# OPTIMIZING PRODUCTIVITY FROM PASTURE-BASED SYSTEMS – QUANTIFICATION OF NUTRIENT SUPPLY

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# OPTIMIZING PRODUCTIVITY FROM PASTURE-BASED SYSTEMS – QUANTIFICATION OF NUTRIENT SUPPLY

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## ABSTRACT

Pasture-based diets can comprise completely of human inedible ingredients, support a resilient business model for the producer, and an animal welfare friendly image. However, increasing environmental challenges will require pasture-based systems to adapt and innovate. To ascertain optimal nutritional strategies to increase the nutrient use efficiency, productivity, and sustainability of these systems, a robust understanding of the nutrient supply from pasture is required. Therefore, the objectives of this research were: 1) to characterize the nutritive value of pasture using new and updated feed chemistry methods, 2) provide a more robust understanding of nutrient supply and microbial dynamics in lactating dairy cows fed pasture-based diets, 3) incorporate new understanding of protozoal dynamics into the microbial sub-model of the Cornell Net Carbohydrate and Protein System (**CNCPS**) v7.0, and 4) investigate nutritional strategies to optimize productivity from pasture-based systems. Results from laboratory analysis demonstrated that the neutral detergent fiber fraction of immature pasture comprises of a large potentially digestible pool that degrades rapidly. In addition, a large proportion of pasture N was estimated to be soluble and highly degradable in the rumen. An in vivo study was performed to determine the effects of rolled barley supplementation on cows fed pasture-based diets, which incorporated the omasal sampling technique, rumen evacuation, microbial isolation, and amino acid (**AA**) analysis.

Cows supplemented with rolled barley did not increase overall performance and reduced ruminal and total-tract neutral detergent fiber digestibility; however, this was not mediated through a reduction in reticulorumen pH. Rolled barley supplementation increased microbial AA flow, which was likely due to the greater amount of fermentable carbohydrate digested in the rumen and a greater efficiency of microbial protein synthesis. Extensive rumen degradation of pasture AA, for both diets, indicated that cows consuming pasture-based diets exhibit a large dependence on microbial AA to support metabolizable AA supply. Protozoa N flow was not affected by diet; however, protozoa supplied a much larger amount of microbial N and exhibited shorter generation time than previously assumed, indicating that protozoa have the capability to grow and leave the rumen at high rates. Reparameterization of the coefficients of the protozoal sub-model in the CNCPS v7.0 considerable improved the ability of the model to predict AA flow, when compared with a literature data set. However, discrepancies between the predicted and observed Met and Lys flows indicated that further refinement is required while all other AA are predicted with fairly high accuracy. Finally, when investigating nutritional strategies to optimize productivity from pasture-based diets, results demonstrated that increased metabolizable protein supply allowed higher milk yield to be achieved, as metabolizable protein was more limiting than metabolizable energy. However, the high soil moisture deficit experienced during the study altered the chemical composition of the pasture, reducing the ability to extrapolate the results to cows consuming typical pasture-based diets. Overall, this research provides an enhanced understanding of the nutrient supply from pasture-based diets and highlights opportunities to increase the productivity and efficiency of pasture-based systems. Future studies incorporating the quantitative techniques described in this research are required to provide further insight into the nutrient supply of pasture-fed cows.

## BIOGRAPHICAL SKETCH

Michael Dineen was raised on a pasture-based dairy farm in County Cork, Ireland. He attended Leap National School from 1996-2004, after which he attended Mount Saint Michael Secondary School (2004-2010). A love for agriculture led him to University College Dublin, where he obtained a B.S. in Animal Science in 2014. During his B.S., Mike became aware of the important role Teagasc (who are an Irish governmental body involved in research, education, and extension) play in the Irish agricultural industry. In June of 2014, he began a Ph.D. program at Teagasc Moorepark in conjugation with Queens University Belfast under the guidance of Dr. Brian McCarthy. However, during this time, an opportunity arose to join the laboratory of Dr. Michael Van Amburgh at Cornell University. Mike concluded his work at Teagasc Moorepark obtaining a Master of Philosophy. His thesis was titled: Impact of *Lolium perenne* L. ploidy and *Trifolium repens* L. inclusion in grazing swards, on milk production of spring calving dairy cattle. In August of 2015, Mike moved to Ithaca, NY and began a Ph.D. program at Cornell University to describe the nutrient supply from pasture-based diets along with the evaluation and development of the Cornell Net Carbohydrate and Protein System.

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This dissertation is dedicated to my loved ones. I have deeply missed my family and eagerly await spending much more time together. Thank you for your ceaseless support and love. To all

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# CHAPTER 1: LITERATURE REVIEW

## 1.1. Introduction and Overview

The global population is expected to increase from 7.7 billion in 2019 to 9.7 billion by 2050 (UN, 2019). Due to this 26% rise in population, coupled with increasing standards of living, the global demand for dairy products is projected to increase by 48% (Alexandratos and Bruinsma, 2012). Concurrently, the demand for human-edible plant resources is also increasing rapidly, potentially reducing the availability of these resources and the associated arable land for livestock production (Mottet et al., 2017). Therefore, to maintain a significant contribution to net food production, ruminant production systems should increase their reliance on the unique ability to convert human inedible resources into human edible food (Kim et al., 2019). Accordingly, ruminant production systems have the opportunity to continue to play a major role in global food and nutrition security, while reducing the environmental impact of other food production systems (White and Hall, 2017; Van Amburgh et al., 2019).

In temperate regions, pastures comprised of cool season grass species (from here on referred to as 'pasture') are an important source of nutrients for livestock (O'Brien et al., 2018). In such systems, the diet of the cow can comprise almost completely of human inedible ingredients resulting in a large to infinite return on human edible resources (Dijkstra et al., 2013; Laisse et al., 2018). In addition, high pasture inclusion levels in dairy cow diets can support a resilient business model for the producer and an animal welfare friendly image (Dillon et al., 2008; van Vuuren and Chilibroste, 2013). Recently, there has been a growing interest in the intensification of pasture-based dairy systems (Läpple and Sirr, 2019). However, there are concerns that this intensification will be associated with adverse environmental effects, such as N losses to air and groundwater, increased global greenhouse gas emissions, and loss of natural ecological habitats (Hoekstra et al.,

2020). It is critical that future intensification of the dairy industry is achieved sustainably, that is, increasing total food production from the current agricultural area, while reducing the industry's overall environmental impact (Godfray et al., 2010; Schulte et al., 2014).

There are opportunities to increase the productivity and efficiency of pasture-based systems by strategically modifying the nutrient supply of the cow (Peyraud and Delagarde, 2013). Potential strategies include, but are not limited to, improved pasture management (O'Donovan et al., 2002), optimization of concentrate supplementation (Baudracco et al., 2010), selection of superior plant genetics (Lee et al., 2012), and the development of binary or multi-species pastures (McCarthy et al., 2020). To select the optimal strategy, quantitative knowledge of how the diet interacts with the ruminant, the nutrients it supplies, and the metabolic requirements of the cow is crucial.

The following review will focus on our current understanding of energy and protein nutrition in lactating dairy cows fed pasture-based diets. Specifically, this review will focus on the nutritional composition of pasture, its digestive behavior, and its interaction with microbial metabolism. Also discussed are 1) evaluation techniques used to elucidate the nutrient supply from pasture and 2) areas of opportunity to alter nutrient supply to optimize productivity and efficiency of lactating dairy cows fed pasture-based diets.

### 1.2. Characterization of the Nutrient Supply from Pasture

The net capture of nutrients (digestibility) from pasture is affected by 1) the amount of indigestible matter and 2) the competition between the relative rates of digestion and passage of the potentially digestible matter. In addition, it is important to consider that the latter can dictate the compartment in which digestion might occur and, ultimately, the profile of nutrients available for absorption (e.g. volatile fatty acids, AA from microbial protein). Many factors can affect the

nutrient supply from pasture such as species and cultivar (Smit et al., 2005; Chen et al., 2019), morphological proportions (Beecher et al., 2015), environmental conditions (Van Soest, 1994), N fertilizer application (Peyraud and Astigarraga, 1998) and seasonal variation (Roche et al., 2009; Douglas et al., 2020). Ideally, to assess the effect of these factors on the nutritive value of pasture, all combinations would be evaluated in controlled cow experiments and the production outcomes recorded. Obviously, this is neither practical nor cost effective, and therefore, nutritionists are reliant on laboratory feed evaluation techniques and mathematical models to estimate the availability of energy and protein in a feed. The overall goal of feed evaluation systems is to create general classification schemes to group feed fractions that behave similarly or uniformly across diets (Lucas, 1964; Van Soest, 1967). Such systems can aid in the elucidation of the nutrient supply and nutritional factors that might limit cow productivity and efficiency (Minson, 1981).

### 1.3. Plant Cell Wall and Digestibility

A widely accepted feed evaluation system that can aid in the characterization of the digestive behaviour of a feed is the detergent fiber system (Van Soest, 1991). Notably, neutral detergent fiber (**NDF**) differentiates plant dry matter (**DM**) into cell solubles that have a constant and essentially complete true digestibility (DM – NDF + ash), and insoluble cell wall material that has a variable true digestibility. Although pectin and β-glucans are components of the plant cell wall, nutritionally they resemble the cell contents, exhibiting complete true digestibility. The non-ideal behaviour (Lucas, 1964) of the insoluble cell wall material has been demonstrated to account for the majority of variation in plant digestibility (Nousiainen et al., 2004). Therefore, knowledge pertaining to plant cell wall development and structure is necessary to gain an understanding of

the factors influencing plant digestibility and consequently, DMI and cow performance (Rinne, 2000; Beecher et al., 2018).

### *1.3.1. Cell Wall Structure*

The plant cell wall is often described as a giant macromolecule comprised of many different molecules including polysaccharides, protein, lignin, and phenolic acids (Van Soest, 1994). During primary cell wall growth, as the plant cells are dividing and expanding, a foundation of cellulose microfibrils, linked together by hydrogen bonds, is constructed resulting in the outer layer of the cell wall (Wilson, 1993). In addition, structural proteins, pectins, xylans, and phenolic acids are deposited; however, at this stage the primary cell wall is devoid of lignin (Jung and Allen, 1995). Furthermore, during primary cell wall growth in grasses, initial polysaccharide crosslinking can occur as ferulic acid and a small amount of *p*-coumaric acid are esterified to arabinoxylans (Vailhé et al., 2000). Upon completion of plant cell elongation, secondary cell wall thickening initiates, beginning from the interior side of the primary wall. Secondary wall thickening can comprise of up to three layers of cellulose microfibrils, which are devoid of pectin and ferulic acid. Secondary cell wall thickening initiates lignin deposition, beginning in the middle lamella and progressively proceeding through the primary cell wall and into the secondary cell wall layers. As lignin deposition occurs, the arabinoxylan ferulate esters, developed during primary cell wall growth, can be etherified or covalently bonded to lignin, resulting in the cross-linking of arabinoxylans to lignin (Iiyama et al., 1990; Grabber et al., 2000). Etherification of *p*-coumaric acid to lignin has also been demonstrated; however, further cross-linking to arabinoxylan does not seem to occur (Lam et al., 1992).

### *1.3.2. Cell Wall Digestibility*

A negative correlation between total lignin concentration and cell wall digestibility has been recognized for quite some time, as lignin is resistant to microbial degradation in the rumen (Jung and Deetz, 1993). While it is unequivocal that cell wall lignification impacts digestibility, the proportion of variation attributable to lignin concentration is debatable, as plants with similar lignin concentrations can differ greatly in digestibility (Jung et al., 1994; Van Amburgh et al., 2015). Changes in lignin composition, measured as the syringyl:guaiacyl ratio, has been suggested to account for increased digestibility observed in brown midrib mutants (Cherney et al., 1991). However, the effect of altered lignin composition on cell wall digestibility has been strongly refuted (Grabber et al., 1997; Jung et al., 1999). Notably, there is growing evidence that the degree of cross-linking of cell wall carbohydrates, among one another and to lignin, is an important factor governing plant digestibility (Grabber et al., 2009; Jung et al., 2011). Arabinoxylan ferulate esters appear to inhibit digestion by hindering the alignment of xylanase with its substrate (Jung et al., 1991). However, ruminal microorganisms have the ability to produce phenolic acid esterases, therefore, the overall impact of these linkages is related to cell wall digestion rate rather than extent (Jung and Allen, 1995). The second ferulate linkage, by means of an ether or covalent bond, results in the cross-linking of arabinoxylans to lignin (Iiyama et al., 1990). The proximity of lignin to arabinoxylans has been suggested to have a more detrimental effect on the extent of cell wall digestion (Jung and Deetz, 1993). Grabber et al. (2009) demonstrated that a reduction in the cross-linking of arabinoxylans to lignin improved the digestion of hemicellulose and attributed over half of the inhibitory effect of lignin on cell wall digestion to these linkages. In agreement, Jung et al. (2011) demonstrated that a seedling leaf ferulate ester mutant, that also exhibits reduced ferulate

ether cross-linking of lignin to arabinoxylan, had increased in vitro cell wall digestibility and improved DMI and milk yield in lactating dairy cows compared with a conventional corn silage.

Within pasture-based systems, there have been attempts to select cultivars based on the digestibility of the plant cell wall (Taweel et al., 2005a; Tas et al., 2006a). These studies reported a narrow range in the rumen pool sizes, clearance, and digestibility among a number of cultivars, concluding that there is low potential for genetic improvement. However, the cultivars compared in the studies were chosen based on contrasting heading dates, rather than differences in cell wall structure. Recently, Raffrenato et al. (2017) demonstrated that while immature and mature grasses contain similar quantities of esterified ferulic acid, mature grasses contained significantly higher quantities of etherified ferulic acid. These etherified linkages likely explain, at least in part, the typical reduction in digestibility observed as pasture grasses mature (Chaves et al., 2006). If mutants suitable for grazing are developed, similar to those previously discussed for corn and sorghum, the rate of decline in cell wall digestibility as the plant ages might be reduced. This would allow greater flexibility in pasture management and likely increase the productivity and efficiency of pasture-based systems.

### *1.3.3. Quantification of NDF Digestibility*

Pasture NDF concentration can vary substantially, from 17.8 to 78.0% of DM (Corson et al., 1999). In addition, a wide range in the total-tract digestibility (**TTD**) of NDF has been reported for pasture-fed lactating dairy cows (64.4 to 90.2% NDF TTD; Rius et al., 2012; Garry, 2016). Both the quantity and the digestion characteristics of NDF can considerably influence DMI, rumen pool size, rumination, and milk production (Cotanch et al., 2014; Zontini et al., 2015). Therefore, it is critical to achieve a robust understanding of NDF concentration and digestibility. The total

collection method has primarily been utilized to measure TTD of pasture NDF **(**McDonald et al., 2002). While an accurate measure of TTD can be achieved from this method, an understanding of the ruminal kinetics of digestion and passage cannot be attained. This reduces our ability to understand mechanisms regulating DMI, the true nutrient supply to the host, and the effect of a suboptimal ruminal environment on NDF digestibility (Huhtanen et al., 2006). Furthermore, investigations utilizing the total collection method frequently incorporate castrated male sheep to measure TTD, as it is easier to separate urine from faeces when compared with lactating dairy cows (Rymer, 2000). However, animal species can affect the measure of TTD, which brings into question the direct extrapolation of the data obtained from sheep to lactating dairy cows (Van Soest, 1994; Garry, 2016).

To attain an understanding of the site of digestion in pasture-fed cows, digesta flow studies incorporating sampling from a duodenal fistula have been performed (O'Mara et al., 1997; Younge et al., 2004). Such studies indicate that the majority of pasture NDF digestion occurs prior to the duodenum, on average, 95% of the total NDF digested (Bargo et al., 2003). However, a number of duodenal sampling studies have reported zero or negative hind-gut NDF digestion (Van Vuuren et al., 1992; Peyraud et al., 1997). This suggests unrepresentative digesta sampling or marker dysfunction within those studies, as negative NDF digestion coefficients are biologically impossible (Huhtanen et al., 2010). In addition, other concerns such as invasive surgical procedures led to the development of a new digesta flow sampling technique. The new technique is less invasive, as digesta is sampled at the omasal canal via a rumen cannula without repeated entry into the omasum (Huhtanen et al., 1997). Furthermore, when combined with multiple marker methods to measure digesta flow, the technique has been demonstrated to have smaller variability in the measurement of ruminal NDF digestibility compared with duodenal sampling (Huhtanen et

al., 2010). To date, one omasal sampling study has been performed in pasture-fed lactating dairy cows (Sairanen et al., 2005). In that study, the NDF TTD was 83.6%, with 92.8% of the NDF digestion occurring in the rumen.

When the omasal sampling technique is combined with the rumen evacuation technique, fractional rates of intake, passage, and digestion can be measured (Robinson et al., 1987) using the following equations:

$$\text{Rate of intake (ki)} = 1/24 \times (\text{intake, kg/d}) / (\text{rumen pool size, kg})$$

$$\text{Rate of passage (kp)} = 1/24 \times (\text{omasal flow, kg/d}) / (\text{rumen pool size, kg})$$

$$\text{Rate of digestion (kd)} = \text{ki} - \text{kp}$$

Using such techniques and calculations, Sairanen et al. (2005) reported a rapid ruminal rate of digestion of the digestible NDF fraction, 9.2%/h, in pasture-fed cows. Furthermore, the authors inferred from measures of the rumen pool size, that rumen distension does not limit the intake of cows fed highly digestible pasture, which is contrast with the findings of Boudon et al. (2009). The use of rumen evacuation and omasal sampling techniques has been extremely limited in studies investigating the supply of nutrients to pasture-fed lactating dairy cows. Incorporating these methods into future studies can provide a more robust understanding of the factors governing ruminal digestion kinetics, DMI, and performance of pasture-fed lactating dairy cows.

A likely reason for the limited use of such techniques is that they are laborious, time consuming, and expensive. Therefore, defined laboratory analytical procedures combined with mathematical models are vital to predict the digestion characteristics of NDF (Chesson, 1993). As no chemical fractionation scheme can quantify the overall impact of lignin concentration and lignin cross-

linking within the cell wall on digestibility, assays incorporating live rumen microorganisms or cell-wall degrading enzymes are required (Van Soest, 1994). Some feed evaluation systems (INRA, 2018) and pasture value indexes (McEvoy et al., 2011; Chapman et al., 2017) rely on in vitro organic matter digestibility methods to indirectly measure plant cell wall digestibility. However, these methods have a number of limitations when used to predict in vivo digestibility. For example, the measures of organic matter digestibility are primarily achieved with cellulolytic enzymes that do not degrade NDF as efficiently as rumen microorganisms (Mertens and Grant, 2007). In addition, the in vitro digestibility methods typically incorporate large pore size filtering apparatus, which has been demonstrated to underestimate the recovery of components of the plant cell wall and overestimate digestibility (Udén, 2006; Raffrenato et al., 2018). Furthermore, a single fermentation time point is performed, reducing the method's ability to adequately describe the dynamic and heterogeneous nature of NDF (Ellis et al., 2005; Huhtanen et al., 2008). Overall, the method results in biased predictions that require correction equations and continuous re-calibration to estimate in vivo digestibility (Beecher et al., 2015; Garry et al., 2018).

