch, g/kg DM	79.8	4.10	-0.511	0.2027	0.0397

C3, mol/100 mol	Forage NDF g/kg DM	39.6	3.08	-0.533	0.0662	0.0002
	Starch, g/kg DM	8.0	3.12	0.666	0.1318	0.0015
	NFC, g/kg DM	-4.5	6.84	0.792	0.1807	0.0023
	NE_L, MJ/kg DM	-37.7	15.03	9.44	2.21	0.0027
	NDF, g/kg DM	45.0	5.83	-0.581	0.1705	0.0038
	NDF:ADF	9.7	5.33	9.81	3.33	0.0121
	Concentrate, %	13.4	2.90	0.234	0.0462	0.0370
	$peNDF_{>8.0}$, g/kg DM	28.3	1.81	-0.01950	0.00851	0.0379
NH_4-N,[1] m*M*	NDF, g/kg DM	-0.3	2.65	0.326	0.078	0.0015

*Only significant relationships are shown ($P < 0.05$).
[1]ADF = acid detergent fiber; C2 = acetate; C3 = propionate; DM = dry matter; N = nitrogen; NH_4-N = ammonium-N; NDF = neutral detergent fiber; NE_L = net energy of lactation; NDF:ADF = NDF to acid detergent fiber ratio; NFC = non-fiber carbohydrates; $peNDF_{>8.0}$ = physically effective NDF concentration estimated as NDF concentration multiplied by proportion of DM retained on 19.0- and 8.0-mm sieves (Lammers et al., 1996); $peNDF_{>1.18}$ = physically effective NDF concentration estimated as the NDF concentration multiplied by the proportion of DM retained on 19.0-, 8.0-, and 1.18-mm sieves (Kononoff et al., 2003a); $SE_{Intercept}$ = standard error of intercept; SE_{Slope} = standard error of slope; X_{gm} = geometric mean of particle size; VFA = volatile fatty acids.
‡List of references in Chapter 5.4.

Table A.10 Best-fit equations for multiple regression* of animal responses and microbial to different dietary factors in dairy cows.

Variable (Y)	Dietary factor (X)	Variable estimates				*P*-values
		Intercept	$SE_{Intercept}$	Slope	SE_{Slope}	
DM[1] intake, kg/d	$peNDF_{>8.0}$,[1] g/kg DM	31.0	1.69	-0.041	0.016	0.0296
	Forage NDF,[1] g/kg DM			-0.143	0.055	0.0316
	$peNDF_{>8.0}^2$, g/kg DM			6.9×10^{-5}	3.7×10^{-5}	0.0854
Rumination time, min/d	$peNDF_{>8.0}$, g/kg DM	299	24.7	1.53	0.31	0.0002
	$peNDF_{>8.0}^2$, g/kg DM			-2.76×10^{-3}	1.03×10^{-3}	0.0118
Milk fat, g/kg	$peNDF_{>8.0}$, g/kg DM	50.4	4.52	1.81×10^{-2}	4.88×10^{-3}	0.0016
	NDF:ADF[1]			-11.0	2.83	0.0018
Milk protein, g/kg	Forage NDF, g/kg DM	32.8	1.32	0.13	0.12	0.2973
	Forage NDF^2, g/kg DM			-6.66×10^{-3}	2.47×10^{-3}	0.0165
Rumen pH	$peNDF_{>8.0}$, g/kg DM	5.41	0.24	3.15×10^{-3}	9.91×10^{-4}	0.0035
	NDF, g/kg DM			1.08×10^{-2}	7.75×10^{-3}	0.1869
	$peNDF_{>8.0}^2$, g/kg DM			-5.78×10^{-6}	2.38×10^{-6}	0.0302
C2[1]:C3[1]	NE_L,[1] MJ/kg DM	-40.7	14.4	13.2	4.10	0.0490
	X_{gm}, mm			0.093	0.023	0.0157
	NE_L^2, MJ/kg DM			-1.01	0.293	0.0108
NH_4-N,[1] m*M*	NDF, g/kg DM	-16.7	5.68	0.564	0.083	0.0002
	NFC,[1] g/kg DM			0.222	0.089	0.0416

*For simplicity, only the best-fit equations that improved further the relationship obtained from linear regressions are shown.

[1]C2:C3 = acetate to propionate ratio; DM = dry matter; NDF = neutral detergent fiber; NE_L = net energy of lactation; NDF = neutral detergent fiber; NDF:ADF = NDF to acid detergent fiber ratio; NE_L = net energy of lactation; NFC = non-fiber carbohydrate; $peNDF_{>8.0}$ = physically effective NDF concentration estimated as NDF concentration multiplied by proportion of DM retained on 19.0- and 8.0-mm sieves (Lammers et al., 1996); $SE_{Intercept}$ = standard error of intercept; SE_{Slope} = standard error of slope.

‡List of references in Chapter 5.4.

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