Recently, an in vitro amylase- and sodium sulfite-treated NDF corrected for ash residue (**aNDFom**) digestibility method has been employed, to overcome a number of these limitations (Mertens, 2002; Raffrenato et al., 2018). The method utilizes rumen microorganisms to efficiently digest aNDFom and incorporates a glass microfiber filter with a pore size of 1.5-µm to increases the recovery of the indigestible cell wall material. The method also utilizes long fermentation time points (240 h), to approximate the extent of digestion, namely undigested aNDFom (**uNDFom**). In addition, after determination of uNDFom, the potentially digestible aNDFom (**pdNDFom**) can be calculated by difference (aNDFom – uNDFom). When combined with a mathematical modelling procedure, the method can be applied at strategic fermentation time points to

differentiate the digestion of aNDFom into multiple rates and fractions (Figure 1.1; Raffrenato et al., 2019). The fast- and slow-degrading fractions, with their respective rates of digestion, along with an undigested fraction can more accurately describe the dynamic and heterogeneous nature of aNDFom. However, during development of the method, the authors noted that in order to comprehensively describe the digestion behavior of immature pasture, further in vitro fermentation time points might be required (Raffrenato et al., 2019).

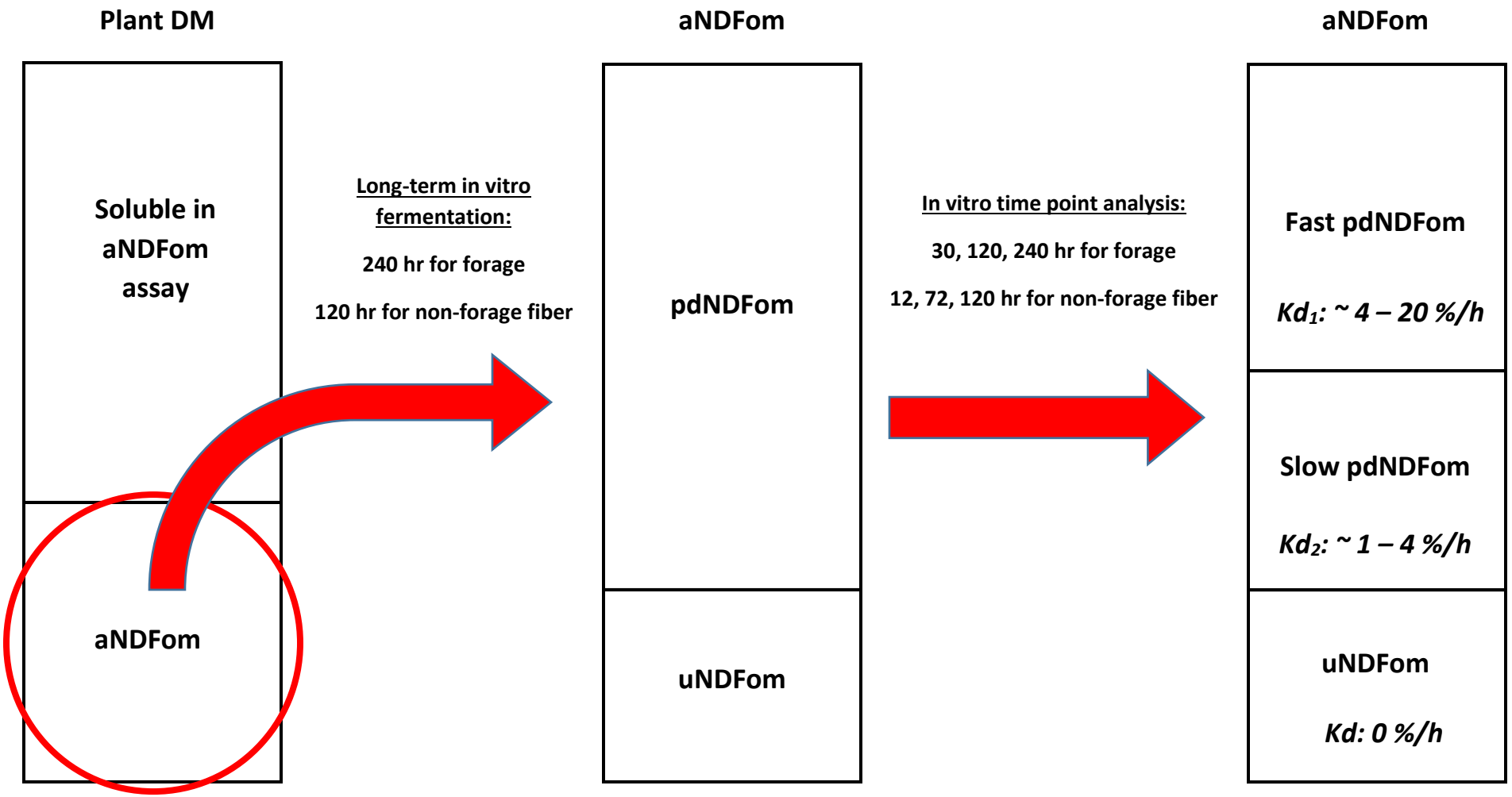


**Figure 1.1.** Fractionation of feed aNDFom as described by Raffrenato et al. (2018, 2019).

### *1.3.4. Soluble Fiber*

The primary components of soluble fiber comprise of pectins and β-glucans. Though they are components of the plant cell wall, they behave similarly to the cell contents due to their lack of covalent linkages with lignin. Accordingly, soluble fiber is completely available to microbial fermentation (Van Soest, 1994) but is, however, indigestible to mammalian enzymes. Pectins are predominantly found in the middle lamella and the primary cell wall. In addition, they are much more abundant in dicots than monocots with the majority of knowledge on pectin in forages pertaining to legumes. Beta-glucans can be recovered in high quantities from oats and barley (40-120 g/kg DM; Engstrom et al., 1992), with small quantities identified in the cell wall of grasses (Van Soest, 1994). Depending on the definition of soluble fiber, fructans can also be included; however, for this review fructans will be classified as non-fiber carbohydrates (discussed further below). Feeds such as citrus pulp, beet pulp, soybean hulls, and alfalfa can contain considerable amounts of soluble fiber (Van Amburgh et al., 1999). Fermentation rates of soluble fiber are typically higher than that of NDF (~10 to 20%/h) reaching complete fermentation in vitro by 9 to 14 h, except for soybean hulls, which take approximately 24 to 34 h (Hall et al., 1998). An important characteristic of soluble fiber is that when fermented, like NDF, acetic acid is the main volatile fatty acid (**VFA)** produced with little or no lactate being generated (Strobe1 and Russell, 1986). Supplementation of pasture-based diets with ingredients high in soluble fiber, when compared with starch-based ingredients, has been suggested to maintain higher rumen pH, NDF digestion, and DMI; however, effects on cow performance are equivocal (Stakelum, 1993; Delahoy et al., 2003).

## 1.4. Non-Fiber Carbohydrates

### *1.4.1. Water-Soluble Carbohydrates*

Water-soluble carbohydrates (**WSC**) are the main energy storage form in the leaves and stems of pasture. The WSC fraction comprises mainly of glucose, fructose, sucrose, and fructans (Turner et al., 2006). After defoliation, the plant relies on and depletes stored WSC reserves to grow new shoots. When 75% of the first new leaf has regrown, the plant has adequate photosynthetic capacity for growth, and the WSC concentration begins to replenish (Fulkerson and Donaghy, 2001). Fructans, polymers of fructose that begin with a sucrose unit (Van Soest, 1994), typically are the greatest contributor to the WSC fraction in pasture (Turner et al., 2006). In a study utilizing high-performance liquid chromatography techniques to quantify fructan concentrations of pasture, a wide range of concentrations were observed (8 to 30% of DM; Longland et al., 2012). Many factors such as grass species, growth stage, light intensity, photosynthetic rate, and environmental temperature can affect the concentration of fructans (Turner et al., 2006). Cool temperatures and drought conditions favour the accumulation of fructans, whereas high temperatures and low light intensity reduce their concentrations (Longland et al., 2012). Furthermore, increasing rates of N fertilizer application have been demonstrated to reduce the concentration of fructans, due to the increased partitioning of energy by the plant towards nitrate reduction and protein synthesis (Bowerman and Goodman, 1971; Loaiza et al., 2017).

The WSC fraction degrades rapidly at 40%/h, supporting high microbial growth rates (Molina, 2002). These carbohydrates can also be converted into energy reserves in the form of glycogen by the rumen microorganisms (Hackmann and Firkins, 2015). Compared with starch, when WSC are fermented by rumen microorganisms, similar amounts of propionate but higher amounts of butyrate are produced (Strobe1 and Russell, 1986). In addition, at lowered rumen pH, lactate can

be produced from the fermentation of the WSC components (Thomas, 1960). Based on these fermentation characteristics, the WSC assay, which wholly extracts fructans, is preferred over that of the ethanol-soluble carbohydrate assay to estimate nutrient supply to the ruminant (Hall, 2014).

In an attempt to increase the productivity and nitrogen use efficiency of pasture-fed cows, grass cultivars have been conventionally bred to exhibit increased WSC concentrations (Humphreys, 1989). However, the data to support increased productivity of lactating dairy cows fed high WSC cultivars compared with standard cultivars is equivocal. Miller et al. (2001) reported increased digestible DMI and milk production in late-lactation cows fed a high WSC cultivar compared with a standard cultivar. In agreement, Moorby et al. (2006) reported increased DMI and milk protein yield in early-lactation cows fed a high WSC cultivar compared with a standard cultivar. However, while both studies reported differences in cultivar WSC concentrations, there was also significant differences in the NDF concentrations between cultivars, which confounds the mechanism that allowed the increased performance to be achieved. In addition, the cultivars offered to the cows in both studies were extremely low in CP concentration (9.2 to 10.6% of DM). The authors attributed the low CP concentrations of the cultivars to the low level of N fertilizer application incorporated into the study design, which was implemented to ensure high WSC concentrations in the cultivars offered to the cows (Miller et al., 2001). The low CP concentrations of the cultivars make it difficult to extrapolate the studies outcomes to pasture-based systems with standard levels of N fertilizer application. In other studies, where more typical CP concentrations were observed, milk production and DMI were similar between high WSC cultivars and standard cultivars (Taweel et al., 2005b, 2006; Tas et al 2006b). In the studies of Tas et al. (2006c) and Cosgrove et al. (2007), variable results were observed, where in one year cows consuming high WSC cultivars increased milk yield and DMI compared with standard cultivars, and in the other year there was no treatment

effect. Finally, in more recent experiments, both Chen et al. (2017) and Merino et al. (2019) reported similar milk production when cows grazed a high WSC cultivar compared with a standard cultivar. Further work is clearly warranted to determine the efficacy of grass cultivars selected for high WSC and the nutritional factors limiting performance of pasture-fed cows.

### *1.4.2. Starch*

In pastures, starch is only present in the seed (Van Soest, 1994). Because of modern pasture-based management strategies (O'Donovan et al., 2002) pastures rarely develop a seed head; therefore, starch is of little importance when quantifying the nutrient supply from pasture-only diets. However, the use of high starch concentrate supplements, such as the grains of wheat, barley, and corn, is a common strategy adopted to increase the total energy intake of pasture-fed cows (Leddin et al., 2010; Moate et al., 2020). The effectiveness of high starch concentrate supplements is variable, as a wide range of milk response (calculated as the difference in milk produced between non-supplemented and supplemented treatments divided by supplement DMI) has been reported in the literature (0.3 to 1.2 kg of milk/kg of supplement DM; Dillon et al., 1997; Grala et al., 2013). Furthermore, linear increases in milk production up to 10 kg of concentrate supplemented per day have been reported (Bargo et al., 2003; Moate et al., 2020), whereas other studies have reported diminishing responses at levels greater than 3 kg/d (Kellaway and Porta, 1993; Roche et al., 2006). Factors such as pasture allowance (Stakelum, 1986; Penno, 2002), pasture chemical composition (Doyle et al., 2005), level of pasture substitution (Dixon and Stockdale, 1999), and stage of lactation and genetic merit of the cows (Kennedy et al., 2003) have been suggested to contribute to this variation in milk response to high starch concentrate supplementation.

Nutrient supply from pasture can be affected by starch supplementation due to negative associative effects on the digestion of NDF (Leddin et al., 2010). Increasing levels of starch supplementation can reduce ruminal pH and affect the cellulolytic microorganisms, resulting in reduced fermentation of the cell wall (Wales et al., 2009; Leddin et al., 2010). Numerous critical ruminal pH thresholds, as to when digestion of cell wall will be impeded, have been proposed (Mould et al., 1983; de Veth and Kolver, 2001). Wales et al. (2009) suggested that the rate of change of rumen fluid pH and the nadir reached is important to consider. Differences in the critical thresholds proposed have been attributed to the type of forage (Grant and Mertens, 1992; deVeth and Kolver, 2001) and quality of forage being investigated (Mould et al., 1983; Huhtanen and Jaakkola, 1994).

Compared with microorganisms that ferment starch, cellulolytic microorganisms are recognized to be less tolerant to lowered rumen pH (Russell and Wilson, 1996). However, there is limited evidence to suggest that lowered rumen pH is the sole cause of reduced NDF digestion. Williams et al. (2005) reported reduced NDF digestion when pasture-fed cows were supplemented with 5 kg DM/day of barley grain, yet no significant difference in rumen fluid pH among treatments was observed. Mould et al. (1983) described a 'carbohydrate effect' that reduces NDF digestion, independent of the effect of reduced rumen pH, suggesting that the type of carbohydrate being fermented in the rumen altered the microbial species present. Furthermore, Weisbjerg et al. (1999), using continuous culture techniques, reported that the amount of rapidly degrading carbohydrate was more important than changes in pH. When replacing pasture at 0, 15, 30, or 45% of total DM with barley and steam-flaked corn in continuous culture fermentations, Wales et al. (2009) concluded that NDF digestion was maximised when grain comprised 24% of the diet, with digestion depressing rapidly thereafter. In agreement, Delagarde et al. (1995) reported

supplementation of pasture diets with 3 kg DM of wheat had no effect on pasture digestibility, whereas Arriaga-Jordan and Holmes (1986) reported reduced pasture digestibility when higher amounts were fed (5 kg DM/day). It is important to keep in mind that insufficient supply of other essential nutrients, such as ammonia-N and branched-chain VFA (i.e. isobutyrate, isovalarate, and 2-methylbutyrate) might also be involved in the reduction of NDF digestion as those substrates are absolutely required by the fiber digesting bacteria (Allison, 1980; McAllan and Smith, 1983; Van Soest, 1994).

The rate of starch digestion has been demonstrated to play a role in the negative associative effect on NDF digestion (Oba and Allen, 2003). Factors such as grain type (wheat, barley, corn, etc), processing of the grain (i.e. cold, dry heat or hydrothermal), and amylose versus amylopectin concentrations have been demonstrated to effect the rate of starch digestion. Indeed, a wide range in the rate of starch digestion has been reported, from 3%/h for resistant sorghum to 40%/h for wheat (Lanzas et al., 2007). Lastly, the rate of starch digestion can directly affect the nutrients supplied to the host, as starch fermented in the rumen supports microbial protein synthesis and VFA production (Reynolds, 2006). Whelan et al. (2012) demonstrated that early-lactation pasture-fed cows supplemented with corn increased milk production compared with supplementation of barley, whereas McKay et al. (2019) demonstrated that late-lactation pasture-fed cows supplemented with barley increased milk production compared with supplementation of corn. A routine laboratory assay with the capability to measure the rate of starch digestion is required to elucidate the complex direct and indirect effects of starch on the performance of lactating dairy cows (Lanzas et al., 2007).

### 1.5. Volatile Fatty Acids, Lactic Acids, and Other Organic Acids

Volatile fatty acids and lactic acid are end-products of fermentation which can comprise up to 6 and 5 to 15% of DM, respectively, in silages (McDonald et al., 1991). Accordingly, in fresh pasture, both of these groups of acids are essentially non-existent. Other organic acids such as citric, malic, and aconitic acid, which are important in the intermediate metabolism of a plant, can accumulate in considerable amounts especially in immature pastures (2 to 9% of DM; Dijkshoorn, 1973). As this group of 'other organic acids' is usually not included in standard feed analysis, they usually contribute to the residual carbohydrate fraction. Although this group is fermentable by rumen microorganisms, favouring the production of acetate (Russell and Van Soest, 1984), gas production analysis experiments measured ruminal degradation rates of 5%/h (Molina, 2002) which is considerably slower than the carbohydrates that typically comprise the residual carbohydrate fraction (i.e. soluble fiber). This can have important ramifications on their ability to support microbial protein synthesis and deserves further investigation in pasture-fed cows.

### 1.6. Lipids

Lipids can be broadly classified into three main groups; 1) storage compounds in seeds (triglycerides), 2) leaf lipids (galactolipids and phosopholipids), and 3) a miscellaneous category (waxes, carotenoids, chlorophyll, essential oils, and other ether-soluble substances; Van Soest, 1994). Leaf lipids are primarily galactolipids that consist of glycerol, galactose, and unsaturated fatty acids. They play an important role in the fluidity of chloroplast membranes, which is especially important in pastures that experience cold temperatures (Routaboul et al., 2000). The ether extract concentration of pasture typically ranges from 3 to 8% of DM (Harfoot, 1981) with fatty acid concentrations of 1 to 4% of DM (Schroeder et al., 2004). Bauchart et al. (1984)

demonstrated that fatty acid concentration of pasture is highest is the spring, declines rapidly during the summer, and subsequently increases during late-summer/autumn. Fatty acid digestibility is also affected by season, starting low in the spring (59%) and gradually rising throughout the grazing season to a peak of 90% (Bauchart et al., 1984). Many factors can affect the fatty acid concentration of pasture such as cultivar (Palladino et al., 2009), stage of maturity (Dewhurst et al., 2001), and fertilizer regime (Witkowska et al., 2008). More recently, fatty acid concentration has been targeted by plant breeders in an attempt to increase the energy density of pasture-based diets (Winichayakul et al., 2020). However, the effectiveness of increased levels of fatty acids and their rumen metabolism require further quantification in pasture-based diets (Schroeder et al., 2004; Wilkinson et al., 2019).

The fatty acid composition of pasture is predominantly comprised of linolenic acid (LNA; C18:3) followed by linoleic acid (LA: C18:2) and palmitic acid (C16:0; Palladino et al., 2009). The high levels of LNA in pasture has been suggested to increase the health promoting n-3 fatty acid and conjugated linoleic acid concentrations in the milk of pasture-fed cows (Kraft et al., 2003; Dewhurst et al., 2006). Biohydrogenation of LNA can produce intermediates such as vaccenic acid (mentioned below) and others that are precursors to CLA in milk. Dewhurst et al. (2006) states 'Dietary 18:3 n − 3 is the ultimate precursor of 18:3 n − 3 in milk, whilst CLA (cis-9, trans-11) derives from dietary 18:2 n − 6 and 18:3 n – 3'. However, the fatty acids consumed by ruminants are extensively altered by the microbial populations resulting in marked difference in the fatty acids available for absorption (Jenkins et al., 2008). Excess unsaturated fatty acids are toxic to rumen microorganisms and can result in lowered NDF digestibility, VFA production, and microbial protein synthesis (Van Soest, 1994). After ingestion of galactolipids, the ruminal microorganisms split off the galactose moieties and hydrolyze the glycerol fatty acids, with

galactose and glycerol being readily fermented (Van Soest, 1994). The liberated unsaturated fatty acids are then hydrogenated by the rumen microorganisms, at varying degrees of success, in an attempt to produce less toxic saturated fatty acids. Linolenic acid is typically hydrogenated to vaccenic acid and then further to stearic acid. However, some LNA and vaccenic acid can escape rumen biohydrogenation and reach the mammary gland. In the mammary gland, vaccenic acid can be desaturated to yield the nutraceutical cis-9, trans-11 conjugated linoleic acid (Lock and Garnsworthy, 2003).

### 1.7. Nitrogen

The N concentration of pasture can vary from 6% of DM in young immature heavily fertilized pastures (Pacheco and Waghorn, 2008) to less than 1% of DM in mature flowering pastures (Beever et al., 2000). Many factors can affect pasture N concentration such as physiological stage, botanical composition, season of the year, and N fertilizer application (Fulkerson et al., 1998; Aufrere et al., 2003; Peyraud and Astigarraga, 1998). There are many compounds in grasses that contain N, such as protein, peptides, amino acids (AA), nucleic acids, nitrates and other secondary metabolites. The primary true proteins found in the leaf and stem comprise of cytoplasmic and chloroplastic proteins (Van Soest, 1994). The nucleoproteins of the nucleus and the extensin proteins of the plant cell wall also contribute to the true protein, however, at much lower concentrations. The cell wall proteins are less soluble than the proteins in the cell contents and are recovered in NDF (Van Soest, 1994). If the cytoplasmic and chloroplastic proteins are denatured by heat (e.g. wilting) they also can be recovered in NDF.

Large accumulations of non-protein N (20 to 42% of total N) can occur in pasture that includes peptides, AA, amines, nitrates and secondary metabolites (Goswami and Willcox, 1969; Mangan

1982). Nitrogen fertilizer can be taken up rapidly by pasture, resulting in a peak N concentration two weeks after fertilizer application and then decreasing over time (Wilman et al., 1975). During this peak N concentration, nitrate-N has been reported to contribute 10 to 15% of the total N (Reid and Strachan, 1974). High levels of nitrate-N can accumulate under cloudy conditions, as photosynthesis energy is required for the synthesis of nitrate reductase (Van Soest, 1994). In addition, insufficient synthesis of nitrate reductase under high N fertilizer application can also cause nitrate-N to accumulate (Peyraud and Astigarraga, 1998). While nitrate-N has no AA value directly to the cow, in the rumen nitrate can be reduced to ammonia and utilized for the synthesis of microbial AA.

Generally, AA supply is thought to be in excess of requirements of pasture-fed cows, as fertilized pastures typically comprise of excess total N (Pacheco and Waghorn, 2008; Griffiths et al., 2019). However, total N concentration is an insufficient indicator of metabolizable AA supply (Ipharraguerre and Clark, 2005). Although leaf proteins are comprised of a well-balanced AA composition, a large proportion of these AA can be extensively degraded in the rumen (Beever and Siddons, 1986). Proteolytic rumen microorganisms are primarily associated with this degradation (Wallace et al., 1997); however, plant proteinases might also be involved in the protein degradation of fresh forage diets (Zhu et al., 1999; Kingston-Smith et al., 2005; Barrett et al., 2007). The overall impact of plant proteinases remains to be elucidated due to confounding effects of plant-associated microorganisms (Attwood, 2005).

In a review of early investigations into the rumen degradation of pasture N, Beever and Siddons (1986) concluded that pasture N escaping ruminal fermentation insignificantly contributed to metabolizable protein (**MP**) supply (3 to 5%). More recent data, suggests a larger and more variable contribution when measured using in situ techniques (17 to 44%; Van Vuuren et al., 1991;

Valk et al., 1996; Chaves et al., 2006). However, the inability to quantify microbial contamination of the residue, and the physical restriction of the feed being investigated within a nylon bag, brings into question the quantitative data obtained from the in situ technique (Broderick and Cochran, 2000). Furthermore, an assumption of the in situ technique is that proteins, peptides, and AA in the soluble fraction are completely degraded (Broderick et al., 2010). However, substantial quantities of peptides and AA have been demonstrated to escape rumen degradation via passage in the liquid phase (Choi et al., 2002; Reynal et al., 2007).

Digesta flow studies have been utilized to quantify ruminal degradation of pasture N and microbial N flow. Within digesta flow studies, microbial markers differentiate total non-ammonia N (**NAN**) flow into microbial N flow and, by difference, non-ammonia, non-microbial N flow (**NANMN**). The NANMN flow is primarily comprised of feed N, with endogenous N also contributing. A number of studies exist in the literature that quantified the N flows in pasture-fed lactating dairy cows utilized the duodenal sampling technique (Berzaghi et al., 1996; O'Mara et al., 1997; Peyraud et al., 1997; Younge et al., 2004) with only one study utilizing the omasal sampling technique (Sairanen et al., 2005). Rumen degradation of pasture N [1 – (NANMN flow/N intake)] reported in the duodenal sampling studies varied from 64.7 to 79.0%, whereas in the omasal sampling study the measure was 87.5%. These differences in rumen degradation of pasture N likely represent the appearance of endogenous N at the duodenum that will contribute to a proportion of the NANMN flow. This results in an underestimation of the rumen degradation of pasture N and was likely much greater when duodenal sampling was performed, as larger endogenous N contributions occur in the duodenum (Lapierre et al., 2008) when compared with the omasum (Ørskov et al., 1986; Ahvenjärvi et al., 2000). The potential extensive rumen degradation of pasture N suggests that pasture-fed dairy cows might be highly dependent on

microbial protein to meet their AA requirements. In addition, there are a number of studies that demonstrate increased milk yield when pasture-fed cows were supplemented with rumen protected protein ingredients (Minson, 1981; O'Mara et al., 2000; Astigarraga et al., 2002). Therefore, the currently held view that N fertilized pastures provide an excess of metabolizable AA requires further investigation.

## 1.8. Microbial Protein Synthesis

### *1.8.1. Microbial Growth*

The contribution from microbial protein to the AA supply in ruminants can vary greatly, from 60 to 85% (Storm et al., 1983; Clark et al., 1992). From the previous discussion, it would appear that pasture-fed cows are remarkably dependent on the efficiency of microbial protein synthesis. Furthermore, the metabolic products from the extensive ruminal fermentation of pasture, has far-reaching implications, as they are the primary energy supply to the ruminant and can differentially affect host metabolism (Bannink et al., 2006; Danes et al., 2020).

Many factors are involved in the yield and efficiency of microbial protein synthesis. In the anaerobic environment of the rumen, energy supply is the primary limiting factor to microbial growth (Tamminga, 1979). Rumen microorganisms obtain their energy through the fermentation of substrates to short-chain fatty acids, as during this process, energy is released and subsequently conserved in the form of adenosine triphosphate (**ATP**). The rumen microorganisms can utilize this ATP for anabolic processes such as protein and fatty acid synthesis. The yield of ATP depends on the type of substrate fermented and the end-products that are produced. The majority of ruminal microorganisms obtain their ATP through the fermentation of carbohydrates (Hungate, 1966). While certain microorganisms can also ferment AA, this fermentation pathway provides much

lower yields of ATP, resulting in low ruminal populations of these microorganisms (Tamminga, 1979; Russell and Strobel, 2005). Furthermore, the fatty acid component of lipids does not provide ATP for microbial growth, as fatty acids cannot be oxidized by the rumen microorganisms (Dijkstra et al., 1998). The balance of microbial species and the pathway of carbohydrate metabolism also influences the yield of ATP. For example, the synthesis of one mole of acetate or butyrate is thought to produce two and three ATPs, respectively, via substrate level phosphorylation (Van Soest, 1994; Russell and Strobel, 2005).

The efficiency of microbial growth can be expressed in a number of different ways. The most commonly used metric in the literature is grams of microbial N per kilogram of organic matter truly digested in the rumen (Broderick et al., 2010). This metric differentiates between the organic matter fermented in the rumen and the organic matter fermented distal to the rumen, providing a more accurate representation of ATP available for ruminal microbial growth. Additionally, as the metric incorporates true digestibility and not apparent, a more accurate estimation of the digestible matter that can support microbial growth is attained (Van Soest, 1994). However, care must be taken when interpreting such a metric as the organic matter can comprise of varying amounts of substrates that have different ATP yielding capabilities (Dijkstra et al., 1998). This can be overcome by expressing the efficiency of microbial growth per kg of ruminally-fermented carbohydrate (Russell and Strobel, 2005). Such subtleties are important when estimating the capability of pasture to support microbial growth or when parameterizing ruminant nutrition models to predict the microbial protein supply to the cow (Dijkstra et al., 1998).

The efficiency of use of ATP can be affected by a number of factors such as the substrate's rate of digestion, availability of essential precursors, and the overall ruminal environment (Van Soest, 1994). The predominant mechanism through which these factors affect microbial efficiency is

through partitioning of the ATP supply towards non-growth functions such as maintenance, energy spilling, and synthesis of reserve carbohydrates (Hackmann and Firkins, 2015). A high rate of digestion provides a greater amount of substrate per unit of time, allowing higher microbial growth rates and reduces the proportion of ATP required to support maintenance, which increases net efficiency overall (Russell and Strobel, 2005). Availability of nutrients such as N, amino-N, and essential minerals have also been demonstrated to limit microbial growth efficiency (Argyle and Baldwin, 1989; Newbold, 1999). Furthermore, low availability of branched-chain VFA has been suggested to limit microbial growth efficiency and while supporting in vivo digestibility data are limited (Hackmann and Firkins, 2015), supplementation of branched-chain VFA has been demonstrated to increased milk yield (Papas et al., 1984). Lastly, in vitro and in vivo measures support a positive relationship between dilution rate and microbial growth efficiency (Isaacson et al., 1975; Harrison et al., 1976). A higher dilution rate has been shown to increase microbial efficiency through reductions in the mean age of the microbial population, death, and protozoal predation (Van Soest, 1994). High liquid dilution rates in pasture-fed cows have been reported (>20%/h; Rius et al., 2012), which might, at least in part, explain their capability to support high microbial growth efficiency (O'Mara et al., 1997).

### *1.8.2. Protozoal Metabolism*

The importance of protozoa to the ruminant host has been debated for decades with contrasting opinions as to whether they are beneficial or detrimental (Williams and Coleman, 1992). It is clear that protozoa have a substantial impact on ruminant physiology (Newbold et al., 2015); however, the majority of this evidence has been obtained from in vitro investigations or studies where faunated and defaunated animals were compared. Methodological limitations of such investigation

and potential confounders have been discussed at length (Newbold et al., 2015; Firkins et al., 2020). Furthermore, few in vivo studies have been performed using cows fed at relatively high feed intakes and production levels (Firkins et al., 2007). While care must be taken when extrapolating results from such investigations, they have led to critical advances in our current understanding of the protozoal species (Firkins et al., 2020).

Ciliated protozoa can be classified into two main sub-groups; entodiniomorphs and isotrichids (i.e. holotrichs), with the former generally found in higher numbers (Hungate, 1966). Their striking appearance under the microscope helps classify the groups as entodiniomorphs generally have cilia surrounding the peristome (sometimes elsewhere), whereas the isotrichids are generally covered in cilia (Williams and Coleman, 1992). Flagellate protozoa also exist in most rumens; however, in much smaller numbers. As a result and due difficulty in culturing, very little information pertaining to the flagellate protozoa exists (Williams and Coleman, 1992). The two main sub-groups of ciliated protozoa also express many metabolic differences. Entodiniomorphs generally ferment starch, cellulose, and hemicellulose and have demonstrated the ability to engulf fibrous particles, chloroplasts, and bacteria (Huws et al., 2012). Isotrichids, on the other hand, prefer mainly sugar and soluble substrates (Williams and Coleman, 1992), and have not demonstrated an ability to engulfed chloroplasts (Huws et al., 2009). Both groups predate bacteria; however, in vitro investigations suggest that entodiniomorphs could be responsible for greater than 90% of bacterial degradation, whereas isotrichids modestly contribute (Belanche et al., 2012). Such difference in metabolic characteristics have important ramifications when assessing the effect of protozoa on the host metabolism under different dietary situations. However, within in vivo investigations it is often difficult to differentiate among protozoa sub-groups and therefore, results are commonly inferred to a ‘single’ protozoa organism (Williams and Coleman, 1992). In an attempt to

differentiate these effects in vivo, some studies have successfully defaunated and refaunated animals with single protozoal species (Belanche et al., 2012).

Extensive microbial protein degradation and recycling can occur within the rumen, which can have large effects on the efficiency of microbial growth (Wells and Russell, 1996). Based on in vitro studies, the majority of bacterial degradation and recycling has been attributed to protozoa (Wallace and McPherson, 1987). However, when in vitro measured rates of bacterial protein breakdown by protozoa are extrapolated to the rumen, the ruminal bacterial pool are theoretically depleted within hours (Hristov and Jouany, 2005). Protozoa grown in vitro have been shown to ingest more bacteria than in vivo (Coleman and Sandford, 1979), which might explain the excessive rates of bacterial protein breakdown. Furthermore, when investigating defaunation in sheep, there was no effect on the absolute amount of bacterial N recycled (Koenig et al., 2000). Those studies did demonstrate, however, that defaunation improved the intraruminal metabolism of N by increasing the ruminal bacterial biomass and flow of bacterial N to the intestine (Koenig et al., 2000). This increased capture of ruminal N within bacterial biomass likely contributes to the consistent reduction of ammonia-N concentration when protozoa are eliminated from the rumen (Newbold et al., 2015). In addition, a reduction in feed protein degradation, in the absence of rumen protozoa, is likely to also contribute to the reduction in rumen ammonia concentrations (Williams and Coleman, 1992). Based on a recent meta-analysis (Newbold et al., 2015), defaunation increases duodenal microbial N flow and efficiency of microbial protein synthesis (Figure 1.2). However, the negative influence of protozoa on microbial growth might be overestimated due to the lack of adequate microbial markers, which might have led to the underestimation of protozoal N in faunated animals (Broderick and Merchen, 1992; Firkins et al., 1998). Furthermore, as digesta obtained from the duodenum has been subjected to abomasal enzymatic digestion, a reduced

ability to separate N fractions occurs (Ahvenjärvi, 2006), which might also lead to an underestimation of microbial and protozoal N flow (Broderick et al., 2010). Using the omasal sampling technique, in combination with gravimetric determination of protozoal N, studies have demonstrated that protozoa N contributed 7 to 17% of the microbial N outflow from the rumen of lactating dairy cows (Ahvenjärvi et al., 2002; Fessenden et al., 2019), emphasizing the importance of accurate protozoal N flow determination.

Defaunation has consistently increased duodenal NAN flow, which cannot be attributed to unreliable microbial markers and likely reflects lower dietary N degradation (Newbold et al., 2015). While this effect seems beneficial under dietary scenarios with limited intestinal AA supply, other unintended consequences of defaunation must be considered. Recently, protozoa have been demonstrated to contribute significantly to the duodenal flow of conjugated linoleic acid (30 to 43%) and vaccenic acid (40%; Yáñez-Ruiz et al., 2006). Furthermore, protozoa might also contribute substantially to LNA escape from the rumen due to their ability to consume chloroplasts (Huws et al., 2009). The ability of protozoa to consume lactate more rapidly than bacteria (Newbold et al., 1986) and engulf, accumulate, and metabolize soluble carbohydrates, without the production of lactic acid, can result in stabilization of rumen pH (Williams and Coleman, 1992). Elimination of protozoa from the rumen might have further negative associative effects such as reduced fiber digestion (Newbold et al., 2015; Figure 1.2). Therefore, more studies are required to elucidate the overall effect of protozoa metabolism on the supply of nutrients to the ruminant.

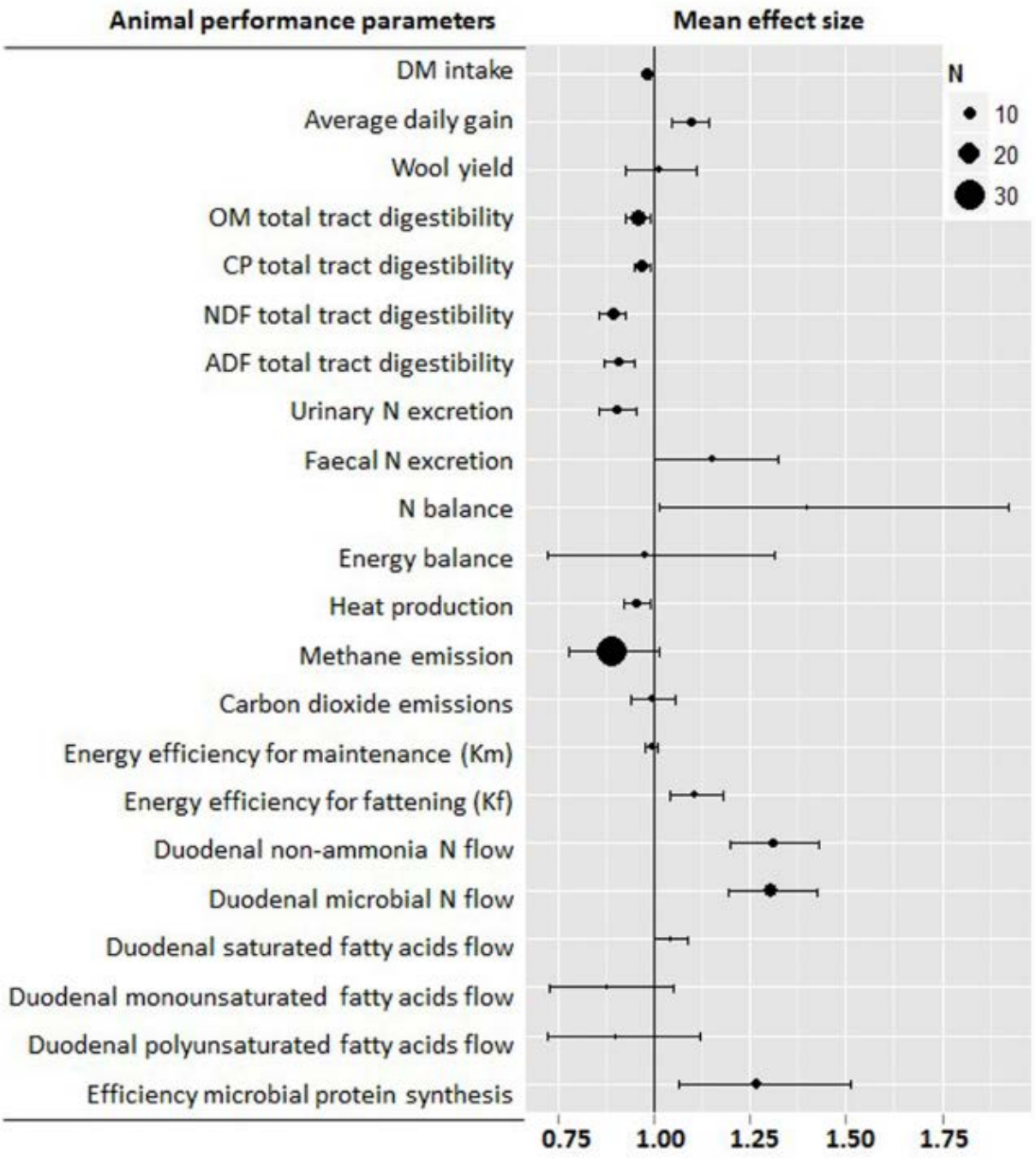


**Figure 1.2.** Results from a meta-analysis of studies investigation the effects of defaunation on animal performance. Reproduced from Newbold et al. (2015).

The most intensely debated topic concerning protozoa metabolism relates to their generation time and passage from the rumen (Firkins et al., 2020). Protozoa generation time is thought to be much longer, compared with bacteria, resulting in higher maintenance requirements and lower energetic efficiency (Williams and Coleman, 1992). The long generation time has also been suggested to result in the elimination of protozoa from the rumen under conditions of rapid turnover (Hungate, 1966; Van Soest, 1994). Relative to the total microbial population, a lower proportion of protozoa flowing out of the rumen, compared with their proportion in the rumen, is interpreted as an ability of protozoa to sequester in the rumen by associating with feed particles and the rumen wall (Bauchop and Clarke, 1976; Hook et al., 2012). Although isotrichids have demonstrated the ability to sequester in the rumen (Diaz et al., 2014), the predominant entodiniomorphids lack the ability to 'attach' and do not sequester in the rumen (Dehority, 1984; Firkins et al., 2020). Furthermore, the majority of studies estimating ruminal protozoal pool size have not simultaneously reported ruminal outflow of protozoa, thus potentially confounding these interpretations (Newbold et al., 2015). More recently, using measures obtained by real-time polymerase chain reaction, Sylvester et al. (2005) demonstrated that the proportion of protozoa in the rumen was similar to that found in the duodenum.

Sylvester et al. (2009) demonstrated that rumen ciliated protozoa could decrease their generation time in response to increasing dilution rate, with other studies reporting similar effects (Harrison et al., 1976; Dehority, 2004). This mechanism might, at least in part, explain the wide range of protozoal generation times (6.0 to 55.6 h), reported by Hook et al. (2012) when compiling literature data. Notably, Firkins et al. (2007) highlighted that many studies investigating protozoal dynamics involved animals at low levels of intake, resulting in lower dilution rate and longer protozoa generation time, when compared with high-producing cows. The authors also suggested

that higher protozoal growth rates in the high-producing cow would result in a larger percentage of dividing forms and more nucleic acid and N per cell, in agreement with the finding of Isaacson et al. (1975) when investigating the effects of dilution rate on microbial composition.

While it is evident that protozoa have a significant impact on ruminant physiology (Newbold et al., 2015) an incomplete understanding of their metabolism currently exists (Firkins et al., 2020). Clearly, more studies simultaneously measuring the omasal flow of protozoa and their rumen pool size are required, as this is the most correct way to represent true protozoal generation time in vivo and their passage from the rumen (Karnati et al., 2007). Cows consuming pasture-based diets exhibit many attributes that could support efficient protozoal growth, such as rapid rumen turnover, moderate rumen pH levels, and an ample supply of soluble carbohydrates and true protein (Clarke, 1965; Williams and Coleman, 1992). Thus, protozoal metabolism likely has a major impact on the supply of nutrients to cows fed pasture-based diets. Furthermore, cows consuming pasture-based diets might be an effective model to enhance our current understanding of protozoal metabolism.

## 1.9. Summary and Objectives

It is difficult to quantify the capacity of a feed to meet the metabolic requirements of the cow, due to dynamic and variable factors, such as plant maturity and environmental conditions. Furthermore, the interaction between ingested pasture and microbial metabolism ultimately dictates the quantity and profile of nutrients available for absorption. Feed evaluations techniques, combined with mathematical models, can aid in the characterization of feed fractions based on similar or uniform behavior across diets, their degradation kinetics, and capacity to support microbial protein synthesis. This mechanistic understanding can enhance our ability to quantify the true nutrient supply to the cow, develop appropriate nutritional strategies to overcome dietary limitations, and ultimately, increase the productivity and efficiency of lactating dairy cows fed pasture-based diets.

Inconsistencies and knowledge gaps currently exist in our understanding of nutrient supply to cows fed pasture-based diets. Therefore, the objectives of this dissertation are to:

1) Characterize the nutritive value of pasture using new and updated feed chemistry methods
2) Provide a more robust understanding of nutrient supply and microbial dynamics in lactating dairy cows fed pasture-based diets
3) Incorporate new understanding of protozoal dynamics into the microbial sub-model of the CNCPS to better characterize protozoal metabolism in lactating dairy cows
4) Investigate nutritional strategies to optimize productivity from pasture-based systems

## 1.10. References


Ahvenjärvi, S. 2006. Measurement of digesta flow entering the omasal canal. Pages 587-589 in Ruminant Physiology: Digestion, Metabolism and Impact of Nutrition on Gene Expression, Immunology and Stress. K. Sejrsen, T. Hvelplund, and M. O. Nielsen, ed. Wageningen Academic Publisher, Wageningen, Netherlands.

Ahvenjärvi, S., A. Vanhatalo, and P. Huhtanen. 2002. Supplementing barley or rapeseed meal to dairy cows fed grass-red clover silage: I. Rumen degradability and microbial flow. J. Anim. Sci. 80:2176– 2187.

Ahvenjärvi, S., A. Vanhatalo, P. Huhtanen, and T. Varvikko. 2000. Determination of forestomach digestion in lactating dairy cows by omasal or duodenal sampling. Br. J. Nutr. 83:67–77.

Alexandratos, N. and J. Bruinsma. 2012. World Agriculture towards 2030/2050: the 2012 revision (No. 12-03). ESA Working paper, Rome, FAO.

Allison, M. 1980. Nitrogen requirements of ruminal bacteria. Pages 128-132 in Protein requirements for cattle. Stillwater: Oklahoma State Univ.

Argyle, J. L., and R. L. Baldwin. 1989. Effects of amino acids and peptides on rumen microbial growth yields. J. Dairy Sci. 72:2017–2027.

Arriaga-Jordan, C. M., and W. Holmes. 1986. The effect of cerealconcentrate supplementation on the digestibility of herbage-based diets for lactating dairy cows. J. Agric. Sci. 106:581–592.

Astigarraga, L., J. L. Peyraud, and L. Delaby. 2002. Effect of nitrogen fertiliser rate and protein supplementation on the herbage intake and the nitrogen balance of grazing dairy cows. Anim. Res. 51:279-293.

Attwood, G. 2005. The contribution of plant-derived proteinases to the breakdown of fresh pasture protein in the rumen. Br. J. Nutr. 93:421-423.

Aufrere, J., D. Graviou, and C. Demarquilly. 2003. Ruminal degradation of protein of cocksfoot and perennial ryegrass as affected by various stages of growth and conservation methods. Anim. Res. 52:245-261.

Bannink, A., J. Kogut, J. Dijkstra, J. France, E. Kebreab, A. M. Van Vuuren, and S. Tamminga. 2006. Estimation of the stoichiometry of volatile fatty acid production in the rumen of lactating cows. J. Theor. Biol. 238:36–51.

Bargo, F., L. D. Muller, E. S. Kolver, and J. E. Delahoy. 2003. Invited review: Production and digestion of supplemented dairy cows on pasture. J. Dairy Sci. 86:1–42.

Barrett, B. A., D. Pacheco, W. C. McNabb, and H. S. Easton. 2007. Opportunities to improve nitrogen utilisation in the rumen by reduction in plant-mediated proteolysis. Proc. N.Z. Grassl. Ass. 69:187–192.

Bauchart, D., R. Verite, and B. Remond. 1984. Long-chain fatty acid digestion in lactating cows fed fresh grass from spring to autumn. Can. J. An. Sci. 330–331

Bauchop, T., and R. T. Clarke. 1976. Attachment of the ciliate Epidinium Crawley to plant fragments in the sheep rumen. Appl. Environ. Microbiol. 32:417–422.

Baudracco, J., N. Lopez-Villalobos, C. Holmes, and K. Macdonald. 2010. Effects of stocking rate, supplementation, genotype and their interactions on grazing dairy systems: a review. N.Z. J. Agric. Res. 53:109-133.

Beecher, M., R. Baumont, M. O'Donovan, T. Boland, J. Aufrere, C. Fleming, N. Galvin, and E. Lewis. 2018. Effects of harvesting perennial ryegrass at different levels of herbage mass on voluntary intake and in vivo digestibility in sheep. Grass Forage Sci. 73:553-561.

Beecher, M., D. Hennessy, T. Boland, M. McEvoy, M. O'Donovan, and E. Lewis. 2015. The variation in morphology of perennial ryegrass cultivars throughout the grazing season and effects on organic matter digestibility. Grass Forage Sci. 70:19-29.

Beever, D. E., N. Offer, and M. Gill. 2000. The feeding value of grass and grass products. Page 140 in Grass: Its Production and Utilization. 3rd ed. A. Hopkins, ed. Br. Grassl. Soc. Blackwell Science.

Beever, D. E. and R. C. Siddons. 1986. Digestion and metabolism in the grazing ruminant. Pages 479–497 in Control of Digestion and Metabolism in Ruminants. L. P. Milligan, W. L. Grovum, and A. Dobson, ed. Prentice Hall, Englewood Cliffs, NJ.

Belanche, A., G. de la Fuente, J. M. Moorby, and C. J. Newbold. 2012. Bacterial protein degradation by different rumen protozoal groups. J. Anim. Sci. 90:4495–4504.

Berzaghi, P., J. H. Herbein, and C. E. Polan. 1996. Intake, site, and extent of nutrient digestion of lactating cows grazing pasture. J. Dairy Sci. 79:1581–1589.

Boudon, A., J. L. Peyraud, P. Faverdin, R. Delagarde, L. Delaby, and A. Chaves. 2009. Effect of rumen fill on intake of fresh perennial ryegrass in young and mature dairy cows grazing or zero-grazing fresh perennial ryegrass. Anim. 3:1706-1720.

Bowerman, A., and P. Goodman. 1971. Variation in nitrate reductase activity in Lolium. Ann. Bot. 35:353-366.

Broderick, G. A., P. Huhtanen, S. Ahvenjärvi, S. M. Reynal, and K. J. Shingfield. 2010. Quantifying ruminal nitrogen metabolism using the omasal sampling technique in cattle - A meta-analysis. J. Dairy Sci. 93:3216-3230.

Broderick, G. A., and R. C. Cochran. 2000. In vitro and in situ methods for estimating digestibility. Pages 53–85 in Feeding Systems and Feed Evaluation Models. M. K. Theodorou and J. France, ed. CABI Publishing, CAB International, Wallingford, UK.

Broderick, G. A., and Merchen, N. R. 1992. Markers for quantifying microbial protein synthesis in the rumen. J. Dairy Sci. 75:2618–2632.

Chapman, D. F., J. R. Bryant, M. E. Olayemi, G. R. Edwards, B. S. Thorrold, W. H. McMillan, G. A. Kerr, G. Judson, T. Cookson, and A. Moorhead. 2017. An economically based evaluation index for perennial and short-term ryegrasses in New Zealand dairy farm systems. Grass Forage Sci. 72:1–21.

Chaves, A. V., G. C. Waghorn, I. M. Brookes, and D. R. Woodfield. 2006. Effect of maturation and initial harvest dates on the nutritive characteristics of ryegrass (Lolium perenne L.). Anim. Feed Sci. Technol. 127:293-318.

Chen, A., R. H. Bryant, and G. R. Edwards. 2019. Morphology and nutritive value of perennial ryegrass cultivars at different phenological stages. Grass Forage Sci. 74:576-581.

Chen, A., R. Bryant, and G. Edwards. 2017. Milk production and composition of dairy cows grazing two perennial ryegrass cultivars allocated in the morning and afternoon. Ani. Prod. Sci. 57:1507-1511.

Cherney, J. H., D. J. R. Cherney, D. E. Akin, and J. D. Axtell. 1991. Potential of brown-midrib, low-lignin mutants for improving forage quality. Adv. Agron. 46:157–198.

Chesson, A. 1993. Mechanistic models of forage cell wall degradation. Pages 347-376 in Forage Cell Wall Structure and Digestibility. H. G. Jung, D. R. Buxton, R. D. Hatfield, and J. Ralph, ed. ASACSSA-SSSA, Madison, WI.

Choi, C. W., S. Ahvenjärvi, A. Vanhatalo, V. Toivonen, and P. Huhtanen. 2002. Quantitation of the flow of soluble non-ammonia nitrogen entering the omasal canal of dairy cows fed grass silage based diets. Anim. Feed Sci. Technol. 96:203-220.

Clark, J. H., T. H. Klusmeyer, and M. R. Cameron. 1992. Microbial protein synthesis and flows of nitrogen fractions to the duodenum of dairy cows. J. Dairy Sci. 75:2304-2323.

Clarke, R. 1965. Diurnal variation in the numbers of rumen ciliate protozoa in cattle. N.Z. J. Agric. Res. 8:1-9.

Coleman, G. and D. C. Sandford. 1979. The engulfment and digestion of mixed rumen bacteria and individual bacterial species by single and mixed species of rumen ciliate protozoa grown in vivo. J. Agri. Sci. 92:729-742.

Corson, D., G. Waghorn, M. Ulyatt, and J. Lee. 1999. NIRS: Forage analysis and livestock feeding. Proc. N.Z. Grassl. Ass. 61:127-132.

Cosgrove, G. P., J. L. Burke, A. F. Death, M. J. Hickey, D. Pacheco, and G. A. Lane. 2007. Ryegrasses with increased soluble carbohydrates: evaluating the potential for dairy grazing dairy cows in New Zealand. Proc. NZ Grassl. Ass. 69:179-185.

Cotanch, K. W., R. J. Grant, M. E. Van Amburgh, A. Zontini, M. Fustini, A. Palmonari, and A. Formigoni. 2014. Applications of uNDF in ration modeling and formulation. Pages 114-131 in Proc. Cornell Nutrition Conference, Syracuse, NY. Cornell Univ., Ithaca, NY.

Danes, M., M. Hanigan, S. A. Apelo, J. Dias, M. Wattiaux, and G. Broderick. 2020. Post-ruminal supplies of glucose and casein, but not acetate, stimulate milk protein synthesis in dairy cows through differential effects on mammary metabolism. J. Dairy Sci. 103:6218–6232.

de Veth, M. J., and E. S. Kolver. 2001. Digestion of ryegrass pasture in response to change in pH in continuous culture. J. Dairy Sci. 84:1449–1457.

Dehority, B. A. 2004. In vitro determination of generation times for Entodinium exiguum, Ophryoscolex purkynjei, and Eudiplodinium maggii. J. Eukaryot. Microbiol. 51:333–338.

Dehority, B. A. 1984. Evaluation of subsampling and fixation procedures used for counting rumen protozoa. Appl. Environ. Microbiol. 48:182–185.

Delagarde, R., and J. Peyraud. 1995. Influence of wheat supplementation on intake and digestibility in dairy cows fed autumn grass ad libitum indoors. Ann. Zootech. 44:210-210.

Delahoy, J., L. Muller, F. Bargo, T. Cassidy, and L. Holden. 2003. Supplemental carbohydrate sources for lactating dairy cows on pasture. J. Dairy Sci. 86:906-915.

Dewhurst, R. J., K. J. Shingfield, M. R. F. Lee, and N. D. Scollan. 2006. Increasing the concentrations of beneficial polyunsaturated fatty acids in milk produced by dairy cows in high-forage systems. Anim. Feed Sci. Technol. 131:168–206.

Dewhurst, R. J., N. D. Scollan, S. J. Youell, J. K. S Tweed , and M. O. Humphreys. 2001. Influence of species, cutting date and cutting interval on the fatty acid composition of grasses. Grass Forage Sci. 56:68–74.

Diaz, H. L., K. N. Barr, K. R. Godden, J. E. Plank, I. Zapata, A. N. Schappacher, M. P. Wick, and J. L. Firkins. 2014. Eukaryotic inhibitors or activators elicit responses to chemosensory compounds by ruminal isotrichid and entodiniomorphid protozoa. J. Dairy Sci. 97:2254–2269.

Dijkshoorn,W. 1973. Organic acids and their role in ion uptake. Pages 163–188 in Chemistry and Biochemistry of Herbage. R. W. Bailey, ed. Academia Press, NY, USA.

Dijkstra, J., J. France, J. Ellis, A. Strathe, E. Kebreab, and A. Bannink. 2013. Production efficiency of ruminants: feed, nitrogen and methane. Pages 10-25 in Sustainable animal agriculture. E Kebreab, ed. CAB International, Wallingford, UK.

Dijkstra, J., J. France, and D. R. Davies. 1998. Different mathematical approaches to estimating microbial protein supply in ruminants. J. Dairy Sci. 81:3370-3384.

Dillon, P., T. Hennessy, L. Shalloo, F. Thorne, and B. Horan. 2008. Future outlook for the Irish dairy industry: a study of international competitiveness, influence of international trade reform and requirement for change. Int. J. Dairy Technol. 61:16-29.

Dixon, R. M., and C. R. Stockdale. 1999. Associative effects between forages and grains: Consequences for feed utilization. Aust. J. Agric. Res. 50:757–773.

Douglas, M., M. Auldist, J. Jacobs, M. Hannah, S. Garcia, and W. Wales. 2020. Quantifying the ruminal degradation of nutrients in three cultivars of perennial ryegrass (Lolium perenne L.) sampled during early spring and summer. Ani. Prod. Sci. 60:370-378.

Doyle, P., S. Francis, and C. Stockdale. 2005. Associative effects between feeds when concentrate supplements are fed to grazing dairy cows: a review of likely impacts on metabolisable energy supply. Aust. J. Agric. Res. 56:1315-1329.

Ellis, W. C., M. Mahlooji, and J. H. Matis. 2005. Models for estimating parameters of neutral detergent fiber digestion by ruminal microorganisms. J. Anim. Sci. 83:1591-1601.

Engstrom, D. F., G. W. Mathison, and L. A. Goonewardene. 1992. Effect of beta-glucan, starch, and fiber content and steam vs dry rolling of barley-grain on its degradability and utilization by steers. Anim. Feed Sci. Technol. 37:33–46.

Fessenden, S. W., T. J. Hackmann, D. A. Ross, E. Block, A. Foskolos, and M. E. Van Amburgh. 2019. Rumen digestion kinetics, microbial yield, and omasal flows of nonmicrobial, bacterial, and protozoal amino acids in lactating dairy cattle fed fermentation by-products or urea as a soluble nitrogen source. J. Dairy Sci. 102:3036–3052.

Firkins, J. L., Z. Yu, T. Park, and J. E. Plank. 2020. Extending Burk Dehority's perspectives on the role of ciliate protozoa in the rumen. Front. Microbiol. 11:123.

Firkins, J. L., Z. Yu, and M. Morrison. 2007. Ruminal nitrogen metabolism: perspectives for integration of microbiology and nutrition for dairy. J. Dairy Sci. 90:1–16.

Firkins, J. L., M. S. Allen, B. S. Oldick, and N. R. St-Pierre. 1998. Modeling ruminal digestibility of carbohydrates and microbial protein flow to the duodenum. J. Dairy Sci. 81:3350–3369.

Fulkerson, W., and D. Donaghy. 2001. Plant-soluble carbohydrate reserves and senescence—Key criteria for developing an effective grazing management system for ryegrass-based pastures: A review. Aust. J. Exp. Agric. 41:261–275.

Fulkerson, W. J., K. Slack, D. W. Hennessy, and G. M. Hough. 1998. Nutrients in ryegrass "Lolium spp.", white clover "Trifolium repens" and kikuyu "Pennisetum clandestinum" pastures in relation to season and stage of regrowth in a subtropical environment. Aus. J. Exp. Agric. 38:227-240.

Garry B, M. O'Donovan, M. Beecher, L. Delaby, C. Fleming, R. Baumont, and E. Lewis. 2018. Predicting in vivo digestibility of perennial ryegrass using the neutral detergent cellulase method: updating the equation. In Sustainable Meat and Milk Production from Grasslands. Pages 194–196 in Proc. of the 27th General Meeting of the European Grassland Federation, Cork, Ireland, 17–21 June, 2018. Wageningen, The Netherlands: Wageningen. Academic Publishers.

Garry, B. 2016. The effect of animal and sward factors on the in vivo organic matter digestibility of perennial ryegrass. PhD dissertation. Sch. Agri. Food Sci., Univ. College Dublin, Belfield, Dublin 4, Ireland.

Godfray, H. C. J., J. R. Beddington, I. R. Crute, L. Haddad, D. Lawrence, J. F Muir, J. Pretty, S. Robinson, S. M. Thomas, and C. Toulmin. 2010. Food security: the challenge of feeding 9 billion people. Sci. 327:812–818.

Goswami, A. K., and J. S. Willcox. 1969. Effect of applying increasing levels of nitrogen to ryegrass. I. Composition of various nitrogenous fractions and free amino acids. J. Sci. Food Agric. 20:592-599.

Grabber, J. H., D. R. Mertens, H. Kim, C. Funk, F. Lua, and J. Ralph. 2009. Cell wall fermentation kinetics are impacted more by lignin content and ferulate cross-linking than by lignin composition. J. Sci. Food Agric. 89:122–129.

Grabber J. H., J. Ralph, and R. D. Hatfield. 2000. Cross-linking of maize walls by ferulate dimerization and incorporation into lignin. J. Agric. Food. Chem. 48:6106–6113

Grabber, J. H., J. Ralph, R. D. Hatfield, and S. Quideau. 1997. p-Hydroxyphenyl, guaiacyl, and syringyl lignins have similar inhibitory effects on wall degradability. J. Agric. Food Chem. 45:2530–2532.

Grant, R. J., and D. R. Mertens. 1992. Influence of buffer pH and raw corn starch addition on in vitro fiber digestion kinetics. J. Dairy Sci. 75:2762-2768.

Griffiths, W. M., C. G. Roach, and D. F. Chapman. 2020. Should forage evaluation indices include a feeding value trait? Grass Forage Sci. 00:1-11.

Hackmann, T. J., and J. L. Firkins. 2015. Maximizing efficiency of rumen microbial protein production. Front. Microbiol. 6:465.

Hall, M. B. 2014. Selection of an empirical detection method for determination of water-soluble carbohydrates in feedstuffs for application in ruminant nutrition. Anim. Feed Sci. Technol. 198:28-37.

Hall, M. B., A. N. Pell, and L. E. Chase. 1998. Characteristics of neutral detergent-soluble fiber fermentation by mixed ruminal microbes. Anim. Feed Sci. Technol. 70:23–39.

Harfoot, C. G. 1981. Lipid metabolism in the rumen. Pages 21–56 in Lipid Metabolism in Ruminant Animals. W. W. Christie. ed. Pergamon Press, New York.

Harrison, D. G., D. E. Beever, D. J. Thomson, and D. F. Osbourn. 1976. Manipulation of fermentation in the rumen. J. Sci. Food Agri. 27:617-620.

Hoekstra, N. J., R. P. O. Schulte, P. J. Forrestal, D. Hennessy, D. J. Krol, G. J. Lanigan, C. Müller, L. Shalloo, D. P. Wall, and K. G. Richards. 2020. Scenarios to limit environmental nitrogen losses from dairy expansion. Sci. Total Environ. 707:134606.

Hook, S. E., J. Dijkstra, A. D. G. Wright, B. W. McBride, and J. France. 2012. Modeling the distribution of ciliate protozoa in the reticulorumen using linear programming. J. Dairy Sci. 95:255-265.

Hristov, A. N. and J. P. Jouany. 2005. Factors affecting the efficiency of nitrogen utilization in the rumen. Pages 117–166 in Nitrogen and Phosphorus Nutrition of Cattle and Environment. A. N. Hristov, and E. Pfeffer, ed. Wallingford: CAB International.

Huhtanen, P., S. Ahvenjärvi, G. A. Broderick, S. M. Reynal, and K. J. Shingfield. 2010. Quantifying ruminal digestion of organic matter and neutral detergent fiber using the omasal sampling technique in cattle—A meta-analysis. J. Dairy Sci. 93:3203-3215.

Huhtanen, P., A. Seppälä, M. Ots, S. Ahvenjärvi, and M. Rinne. 2008. In vitro gas production profiles to estimate extent and effective first-order rate of neutral detergent fiber digestion in the rumen. J. Anim. Sci. 86:651-659.

Huhtanen, P., S. Ahvenjärvi, M. R. Weisbjerg, and P. Norgaard. 2006. Digestion and passage of fibre in ruminants. Pages 87-135 in Ruminant Physiology: Digestion, Metabolism and Impact of Nutrition on Gene Expression, Immunology and Stress. K. Sejrsen, T. Hvelplund, and M. O. Nielsen, ed. Wageningen Academic Publisher, Wageningen, Netherlands.

Huhtanen, P., P. G. Brotz, and L. D. Satter. 1997. Omasal sampling technique for assessing fermentative digestion in the forestomach of dairy cows. J. Anim. Sci. 75:1380–1392.

Huhtanen, P., and S. Jaakkola. 1994. Influence of grass maturity and diet on ruminal dry matter and neutral detergent fibre digestion kinetics. Arch. Tierernahr. 47:153–167.

Humphreys, M. O. 1989. Water-soluble carbohydrates in perennial ryegrass breeding. II. Cultivar and hybrid progeny performance in cut plots. Grass Forage Sci. 44:237-244.

Hungate, R. E. 1966. Rumen and its microbes. Academic Press, New York, NY.

Huws, S. A., M. R. F. Lee, A. H. Kingston-Smith, E. J. Kim, M. B. Scott, J. K. S. Tweed, and N. D. Scollan. 2012. Ruminal protozoal contribution to the duodenal flow of fatty acids following feeding of steers on forages differing in chloroplast content. Br. J. Nutr. 108:2207–2214.

Huws, S. A., E. J. Kim, A. H. Kingston-Smith, M. R. F. Lee, S. M. Muetzel, C. J. Newbold, R. J. Wallace, and N. D. Scollan. 2009. Rumen protozoa are rich in polyunsaturated fatty acids due to the ingestion of chloroplasts. FEMS Microbiol. Ecol. 69:461–471.

Iiyama, K., T. B. T. Lam, and B. A. Stone. 1990. Phenolic acid bridges between polysaccharides and lignin in wheat internodes. Phytochem. 29:733–737.

Institut National de Recherche Agronomique. 2018. Feeding system for ruminants. Wageningen Academic Publishers, Wageningen, The Netherlands.

Ipharraguerre, I. R., and J. H. Clark. 2005. Impacts of the source and amount of crude protein on the intestinal supply of nitrogen fractions and performance of dairy cows. J. Dairy Sci. 88:22-37.

Isaacson, H. R., F. C. Hinds, M. P. Bryant, and F. N. Owens. 1975. Efficiency of energy utilization by mixed rumen bacteria in continuous culture. J. Dairy Sci. 58:1645–1659.

Jenkins, T. C., R. J. Wallace, P. J. Moate, and E. E. Mosley. 2008. Board-invited review: Recent advances in biohydrogenation of unsaturated fatty acids within the rumen microbial ecosystem. J. Anim. Sci. 86:397–412.

Jung, H., D. R. Mertens, and R. Phillips. 2011. Effect of reduced ferulate-mediated lignin/arabinoxylan cross-linking in corn silage on feed intake, digestibility, and milk production. J. Dairy Sci. 94:5124-5137.

Jung, H. G., W. Ni, C. C. S. Chapple, and K. Meyer. 1999. Impact of lignin composition on cell-wall degradability in an Arabidopsis mutant. J. Sci. Food Agric. 79:922–928.

Jung, H. and M. Allen. 1995. Characteristics of plant cell walls affecting intake and digestibility of forages by ruminants. J. Anim. Sci. 73:2774-2790.

Jung, H. G., R. R. Smith, and C. S. Endres. 1994. Cell-wall composition and degradability of stem tissue from lucerne divergently selected for lignin and in vitro dry matter disappearance. Grass Forage Sci. 49:295-304.

Jung, H. G., and D. A. Deetz. 1993. Cell wall lignification and degradability. Pages 315-346 in Forage Cell Wall Structure and Digestibility. H. G. Jung, D. R. Buxton, R. D. Hatfield, and J. Ralph, ed. ASA-CSSA-SSSA, Madison, WI.

Jung, H. G., J. Ralph, and R. D. Hatfield. 1991. Degradability of phenolic acid-hemicellulose esters: A model system. J. Sci. Food Agric. 56:469-478.

Karnati, S. K. R., J. T. Sylvester, S. M. Noftsger, Z. Yu, N. R. St-Pierre, and J. L. Firkins. 2007. Assessment of ruminal bacterial populations and protozoal generation time in cows fed different methionine sources. J. Dairy Sci. 90:798–809.

Kellaway, R., and S. Porta. 1993. Feeding concentrates: supplements for dairy cows. Dairy Res. Dev. Corp., Australia.

Kennedy, J., P. Dillon, L. Delaby, P. Faverdin, G. Stakelum, and M. Rath. 2003. Effect of genetic merit and concentrate supplementation on grass intake and milk production with holstein friesian dairy cows. J. Dairy Sci. 86:610-621.

Kim, S. W., J. F. Less, L. Wang, T. Yan, V. Kiron, S. J. Kaushik, and X. G. Lei. 2019. Meeting global feed protein demand: challenge, opportunity, and strategy. Annu. Rev. Anim. Biosci. 7:221–243.

Kingston-Smith, A.H., R. J. Merry, D. K. Leemans, H. Thomas, and M. K. Theodorou. 2005. Evidence in support of a role for plant-mediated proteolysis in the rumens of grazing animals Br. J. Nutr. 93:73-79.

Koenig, K. M., C. J. Newbold, F. M. McIntosh, and L. M. Rode. 2000. Effects of protozoa on bacterial nitrogen recycling in the rumen. J. Anim. Sci. 78:2431–2445.

Kraft, J., M. Collomb, P. Möckel, R. Sieber, and G. Jahreis. 2003. Differences in CLA isomer distribution of cow's milk lipids. Lipids 38:657–664.

Laisse, S., R. Baumont, P. Veysset, M. Benoit, P. Madrange, B. Rouillé, and J. L. Peyraud. 2018. The net contribution of ruminant production to the protein supply for humans. Pages 718–720 in General meeting of the European Grassland Federation. Cork, Ireland.

Lam, T. B. T., K. Iiyama, and B. A. Stone. 1992. Cinnamic acid bridges between cell wall polymers in wheat and Phalaris internodes. Phytochem. 31:1179-1183.

Lanzas, C., C. J. Sniffen, S. Seo, L. O. Tedeschi, and D. G. Fox. 2007. A feed carbohydrate fractionation scheme for formulating rations for ruminants. Anim. Feed Sci. Technol. 136:167–190.

Lapierre, H., D. R. Ouellet, R. Berthiaume, R. Martineau, G. Holtrop, and G. E. Lobley. 2008. Distribution of $^{15}N$ in amino acids during $^{15}N$-Leucine infusion: Impact on the estimation of endogenous flows in dairy cows. J. Dairy Sci. 91:2702–2714.

Läpple, D., and G. Sirr. 2019. Dairy intensification and quota abolition: A comparative study of production in Ireland and the Netherlands. EuroChoices (Uckfield) 18:26–32.

Leddin, C., C. Stockdale, J. Hill, J. Heard, and P. Doyle. 2010. Increasing amounts of crushed wheat fed with Persian clover herbage reduced ruminal pH and dietary fibre digestibility in lactating dairy cows. Ani. Prod. Sci. 50:837-846.

Lee, J. M., C. Matthew, E. R. Thom, and D. F. Chapman. 2012. Perennial ryegrass breeding in New Zealand: a dairy industry perspective. Crop Pasture Sci. 63:107-127.

Loaiza, P., O. Balocchi, and A. Bertrand. 2017. Carbohydrate and crude protein fractions in perennial ryegrass as affected by defoliation frequency and nitrogen application rate. Grass Forage Sci. 72:556-567.

Lock, A. L., and P. C. Garnsworthy. 2003. Seasonal variation in milk conjugated linoleic acid and Δ9-desaturase activity in dairy cows. Livest. Sci. 79:47–59.

Longland, A. C., M. S. Dhanoa, and P. A. Harris. 2012. Comparison of a colorimetric and a high-performance liquid chromatography method for the determination of fructan in pasture grasses for horses. J. Sci. Food Agric. 92:1878–1885.

Lucas, H. L. 1964. Stochastic elements in biological models; their sources and significance. In: Stochastic Models in Medicine and Biology. J. Gurland, ed. Univ. Wisconsin Press, Madison. Page 355.

Mangan, J. L. 1982. The nitrogenous constituents of fresh forages. Occasional Publication, Br. Soc. Ani. Prod. 6:25-40.

McAllan, A., and R. Smith. 1983. Factors influencing the digestion of dietary carbohydrates between the mouth and abomasum of steers. Br. J. Nutr 50:445-454.

McCarthy, K., C. McAloon, M. Lynch, K. Pierce, and F. Mulligan. 2020. Herb species inclusion in grazing swards for dairy cows—A systematic review and meta-analysis. J. Dairy Sci. 103:1416-1430.

McDonald, P., R. A. Edwards, J. F. D. Greenhalgh, and C. A. Morgan. 2002. Evaluation of foods: Digestibility. Animal Nutrition. 6th ed. Essex, England: Pearson Education Limited, 245-262.

McDonald, P., A. R. Henderson, and S. J. E. Heron. 1991. The Biochemistry of Silage, 2nd ed. Cambrian Printers Ltd., Aberystwyth, UK.

McEvoy, M., M. O'Donovan, and L. Shalloo. 2011. Development and application of an economic ranking index for perennial ryegrass cultivars. J. Dairy Sci. 94:1627–1639.

McKay, Z., M. Lynch, F. Mulligan, G. Rajauria, C. Miller, and K. Pierce. 2019. The effect of concentrate supplementation type on milk production, dry matter intake, rumen fermentation, and nitrogen excretion in late-lactation, spring-calving grazing dairy cows. J. Dairy Sci. 102:5042-5053.

Merino, V. M., O. A. Balocchi, and M. J. Rivero. 2019. Milk production, milk quality, and behaviour of dairy cows grazing on swards with low and high water-soluble carbohydrates content in autumn: a pilot trial. Animals. 9:1012.

Mertens, D. R. and R. J. Grant. 2007. Digestibility and intake. Pages 487–507 in Forages Volume II: The Science of Grassland Agriculture, 6th ed. R. F. Barnes, C. J. Nelson, K. J. Moore, M. Collins, ed. Iowa State Univ. Press: Ames, IA.

Mertens, D. R. 2002. Gravimetric determination of amylase-treated neutral detergent fiber in feeds with refluxing in beakers or crucibles: collaborative study. J. AOAC Int. 85:1217-1240.

Miller, L. A., J. M. Moorby, D. R. Davies, M. O. Humphreys, N. D. Scollan, J. C. MacRae, and M. K. Theodorou. 2001. Increased concentration of water-soluble carbohydrate in perennial ryegrass (Lolium perenne L.): milk production from late-lactation dairy cows. Grass Forage Sci. 56:383-394.

Minson, D. 1981. The effects of feeding protected and unprotected casein on the milk production of cows grazing ryegrass. J. Agric. Sci. 96:239-241.

Moate, P., M. Deighton, J. Jacobs, B. Ribaux, G. Morris, M. Hannah, D. Mapleson, M. Islam, W. Wales, and S. Williams. 2020. Influence of proportion of wheat in a pasture-based diet on milk yield, methane emissions, methane yield, and ruminal protozoa of dairy cows. J. Dairy Sci. 103:2373-2386.

Molina, D. O. 2002. Prediction of intake of lactating cows in the tropics and the energy value of organic acids. Ph.D. Dissertation. Cornell Univ., Ithaca, NY, USA.

Moorby, J. M., R. T. Evans, N. D. Scollan, J. C. MacRae, and M. K. Theodorou. 2006. Increased concentration of water-soluble carbohydrate in perennial ryegrass (Lolium perenne L.). Evaluation in dairy cows in early lactation. Grass Forage Sci. 61:52-59.

Mottet, A., C. De Haan, A. Falcucci, G. Tempio, C. Opio, and P. Gerber. 2017. Livestock: On our plates or eating at our table? A new analysis of the feed/food debate. Global Food Security 14:1-8.

Mould, F., E. Ørskov, and S. Mann. 1983. Associative effects of mixed feeds. I. Effects of type and level of supplementation and the influence of the rumen fluid pH on cellulolysis in vivo and dry matter digestion of various roughages. Anim. Feed Sci. Technol. 10:15-30.

Newbold, C. J. 1999. The need for nitrogen. Br. J. Nutr. 82:81–82.

Newbold, C. J., G. de la Fuente, A. Belanche, E. Ramos-Morales, and N. R. McEwan. 2015. The role of ciliate protozoa in the rumen. Front. Microbiol. 6:1313.

Newbold, C. J., D. G. Chamberlain, and A. G. Williams. 1986. The effects of defaunation on the metabolism of lactic acid in the rumen. J. Sci. Food Agric. 37:1083–1090.

Nousiainen, J., S. Ahvenjärvi, M. Rinne, M. Hellämäki, and P. Huhtanen. 2004. Prediction of indigestible cell wall fraction of grass silage by near infrared reflectance spectroscopy. Anim. Feed Sci. Technol. 115:295-311.

O'Mara, F. P., G. K. Stakelum, P. Dillon, J. J. Murphy, and M. Rath. 1997. Rumen fermentation and nutrient flows for cows fed grass and grass supplemented with molassed beet pulp pellets. J. Dairy Sci. 80:2466-2474.

Oba, M. and M. Allen. 2003. Effects of corn grain conservation method on ruminal digestion kinetics for lactating dairy cows at two dietary starch concentrations. J. Dairy Sci. 86:184-194.

O'Brien, D., B. Moran, and L. Shalloo. 2018. A national methodology to quantify the diet of grazing dairy cows. J. Dairy Sci. 101:8595-8604.

O'Donovan, M., P. Dillon, M. Rath, and G. Stakelum. 2002. A comparison of four methods of herbage mass estimation. Ir. J. Agric. Food Res. 17-27.

O'Mara, F. P., J. J. Murphy, and M. Rath. 2000. The effect of concentrate supplements differing in ruminal protein degradability on milk production and blood metabolite concentrations of dairy cows grazing perennial ryegrass pasture. Livest. Prod. Sci. 64:183-191.

Ørskov, E. R., N. A. Macleod, and D. J. Kyle. 1986. Flow of nitrogen from the rumen and abomasum in cattle and sheep given protein free nutrients by intragastric infusion. Br. J. Nutr. 56:241–248.

Pacheco, D. and G.C. Waghorn. 2008. Dietary nitrogen − definitions, digestion, excretion and consequences of excess for grazing ruminants. Proc. N. Z.Grassl. Assoc. 70:107–116.

Palladino, R., M. O'Donovan, E. Kennedy, J. Murphy, T. Boland, and D. Kenny. 2009. Fatty acid composition and nutritive value of twelve cultivars of perennial ryegrass. Grass Forage Sci. 64:219-226.

Papas, A. M., S. R. Ames, R. M. Cook, C. J. Sniffen, C. E. Polan, and L. Chase. 1984. Production responses of dairy cows fed diets supplemented with ammonium salts of iso C-4 and C-5 acids. J. Dairy Sci. 67:276–293.

Penno, J. W. 2002. The response by grazing dairy cows to supplementary feeds. PhD. Animal Science, Institute of Veterinary, Animal and Biomedical Sciences, Massey Univ., Palmerston North, N.Z.

Peyraud, J. L. and R. Delagarde. 2013. Managing variations in dairy cow nutrient supply under grazing. Anim. 7:57-67.

Peyraud, J. and L. Astigarraga. 1998. Review of the effect of nitrogen fertilization on the chemical composition, intake, digestion and nutritive value of fresh herbage: consequences on animal nutrition and N balance. Anim. Feed Sci. Technol. 72:235-259.

Peyraud, J., L. Astigarraga, and P. Faverdin. 1997. Digestion of fresh perennial ryegrass fertilized at two levels of nitrogen by lactating dairy cows. Anim. Feed Sci. Technol. 64:155-171.

Raffrenato, E., C. Nicholson, and M. E. Van Amburgh. 2019. Development of a mathematical model to predict pool sizes and rates of digestion of 2 pools of digestible neutral detergent fiber and an undigested neutral detergent fiber fraction within various forages. J. Dairy Sci. 102:351-364.

Raffrenato, E., D. A. Ross, and M. E. Van Amburgh. 2018. Development of an in vitro method to determine rumen undigested aNDFom for use in feed evaluation. J. Dairy Sci. 101:9888–9900.

Raffrenato, E., R. Fievisohn, K. W. Cotanch, R. J. Grant, L. E. Chase, and M. E. Van Amburgh. 2017. Effect of lignin linkages with other plant cell wall components on in vitro and in vivo neutral detergent fiber digestibility and rate of digestion of grass forages. J. Dairy Sci. 100:8119–8131.

Reid, D. and N. Strachan. 1974. The effects of a wide range of nitrogen rates on some chemical constituents of the herbage from perennial ryegrass swards with and without white clover. J. Agri. Sci. 83:393-401.

Reynal, S., I. Ipharraguerre, M. Lineiro, A. Brito, G. Broderick, and J. Clark. 2007. Omasal flow of soluble proteins, peptides, and free amino acids in dairy cows fed diets supplemented with proteins of varying ruminal degradabilities. J. Dairy Sci. 90:1887-1903.

Reynolds, C. 2006. Production and metabolic effects of site of starch digestion in dairy cattle. Anim. Feed Sci. Technol. 130:78-94.

Rinne, M. 2000. Influence of the timing of the harvest of primary grass growth on herbage quality and subsequent digestion and performance in the ruminant animal. PhD thesis. Univ. of Helsinki, Finland. Department of Animal Science Publications 54.

Rius, A., S. Kittelmann, K. Macdonald, G. Waghorn, P. Janssen, and E. Sikkema. 2012. Nitrogen metabolism and rumen microbial enumeration in lactating cows with divergent residual feed intake fed high-digestibility pasture. J. Dairy Sci. 95:5024-5034.

Robinson, P., S. Tamminga, and A. Van Vuuren. 1987. Influence of declining level of feed intake and varying the proportion of starch in the concentrate on rumen ingesta quantity, composition and kinetics of ingesta turnover in dairy cows. Livest. Prod. Sci. 17:37-62.

Roche, J., L. Turner, J. Lee, D. Edmeades, D. Donaghy, K. Macdonald, J. Penno, and D. Berry. 2009. Weather, herbage quality and milk production in pastoral systems. 2. Temporal patterns and intra-relationships in herbage quality and mineral concentration parameters. Ani. Prod. Sci. 49:200-210.

Roche, J. R., D. P. Berry, and E. S. Kolver. 2006. Holstein-Friesian strain and feed effects on milk production, body weight, and body condition score profiles in grazing dairy cows. J. Dairy Sci. 89:3532-3543.

Routaboul, J., C. Skidmore, J. G. Wallis, and J. Browse. 2012. Arabidopsis mutants reveal that short- and long-term thermotolerance have different requirements for trienoic fatty acids. J. Exp. Bot. 63:1435–1443.

Russell, J. B., and H. J. Strobel. 2005. Microbial energetics. Pages 229–261 in Quantitative Aspects of Ruminant Digestion and Metabolism, 2nd Ed. J. Dijkstra, J. M. Forbes, and J. France, ed. CABI Publishing, Cambridge, MA.

Russell, J. B, and D. B. Wilson. 1996. Why are ruminal cellulolytic bacteria unable to digest cellulose at low pH? J. Dairy Sci. 78:1503–1509.

Russell, J. B., and P. J. Van Soest, 1984. In vitro ruminal fermentation of organic acids common in forage. Appl. Environ. Microbiol. 47:155–159.

Rymer, C. 2000. The measurement of forage digestibility in vivo. Pages 113-134 in Forage Evaluation in Ruminant Nutrition. D. I. Givens, E. Owen, R. F. E. Axford, and H. M. Omed, ed. Oxon, UK and New York, USA: CABI Publishing.

Sairanen, A., H. Khalili, J. I. Nousiainen, S. Ahvenjärvi, and P. Huhtanen. 2005. The effect of concentrate supplementation on nutrient flow to the omasum in dairy cows receiving freshly cut grass. J. Dairy Sci. 88:1443-1453.

Schroeder, G., G. A. Gagliostro, F. Bargo, J. Delahoy, and L. Muller. 2004. Effects of fat supplementation on milk production and composition by dairy cows on pasture: a review. Livest. Prod. Sci. 86:1-18.

Schulte, R. P. O., R. E. Creamer, T. Donnellan, N. Farrelly, R. Fealy, C. O'Donoghue, and D. O'hUallachain. 2014. Functional land management: a framework for managing soil-based ecosystem services for the sustainable intensification of agriculture. Environ. Sci. Policy. 38:45–58.

Smit, H., B. Tas, H. Taweel, S. Tamminga, and A. Elgersma. 2005. Effects of perennial ryegrass (Lolium perenne L.) cultivars on herbage production, nutritional quality and herbage intake of grazing dairy cows. Grass Forage Sci. 60:297-309.

Stakelum, G. K. N., 1993. Supplementary Feeding and Herbage Intake of the Dairy Cow. Ph.D. National Univ. of Ireland, Dublin, Ireland.

Stakelum, G. 1986. Herbage intake of grazing dairy cows: 3. Effects of herbage mass, herbage allowance and concentrate feeding on the herbage intake of dairy cows grazing on mid-summer pasture. Ir. J. Agric. Res. 179-189.

Storm, E., E. R. Orskov, and R. Smart. 1983. The nutritive value of rumen microorganisms in ruminants. 2. The apparent digestibility and net utilization of microbial N for growing lambs. Br. J. Nutr. 50:471–478.

Strobel, H. J., and J. B. Russell. 1986. Effect of pH and energy spilling on bacterial protein synthesis by carbohydrate limited cultures of mixed rumen bacteria. J. Dairy Sci. 69:2941–2947.

Sylvester, J. T., S. K. R. Karnati, B. A. Dehority, M. Morrison, G. L. Smith, N. R. St-Pierre, and J. L. Firkins. 2009. Rumen ciliated protozoa decrease generation time and adjust 18S ribosomal DNA copies to adapt to decreased transfer interval, starvation, and monensin. J. Dairy Sci. 92:256-269.

Sylvester, J. T., S. K. R. Karnati, Z. Yu, C. J. Newbold, and J. L. Firkins. 2005. Evaluation of a real-time PCR assay quantifying the ruminal pool size and duodenal flow of protozoal nitrogen. J. Dairy Sci. 88:2083–2095.

Tamminga, S. 1979. Protein degradation in the forestomachs of ruminants. J. Anim. Sci. 49:1615–1630.

Tas, B., H. Taweel, H. Smit, A. Elgersma, J. Dijkstra, and S. Tamminga. 2006a. Rumen degradation characteristics of perennial ryegrass cultivars during the growing season. Anim. Feed Sci. Technol. 131:103-120.

Tas, B. M., H. Z. Taweel, H. J. Smit, A. Elgersma, J. Dijkstra, and S. Tamminga. 2006b. Effects of perennial ryegrass cultivars on milk yield and nitrogen utilization in grazing dairy cows. J. Dairy Sci. 89:3494-3500.

Tas, B. M., H. Z. Taweel, H. J. Smit, A. Elgersma, J. Dijkstra, and S. Tamminga. 2006c. Utilisation of N in perennial ryegrass cultivars by stall-fed lactating dairy cows. Livest. Sci. 100:159-168.

Taweel, H. Z., B. M. Tas, H. J. Smit, A. Elgersma, J. Dijkstra, and S. Tamminga. 2006. Grazing behaviour, intake, rumen function and milk production of dairy cows offered Lolium perenne containing different levels of water-soluble carbohydrates. Livest. Sci. 102:33-41.

Taweel, H., B. Tas, H. Smit, A. Elgersma, J. Dijkstra, and S. Tamminga. 2005a. Improving the quality of perennial ryegrass (Lolium perenne L.) for dairy cows by selecting for fast clearing and/or degradable neutral detergent fiber. Livest. Prod. Sci. 96:239-248.

Taweel, H., B. Tas, H. Smit, A. Elgersma, J. Dijkstra, and S. Tamminga. 2005b. Effects of feeding perennial ryegrass with an elevated concentration of water-soluble carbohydrates on intake, rumen function and performance of dairy cows. Anim. Feed Sci. Technol. 121:243-256.

Thomas, G. J. 1960. Metabolism of the soluble carbohydrates of grasses in the rumen of the sheep. J. Agric. Sci. 54:360–372.

Turner, L. B., A. J. Cairns, I. P. Armstead, J. Ashton, K. Skøt, D. Whittaker, and M. O. Humphreys. 2006. Dissecting the regulation of fructan metabolism in perennial ryegrass (Lolium perenne) with quantitative trait locus mapping. New Phytologist 169:45-58.

Udén, P. 2006. Recovery of insoluble fiber fractions by filtration and centrifugation. Anim. Feed Sci. Technol. 129:316–328.

United Nations, Department of Economic and Social Affairs, Population Division 2019. World Population Prospects 2019.

Vailhé, M. A. B., G. J. Provan, L. Scobbie, A. Chesson, M. P. Maillot, A. Cornu, and J. M. Besle. 2000. Effect of phenolic structures on the degradability of cell walls isolated from newly

extended apical internode of tall fescue (Festuca arundinacea Schreb.). J. Agric. Food Chem. 48:618–623.

Valk, H., I. E. Kappers, and S. Tamminga. 1996. In sacco degradation characteristics of organic matter, neutral detergent fibre and crude protein of fresh grass fertilized with different amounts of nitrogen. Anim. Feed Sci. Technol. 63:63–87.

Van Amburgh, M. E., K. Russomanno, R. Higgs, and L. Chase. 2019. Invited Review: Modifications to the Cornell Net Carbohydrate and Protein System related to environmental issues—Capability to evaluate nitrogen and phosphorus excretion and enteric carbon dioxide and methane emissions at the animal level. Appl. Ani. Sci. 35:101-113.

Van Amburgh, M. E., R. Grant, K. Cotanch, A. Zontini, D. Ross, and A. Foskolos. 2015. NDF–Making something old, new again. Pages 1–7 in Herd Health Nutr. Conference, Syracuse, NY. Cornell Univ., Ithaca, NY.

Van Amburgh M. E., J. Voorhees, and J. Robertson. 1999. Total dietary and soluble fiber content of selected ruminant feeds. Pages 196–202 in Proc. Cornell Nutrition Conf., Syracuse, NY. Cornell Univ., Ithaca, NY.

Van Soest, P. J. 1994. Nutritional ecology of the ruminant. 2nd Ed. Cornell Univ. Press, Ithaca, NY.

Van Soest, P. J., J. B. Robertson, and B. A. Lewis. 1991. Methods for dietary fiber, neutral-detergent fiber and nonstarch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74:3583-3597.

Van Soest, P. J. 1967. Development of a comprehensive system of feed analysis and its application to forages. J. Anim. Sci. 26:119-128.

Van Vuuren, A. and P. Chilibroste. 2013. Challenges in the nutrition and management of herbivores in the temperate zone. Anim. 7:19-28.

Van Vuuren, A. M., F. Krol-Kramer, R. A. Van Der Lee, and H. Corbijn. 1992. Protein digestion and intestinal amino acid in dairy cows fed fresh Lolium perenne with different nitrogen contents. J. Dairy Sci. 75:2215-2225.

Van Vuuren, A .M., S. Tamminga, and R. S. Ketelaar. 1991. In-sacco degradation of organic matter and crude protein of fresh grass (Lolium perenne) in the rumen of grazing dairy cows. J. Agric. Sci. 116:429–436.

Wales, W., E. Kolver, and A. Egan. 2009. Digestion during continuous culture fermentation when replacing perennial ryegrass with barley and steam-flaked corn. J. Dairy Sci. 92:189-196.

Wallace, R. J., R. Onodera, and M. A. Cotta. 1997. Metabolism of nitrogen-containing compounds. Pages 283–328 in The Rumen Microbial Ecosystem. P. N. Hobson, and C. S. Stewart, ed. London: Chapman and Hall

Wallace, R. J., and C. A. McPherson. 1987. Factors affecting the rate of breakdown of bacterial protein in rumen fluid. Br. J. Nutr. 58:313–323.

Weisbjerg, M. R., H. Gado, T. Hvelplund, and B. B. Jensen. 1999. The effect of easily fermentable carbohydrates and pH on fibre digestibility and vfa pattern in an in vitro continous culture system. South African J. Anim. Sci. 29:112–13.

Wells, J. E. and J. B. Russell. 1996. Why do many ruminal bacteria die and lyse so quickly? J. Dairy Sci. 79:1487-1495.

Whelan, S. J., K. M. Pierce, B. Flynn, and F. J. Mulligan. 2012. Effect of supplemental concentrate type on milk production and metabolic status in early-lactation dairy cows grazing perennial ryegrass-based pasture. J. Dairy Sci. 95:4541–4549.

White, R. R. and M. B. Hall. 2017. Nutritional and greenhouse gas impacts of removing animals from US agriculture. Proc. of the National Academy of Sciences 114:E10301-E10308.

Wilkinson, J. M., M. R. F. Lee, M. J. Rivero, and A. T. Chamberlain. 2020. Some challenges and opportunities for grazing dairy cows on temperate pastures. Grass Forage Sci. 75:1–17.

Williams, A. G., and G. S. Coleman. 1992. The Rumen Protozoa. New York, NY: Springer-Verlag.

Williams, Y., W. Wales, P. Doyle, A. Egan, and C. Stockdale. 2005. Effects of grain or hay supplementation on the chewing behaviour and stability of rumen fermentation of dairy cows grazing perennial ryegrass-based pasture in spring. Aus. J. Exp. Agric. 45:1519-1528.

Wilman, D., 1975. Nitrogen and Italian ryegrass: 2. Growth up to 14 weeks: nitrogen, phosphorus and potassium content and yield. J. Br. Grassl. Soc. 31:243–247.

Wilson, J. R. 1993. Organization of forage plant tissues. Pages 1-32 in Forage Cell Wall Structure and Digestibility. H. G. Jung, D. R. Buxton, R. D. Hatfield, and J. Ralph, ed. ASA-CSSA-SSSA, Madison, WI

Winichayakul, S., Z. Beechey-Gradwell, S. Muetzel, G. Molano, T. Crowther, S. Lewis, H. Xue, J. Burke, G. Bryan, and N. Roberts. 2020. In vitro gas production and rumen fermentation

profile of fresh and ensiled genetically modified high–metabolizable energy ryegrass. J. Dairy Sci. 3:2405–2418.

Witkowska, I. M., A. C. Weberebe., G. Gort, and A. Elgersma. 2008. Fatty acids in perennial ryegrass from contrasting nitrogen fertilization – regrowth interval treatments in response to morphological and climatic variables during the growing season. Agron. J. 100:1371–1379.

Yáñez-Ruiz, D. R., Scollan, N. D., Merry, R. J., and Newbold, C. J. 2006. Contribution of rumen protozoa to duodenal flow of nitrogen, conjugated linoleic acid and vaccenic acid in steers fed silages differing in their water-soluble carbohydrate content. Br. J. Nutr. 96:861–869.

Younge, B., J. Murphy, and M. Rath. 2004. Nutrient metabolism in the rumen and milk production in cows fed on grass-silage and fresh grass based diets. Livest. Prod. Sci. 88:43-54.

Zhu, W. Y., A. H. Kingston-Smith, D. Troncoso, R. J. Merry, D. R. Davies, G. Pichard, H. Thomas, and M. K. Theodorou. 1999. Evidence of a role for plant proteases in the degradation of herbage proteins in the rumen of grazing cattle. J. Dairy Sci. 82:2651-2658.

Zontini, A., A. Foskolos, D. A. Ross, J. Metcalf, P. H. Doane, and M. E. Van Amburgh. 2015. Research Update: Formulating diets for lactating cattle using multiple pools of NDF digestibility. Pages 189–196 in Proc. Cornell Nutrition Conference, Syracuse, NY. Cornell Univ., Ithaca, NY.

# CHAPTER 2: CHARACTERIZATION OF THE NUTRITIVE VALUE OF PERENNIAL RYEGRASS (*LOLIUM PERENNE* L.) DOMINATED PASTURES USING UPDATED CHEMICAL METHODS WITH APPLICATION FOR THE CORNELL NET CARBOHYDRATE AND PROTEIN SYSTEM

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## 2.1. ABSTRACT

Ruminant nutrition models, such as the Cornell Net Carbohydrate and Protein System (**CNCPS)**, have evolved over time resulting in increased precision and accuracy with which they can predict animal performance. In tandem with this evolution, new and more precise methodologies have been developed to describe the feed chemistry inputs required to conduct model simulations. However, these updated methods have not been applied to pasture-based systems. The objective of this study was to characterize the nutritive value of perennial ryegrass (*Lolium perenne* L.; **PRG**) dominated pastures using the new and updated feed chemistry methods. Fifty-five samples from three independent experiments were used in this study. The data generated demonstrated that the amylase- and sodium sulphite-treated NDF corrected for ash residue (**aNDFom**) method was significantly lower than the amylase- and sodium sulphite-treated NDF (**aNDF**) method; hence, due to ash contamination aNDF consistently overestimated the true

measure of cell wall contents in PRG. Results from multiple time point in vitro aNDFom digestibility analysis indicated that immature PRG comprises of a large potentially digestible pool that degrades at a rapid rate. A 12-h time point combined with 30, 120, and 240 h better describes the aNDFom degradation curve of PRG. In addition, the mean undigested aNDFom at 240 h of fermentation was affected by PRG category; whereby, spring and summer were lowest (98 and 111 g/kg of aNDFom, respectively), autumn intermediate (155 g/kg of aNDFom), and drought stressed pastures the highest (200 g/kg of aNDFom). The extent of in vitro aNDFom digestion was not reached prior to 240 h; however, a strong Pearson correlation coefficient between 120-h and 240-h time points (0.98) indicated the potential to predict undigested aNDFom from the 120-h time point. Based on the N fractionation scheme employed by the CNCPS, a large proportion of PRG N is soluble and highly degradable in the rumen. An assay capable of quantitatively describing the rumen degradation of PRG N is required. Using a single 21-h HCl hydrolysis to determine amino acid (**AA**) concentration underestimated the concentration of Ile and Val indicating that multiple-hydrolysis time procedures are required to predict the true AA concentration of PRG. In conclusion, the updated feed chemistry procedures conducted in this study, allowed a more accurate characterization of the nutritive value of PRG. This increased understanding in combination with formulation models, such as the CNCPS, can provide more robust information on how PRG interacts with the ruminant animal, in order to increase the efficiency and productivity of pasture-based systems.

**Key words:** neutral detergent fibre, pasture, CNCPS, digestibility, amino acid.

## 2.2. INTRODUCTION

In temperate regions, pasture-based dairy systems are economically and environmentally sustainable (Dillon et al., 2008; Lorenz et al., 2019); however, opportunities exist to further enhance the productivity and efficiency of such systems (Peyraud and Delagarde, 2013). Nutritional models, such as the Cornell Net Carbohydrate and Protein System (**CNCPS;** Van Amburgh et al., 2015), can be applied to help select optimal dietary strategies by estimating cattle nutrient requirements while simultaneously assessing the adequacy of the diet. To obtain useful information from any biological model, however, accurate and comprehensive feed chemistry is required (Huhtanen et al., 2006a; Higgs et al., 2015).

Current methodology to assess the digestibility of grazing-swards depend upon the use of commercial enzymes incorporated into in vitro methods (Beecher et al., 2015). Such cellulolytic enzymes do not degrade amylase - and sodium sulphite-treated NDF corrected for ash residue (**aNDFom**) as efficiently as rumen microbes (Mertens and Grant, 2007), which might result in biased predictions of in vivo digestibility and the requirement to continuously re-calibrate correction equations (Beecher et al., 2015). Furthermore, a single fermentation time point is performed, reducing the method's ability to capture the dynamic and heterogeneous nature of aNDFom (Ellis et al., 2005; Huhtanen et al., 2008). Recently, an in vitro method was developed utilizing rumen fluid, a small pore size filter paper, and multiple fermentation time points to generate a comprehensive in vitro description of aNDFom degradation (Raffrenato et al., 2018, 2019). A composite decay mathematical model (Raffrenato et al., 2019) utilizes the in vitro fermentation output and describes aNDFom digestion by estimating fast- and slow-degrading fractions, their respective rates of digestion, and an undigested fraction. Numerous animal studies have described the influence of these fractions and rates on animal variables such as DMI, rumen

pool size, rumination, and milk production (Cotanch et al., 2014; Zontini et al. 2015). A comprehensive description of aNDFom degradation dynamics is therefore critical to understand rumen turnover, metabolisable energy supply, and post ruminal amino acid (**AA**) flows (Vieira et al., 2008; Higgs et al., 2015).

In the CNCPS, the AA supply from dietary protein is based on the feed's solubility in buffers and detergent solutions, the AA composition of the intact feed, fraction specific degradation rates, and intestinal digestibility coefficients (Lanzas et al., 2007; Higgs et al., 2015). Methodology to comprehensively fractionate this dietary protein is routinely incorporated into wet chemistry analysis for indoor feeding systems (Licitra et al., 1996). However, our current understanding of the fractionation of perennial ryegrass (*Lolium perenne* L.; **PRG**) protein and its potential impact on the AA supply to the grazing dairy cow is limited (Bryant et al., 2012).

Traditionally, the AA composition of proteins have been obtained at a single time point, after a 21- or 24-h hydrolysis, which was determined as a compromise between maximal AA release from the protein matrix and acid-labile AA degradation (Rutherfurd, 2009). However, these short hydrolysis time-points are insufficient to release all AA from the matrix and underestimates the true concentrations of some AA (Robel and Crane, 1972; Rutherfurd et al., 2008). This underestimation has been suggested, at least in part, to potentially explain a systematically lower estimate of digestible duodenal AA flow compared with measured net portal absorption in lactating dairy cows (Lapierre et al., 2019). Non-ruminant production systems have incorporated extended hydrolysis times into their AA analysis methods, especially for the determination of branched-chain AA (**BCAA**; NRC Swine, 2012). However, extended hydrolysis times are both extremely laborious and expensive. A potential method to overcome these obstacles might be through the use

of log-logistic mathematical models, to develop correction factors that can be applied to a single hydrolysis time-point analysis (Lapierre et al., 2019).

Such methodologies have not been incorporated into pasture-based systems leading to a lack of comprehensive feed chemistry analysis that precludes adoption of the CNCPS. Therefore, the objective of this study was to characterize the nutritive value of PRG dominated pastures using these new and updated feed chemistry methods.

## 2.3. MATERIALS AND METHODS

The cannulated cows used in this experiment as rumen fluid donors were cared for according to the guidelines of the Cornell University's Institutional Animal Care and Use Committee and were all high producing, lactating dairy cattle fed a diet balanced for 45 kg of metabolisable energy and metabolisable protein allowable milk.

### *2.3.1. Sample Collection*

A total of 55 pasture samples were obtained from three experimental data sets that were carried out at the Teagasc, Animal and Grassland Research and Innovation Center, to perform a number of separate feed chemistry investigations [McClearn et al., 2018 (n = 20); Chapter 3 (n = 24); Chapter 6 (n = 11)]. The pasture samples were collected from predominately PRG dominated pastures, with the exception of McClearn et al. (2018) where 50% of the samples contained on average 180 g/kg DM of white clover (*Trifolium repens* L.). The pasture samples obtained were representative of the forages being consumed by the grazing experimental cows, cut at 4 cm above ground level. Samples were obtained using a Gardena hand shears (Accu 60, Gardena International GmbH, Ulm, Germany) except for Chapter 3 where samples were mechanically cut, at 4 cm above

ground level, with a GrassTech Grazer GT80 (Future Grass Technology, Borris Business Park, Clonegoose, Borris, Co. Carlow, Ireland, R95 E032). Immediately after harvesting, samples were frozen at -20ºC. Subsequently, the samples were first bowl-chopped and then freeze dried (LS40+chamber, MechaTech Systems Ltd., Bristol, U.K.) at -55°C for 120 h. Dried samples were ground through a 1-mm screen using a Cyclotech 1093 Sample Mill (Foss, DK-3400 Hillerød, Denmark) and stored for subsequent nutrient composition analysis. In all experiments, best grazing management practices were performed as described by McCarthy et al. (2013) to help maintain high pasture digestibility. Pre-grazing pasture mass (above 4 cm horizon) was determined by harvesting two strips (1.2 m x 10 m) with an Etesia mower (Etesia UK. Ltd., Warwick, UK.). The cut forage was collected, weighed, and a sub-sample removed to determine DM concentration. One hundred grams of the sampled forage was dried for 16 h at 90°C for DM determination. The 55 pasture samples were categorized into four groups; the first three based on the season of sampling [spring = 01 February to 30 April (n = 8); summer = 01 May to 31 July (n = 31); autumn = 01 August to 30 November (n = 8)]. A number of pasture samples from Chapter 6 were categorized as a separate group ‘drought’ (n = 8). These samples were obtained from pastures in the summer of 2018 that were in high moisture deficit. Drought conditions in Ireland are defined as a period of 15 or more consecutive days to none of which is credited 0.2 mm or more of precipitation (Met Eireann, 2018). These samples represented a chemically distinct population in comparison with their corresponding ‘summer’ samples (Table 2.1).

**Table 2.1.** Pre-grazing yield (measured above 4 cm) and chemical composition (g/kg DM unless otherwise stated) of perennial ryegrass samples.

| | Total | | | | Category mean[a] | | | |
|---|---|---|---|---|---|---|---|---|
| Sample[b] | Mean | SD | Min. | Max. | Spring | Summer | Autumn | Drought |
| Pre-grazing yield, kg DM/ha | 1688 | 355 | 1257 | 2575 | 1704 | 1604 | 1495 | 2190 |
| DM (fresh weight basis) | 203 | 46 | 137 | 366 | 155 | 201 | 176 | 283 |
| CP | 176 | 38 | 107 | 254 | 214 | 177 | 191 | 122 |
| Starch | 15 | 11 | 1 | 64 | 4 | 19 | 11 | 12 |
| aNDFom | 360 | 43 | 255 | 459 | 325 | 354 | 355 | 433 |
| ADF | 220 | 24 | 181 | 268 | 205 | 211 | 236 | 254 |
| ADL, g/kg aNDFom | 53 | 13 | 24 | 84 | 51 | 47 | 61 | 69 |
| Ether extract | 32 | 6 | 20 | 50 | 34 | 32 | 35 | 28 |
| Ash | 87 | 20 | 57 | 134 | 103 | 77 | 105 | 87 |

[a]Spring = 01 February to 30 April (n = 8); Summer = 01 May to 31 July (n = 31); Autumn = 01 August to 30 November (n = 8); Drought = a period of 15 or more consecutive days to none of which is credited 0.2 mm or more of precipitation (n = 8).

[b]aNDFom = amylase- and sodium sulphite-treated NDF corrected for ash residue; ADF = acid detergent fibre; ADL = acid detergent lignin (sa)

### *2.3.2. Commercial Laboratory Analyses*

All samples were submitted to a commercial feed analysis laboratory Cumberland Valley Analytical Services (Waynesboro, PA, USA). The 'Cornell Penn Miner Plus Analysis' was performed using wet chemistry procedures. Analytical procedures can be viewed at https://www.foragelab.com/Lab-Services/Forage-and-Feed/Lab-Procedures/ (accessed Jan. 16, 2020). Samples were additionally analyzed by Cumberland Valley Analytical Services for aNDFom (Mertens, 2002) which is not included in the 'Cornell Penn Miner Plus Analysis'.

### *2.3.3. In vitro aNDFom Digestibility Analysis*

Forty-six samples were analyzed to determine the in vitro aNDFom digestibility of pasture grasses at Cornell University, Ithaca, NY, following the procedures described by Raffrenato et al. (2018). Briefly, 0.5 g DM of sample was placed in 125-mL Erlenmeyer flasks with 40 mL of buffer (Goering and Van Soest, 1970). Before inoculation, the flasks were placed in a water bath at 39ºC under continuous $CO_2$ until the resazurin in the buffer solution turned clear. Subsequently, 10 mL of filtered rumen fluid, from 2 lactating cows fed a total mixed ration at the Cornell University Research Center, was transferred to the flasks. The flasks were capped and maintained at 39ºC under anaerobic conditions. A screening procedure, utilizing 12 samples from McClearn et al. (2018), was carried out to determine which time points were necessary to characterize the digestion curve of the lowly-lignified, three-leaf stage pasture grasses. During the screening evaluations, samples were fermented for 6, 12, 24, 48, 120 and 240 h. After several evaluations, the time points of 30, 120, 240 h were selected, along with a 12-h time point to best describe the aNDFom digestion of all fractions within the forages. As indicated by Raffrenato et al. (2019), to fully represent the digestion the fermentation time points should fall in the linear portion of the decay

curve to better fully represent the pool. Therefore, for the high quality pasture grass, the 12-h value is more representative of the faster digesting pool. At the respective time point, flasks were removed from the water bath and placed on ice to stop fermentation. The flask contents along with an initial unfermented sample were then analyzed for aNDFom (Mertens, 2002) using a 1.5 µm pore size glass microfibre filter (VWR 691, 516-0036; VWR International, Hannover, Germany) as suggested by Raffrenato and Van Amburgh (2011). All fermentations were conducted in duplicate. A large quantity of sample was consumed during the in vitro fermentation analyses greatly depleting the remaining sample quantity. Thus, the samples of McClearn et al. (2018) were not included in any other subsequent analysis.

### *2.3.4. Sugar Determination*

Twenty-eight samples selected from the studies of Chapters 3 and 6 were analyzed for water-soluble carbohydrates (**WSC**) at Cornell University, Ithaca, NY. Chemical analysis was performed using the phenol-sulfuric acid assay (DuBois et al., 1956) with sucrose standards, as described by Hall (2014).

### *2.3.5. Nitrogen Fractionation and Amino Acid Analysis*

Seven samples from the adaption periods of Chapter 3 were utilized to conduct an in-depth N fractionation and multiple-hydrolysis time AA analysis. An additional nine samples were included in the N fractionation investigation from the data collection periods of Chapter 3 that had sufficient sample quantity remaining after all other analyses required for the experiment. Therefore, 16 samples were included in the N-fractionation analysis. The procedures of Licitra et al. (1996) were performed to partition the feed N into the fractions required for input into the CNCPS model.

Neutral detergent insoluble N (**NDIN**) was determined excluding sodium sulphite. Total feed N was determined by combustion analysis (Leco FP-528 N Analyzer, Leco Corp., St. Joseph, MI) and Kjeldahl methods (Tecator Digestor 20 and Kjeltec 2300 Analyzer, Foss Analytical AB, Höganäs, Sweden). The AA concentration was determined using an Agilent 1100 series HPLC (Agilent Technologies, Santa Clara, CA) fitted with a sodium cation exchange column (Cat. no 1154110T, Pickering Laboratories, Mountain View, CA) after hydrolysis in a 110ºC block heater for 21, 72, and 168 h, except for Trp. Tryptophan has previously been shown to decrease in concentration after 16 h of hydrolysis (Ross, 2004) therefore, the time points of 16 and 21 h were selected. For Met and Cys, additional aliquots containing 2 mg N and the internal standard, norleucine, were pre-oxidized with 1 mL performic acid (0.9 mL of 88% formic acid, 0.1 mL of 30% $H_2O_2$ and 5 mg phenol) for 16 h at 4°C prior to acid hydrolysis (Elkin and Griffith, 1984). Multiple-hydrolysis times were utilized to assess the release of each AA from the forage matrix as discussed previously for milk proteins (Rutherfurd et al., 2008) and microbial protein (Fessenden et al., 2017). All AA were analyzed as described by Fessenden et al. (2017). Analyses were performed in duplicate for each sample.

### *2.3.6. Statistical Analysis*

The mean values calculated from replicate analyses of each sample for each wet chemistry procedure were used to perform statistical analyses. Data were analyzed using SAS version 9.4 (SAS Institute Inc. Cary, NC). The MEANS procedure was used in Table 2.1 for descriptive statistics of the overall analyses. In order to test the differences between the means (paired comparisons), the Student's t-distribution was used (TTEST procedure). The means compared were 1) amylase- and sodium sulphite-treated NDF (**aNDF**) versus aNDFom; 2) the effect of

multiple in-vitro time points, with or without the 12-h time point, on aNDFom digestion characteristics; 3) aNDFom fermentation residues at 120 versus 240 h; and 4) soluble fibre concentration (g/kg DM) utilizing WSC versus that calculated with ethanol-soluble carbohydrate (**ESC**). To derive Pearson correlation coefficients between the in vitro aNDFom digestibility time point analysis, the CORR procedure was used. When analyzing the effect of sample category, the SAS MIXED procedure was used. The model included tests for the fixed effect of category and random effect of sample within category. When analyzing the effect of hydrolysis time or determination method, the SAS MIXED procedure was used (Figure 2.4; Table 2.7). The models included tests for the fixed effect of hydrolysis time or determination method and random effect of sample within hydrolysis time or determination method. Means separation was performed using the Tukey–Kramer test. Statistical significance was considered at $P \leq 0.05$ and trends were considered at $0.05 < P \leq 0.10$.

## 2.4. RESULTS

### *2.4.1. Estimation of Insoluble Fibre Concentration and Digestibility*

A comparison of two neutral detergent fibre assays to quantify the plant cell wall concentration of PRG samples, categorized by season or drought condition, is presented in Table 2.2. The aNDFom concentration was lower for all four categories ($P < 0.01$) compared with aNDF concentration. The Fe concentrations were elevated in samples obtained during spring and autumn (1,311 and 933 mg/kg DM, respectively) in comparison with the summer and drought (466 and 312 mg/kg DM, respectively; $P < 0.01$).

**Table 2.2.** A comparison of aNDF[a] versus aNDFom[a] concentration (g/kg DM) of perennial ryegrass categorized by season[b] or drought condition[c].

| | | Method | | | | |
|---|---|---|---|---|---|---|
| Sample | n | aNDF | aNDFom | Difference | SED | P-Value |
| Spring | 8 | 359 | 325 | -34 | 3.4 | <0.01 |
| Summer | 31 | 365 | 354 | -11 | 2.7 | <0.01 |
| Autumn | 8 | 384 | 355 | -29 | 2.1 | <0.01 |
| Drought | 8 | 449 | 433 | -15 | 2.5 | <0.01 |

[a]aNDF = amylase- and sodium sulphite-treated NDF; aNDFom = amylase- and sodium sulphite-treated NDF corrected for ash residue.
[b]Spring = 01 February to 30 April; summer = 01 May to 31 July; autumn = 01 August to 30 November.
[c]A period of 15 or more consecutive days to none of which is credited 0.2 mm or more of precipitation.

For the screening step in determining the fermentation time points to use to provide adequate degradation information, the average in vitro aNDFom residues of 12 PRG samples, equally represented among the seasons of the year, after fermentation for 6, 12, 24, 48, 120 and 240 h, are shown in Figure 2.1. Notably, 714 ± 38 g/kg aNDFom rapidly digested before 24 h of fermentation and the PRG contained a low proportion of undigested aNDFom after 240-h fermentation (**$uNDFom_{240h}$**; 122 ± 28 g/kg aNDFom).

Including a 12-h fermentation time point in the procedure of Raffrenato et al. (2018) reduced the size of the potentially digestible aNDFom fast fraction (**$pdNDFom_1$**; $P < 0.05$) and accordingly increased the size of the potentially digestible aNDFom slow fraction (**$pdNDFom_2$**; $P < 0.05$) compared with not including the 12-h time point (Table 2.3). Furthermore, the overall rate of digestion (**kd**; weighted average of $pdNDFom_1$ and $pdNDFom_2$ digestion rates based on the calculated size of the respective fractions) was higher ($P < 0.01$) when the 12-h time point was included versus not included.

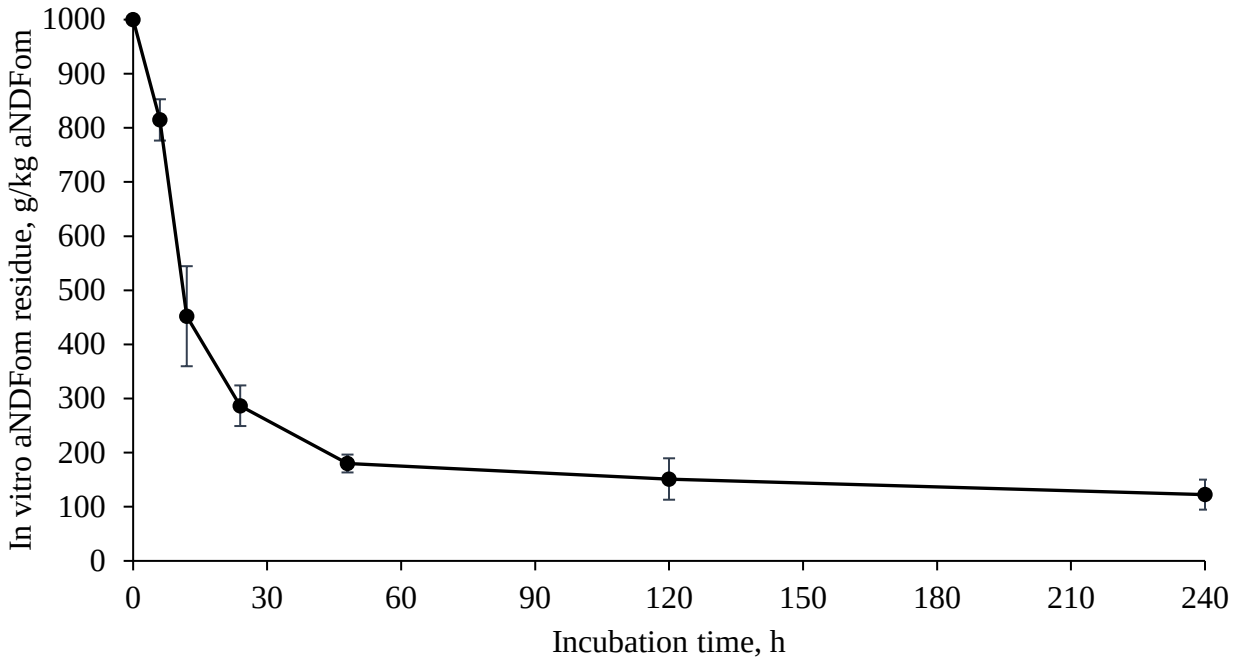


**Figure 2.1.** In vitro aNDFom fermentation residues (g/kg aNDFom) after fermentation for 6, 12, 24, 48, 120 and 240 h. Data are mean ± SD (error bars), averaged among 12 perennial ryegrass samples.

**Table 2.3.** The fraction sizes (g/kg aNDFom) and rates of digestion[a] (kd; $h^{-1}$) obtained from in vitro aNDFom digestibility procedures (Raffrenato et al., 2018, 2019) for perennial ryegrass without and with the inclusion of a 12-h fermentation time point

| | Time points | | | | |
|---|---|---|---|---|---|
| Item | 30-120-240 h | 12-30-120-240 h | Difference | SED | P-Value |
| $pdNDFom_1$ | 672 | 641 | -32 | 12.7 | <0.05 |
| $pdNDFom_2$ | 196 | 227 | 32 | 12.8 | <0.05 |
| $uNDFom_{240h}$ | 132 | 132 | - | - | - |
| $k_1$ | 0.154 | 0.184 | 0.031 | 0.022 | 0.18 |
| $k_2$ | 0.021 | 0.022 | 0.001 | 0.001 | 0.18 |
| kd | 0.068 | 0.074 | 0.006 | 0.001 | <0.01 |

[a]$pdNDFom_1$ = potentially digestible aNDFom fast fraction, g/kg aNDFom; $pdNDFom_2$ = potentially digestible aNDFom slow fraction, g/kg aNDFom; $uNDFom_{240h}$ = undigested aNDFom after 240 h of in vitro fermentation, g/kg aNDFom; $k_1$ = digestion rate of the fast fraction, $h^{-1}$; $k_2$ = digestion rate of the slow fraction, $h^{-1}$; kd = digestion rate of pdNDFom, $h^{-1}$; n = 46.

The effect of season and drought condition on the fraction sizes and rates of digestion of PRG are shown in Table 2.4. The $uNDFom_{240h}$ (g/kg aNDFom) was affected by PRG category ($P < 0.01$), whereby spring and summer were lowest, autumn intermediate, and drought highest (Table 2.4). In comparison with spring, the drought samples had a lower $pdNDFom_1$ fraction. The kd was fastest for spring, intermediate for summer and slowest for drought samples (Table 2.4). Additionally, the kd of autumn samples tended to be slower than that of spring ($P = 0.06$), similar to summer, and faster than drought samples ($P < 0.01$).

**Table 2.4.** The fraction sizes (g/kg aNDFom) and rates of digestion[a] (kd; $h^{-1}$) obtained from in vitro aNDFom digestibility procedures (Raffrenato et al., 2018, 2019) with the added 12-h time point for perennial ryegrass categorized by season[b] or drought condition[c]

| | Category | | | | | |
|---|---|---|---|---|---|---|
| Item[d] | Spring | Summer | Autumn | Drought | SEM | P-Value |
| $pdNDFom_1$ | $717^{x}$ | $653^{xy}$ | $695^{xy}$ | $477^{y}$ | 56.5 | <0.05 |
| $pdNDFom_2$ | 185 | 236 | 150 | 323 | 54.2 | 0.21 |
| $uNDFom_{240h}$ | $98^{x}$ | $111^{x}$ | $155^{y}$ | $200^{z}$ | 9.3 | <0.01 |
| $k_1$ | 0.206 | 0.172 | 0.153 | 0.228 | 0.029 | 0.32 |
| $k_2$ | $0.028^{x}$ | $0.024^{xz}$ | $0.014^{y}$ | $0.016^{yz}$ | 0.002 | <0.01 |
| kd | $0.098^{x}$ | $0.073^{y}$ | $0.077^{xy}$ | $0.050^{z}$ | 0.005 | <0.01 |

[a]$pdNDFom_1$ = potentially digestible aNDFom fast fraction, g/kg aNDFom; $pdNDFom_2$ = potentially digestible aNDFom slow fraction, g/kg aNDFom; $uNDFom_{240h}$ = undigested aNDFom after 240 h of in vitro fermentation, g/kg aNDFom; $k_1$ = digestion rate of the fast fraction, $h^{-1}$; $k_2$ = digestion rate of the slow fraction, $h^{-1}$; kd = digestion rate of pdNDFom, $h^{-1}$.

[b]Spring = 01 February to 30 April; summer = 01 May to 31 July; autumn = 01 August to 30 November.

[c]A period of 15 or more consecutive days to none of which is credited 0.2 mm or more of precipitation.

[d]n = 8, 22, 8, and 8 for spring, summer, autumn, and drought, respectively.

[x-z]Means within row with different superscripts are significantly different ($P < 0.05$).

Analysis of undigested aNDFom (g/kg aNDFom) at 120 and 240 h of in vitro fermentation are shown in Table 2.5. A lower determination of uNDFom was observed at 240 h in comparison with 120 h for summer, autumn, and drought samples ($P < 0.01$); however, no difference was observed for spring ($P = 0.12$). Pearson correlation coefficients between the 12-h, 30-h, and 120-h time points with the 240-h time point were 0.70, 0.74, and 0.98, respectively. When $uNDFom_{240h}$ (g/kg aNDFom), was regressed on acid detergent lignin (sa; g/kg of aNDFom) an overall slope of 2.92 was observed with a coefficient of determination ($R^2$) of 0.68 and accuracy (RMSE) of 26.5 g/kg aNDFom (Figure 2.2).

**Table 2.5.** Comparison of undigested aNDFom[a] (g/kg aNDFom) determined after 120 or 240 h of in vitro fermentation for perennial ryegrass categorized by season[b] or drought[c] condition.

| | | Method | | | | |
|---|---|---|---|---|---|---|
| Sample | n | 120 h | 240 h | Difference | SED | P-Value |
| Spring | 8 | 109 | 100 | -10 | 5.5 | 0.12 |
| Summer | 22 | 129 | 111 | -18 | 1.3 | <0.01 |
| Autumn | 8 | 186 | 155 | -31 | 5.6 | <0.01 |
| Drought | 8 | 231 | 200 | -31 | 1.9 | <0.01 |

[a]aNDFom = amylase- and sodium sulphite-treated NDF corrected for ash residue.
[b]Spring = 01 February to 30 April; summer = 01 May to 31 July; autumn = 01 August to 30 November.
[c]A period of 15 or more consecutive days to none of which is credited 0.2 mm or more of precipitation.

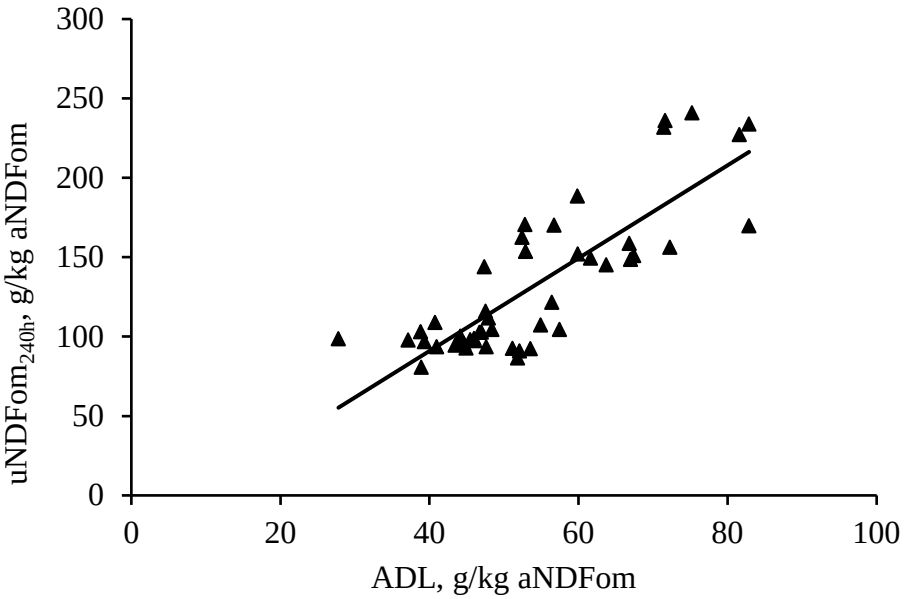


**Figure 2.2.** The undigested aNDFom ($uNDFom_{240h}$), on an aNDFom basis, regressed on acid detergent lignin (ADL; sa), on an aNDFom basis ($y = 2.9234x – 25.991$; $R^2 = 0.68$; RMSE = 26.5 g/kg aNDFom; n = 46) of perennial ryegrass.

### *2.4.2. Nitrogen Fractionation and its Relationship with Soluble Carbohydrates*

The PRG N was fractionated using wet chemistry procedures and data are presented in Table 2.6. The Kjeldahl N concentrations were slightly lower than that of the combustion analysis ($y = 0.9553x + 0.0004$; $R^2 = 0.99$; RMSE = 0.0005; n = 16). In the PRG samples, 783 g/kg total N was determined as true protein (Table 2.6) or conversely, 217 g/kg total N was determined as non-protein N (**NPN**), when utilizing tungstic acid as a precipitating agent. Perennial ryegrass insoluble N in borate phosphate buffer was 680 g/kg total N (Table 2.6). Lower proportions of the total PRG N were associated with the neutral detergent insoluble (139 g/kg total N) and acid detergent insoluble fractions (26 g/kg total N). The results of the procedures were also utilized to calculate the most recent CNCPS N fractionation scheme and are presented in Table 2.6. A more pronounced relationship was observed among WSC and N concentrations compared with ESC and N concentrations in the PRG samples (Figure 2.3). Calculated soluble fibre concentration was lower when utilizing the WSC assay in comparison with utilizing the ESC assay (124 g/kg vs. 216 g/kg DM, respectively; $P < 0.01$; n = 28).

**Table 2.6.** The nitrogen fractionation of perennial ryegrass (mean ± SD) and the CNCPS N fractionation scheme as described by Higgs et al. (2015) for perennial ryegrass.

| | PRG[b] | |
|---|---|---|
| Item[a] | N, g/kg DM | N , g/kg total N |
| Leco N | 26.7± 4.5 | - |
| Kjeldahl N | 25.7 ± 4.3 | 969 ± 18 |
| Tungstic acid perceptible N residue | 20.9 ± 4.0 | 783 ± 45 |
| Buffer insoluble N residue | 18.2 ± 3.9 | 680 ± 49 |
| NDIN | 3.7 ± 0.8 | 139 ± 20 |
| ADIN | 0.7 ± 0.1 | 26 ± 5 |
| CNCPS N fractionation[c] | | |
| PA1 | - | ND[d] |
| PA2 | - | 320 ± 49 |
| PB1 | - | 541 ± 38 |
| PB2 | - | 113 ± 19 |
| PC | - | 26 ± 5 |

[a]N = nitrogen; NDIN = neutral detergent insoluble nitrogen; ADIN = acid detergent insoluble nitrogen.
[b]PRG = perennial ryegrass (*Lolium perenne* L.); n = 16.
[c]PA1 = Ammonia; PA2 = Soluble true protein; PB1 = Insoluble true protein; PB2 = Fibre-bound protein; PC = Indigestible protein (Higgs et al., 2015).
[d]ND = not determined.

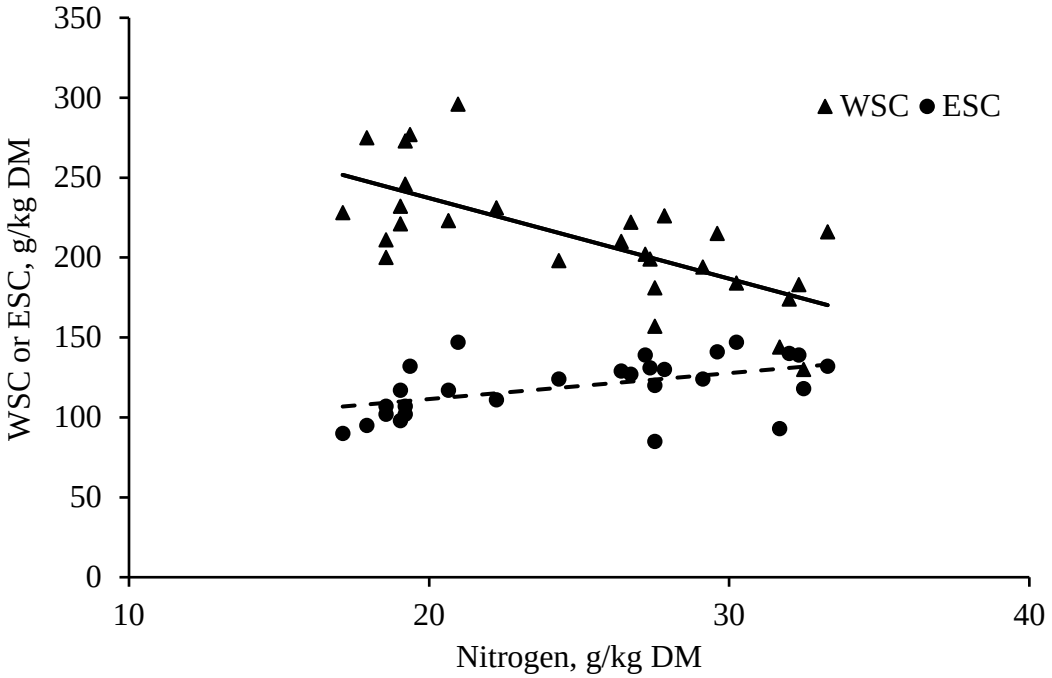


**Figure 2.3.** Water-soluble carbohydrate concentration (g/kg DM; ▲; y = -5.04x + 338.04; $R^2$ = 0.49; RMSE = 28.5 g/kg DM; n = 28) or ethanol-soluble carbohydrate concentration (g/kg DM; ●; y = 1.62x + 78.99; $R^2$ = 0.24; RMSE = 16.1 g/kg DM; n = 28) regressed on nitrogen concentration (g/kg DM) of perennial ryegrass.

### *2.4.3. Amino Acid Determination*

The release of the BCAA (Ile, Leu, and Val) and Ser from the protein matrix over a time-course hydrolysis, averaged among 7 PRG samples, are shown in Figure 2.4. Both Ile and Val increased in concentration after a 168-h hydrolysis compared with a 21-h hydrolysis. The concentration of Ser decreased after a 168-h hydrolysis compared with a 21-h hydrolysis. Leucine was not affected by hydrolysis time (Figure 2.4). The concentrations of Arg, His, Lys, Met, Phe, Thr, Trp, Ala, Asp, Cys, Glu, Gly, Pro, and Tyr were not affected by hydrolysis time (data not shown). The effect of AA determination method is presented in Table 2.7. The concentrations of Ile and Val increased when the AA concentrations were determined using the maximum concentrations recovered after multiple hydrolysis times compared with using a single 21-h hydrolysis time or applying a hydrolysis-time correction factor to the single 21-h hydrolysis time. The concentrations of the remaining AA were not significantly affected by time.

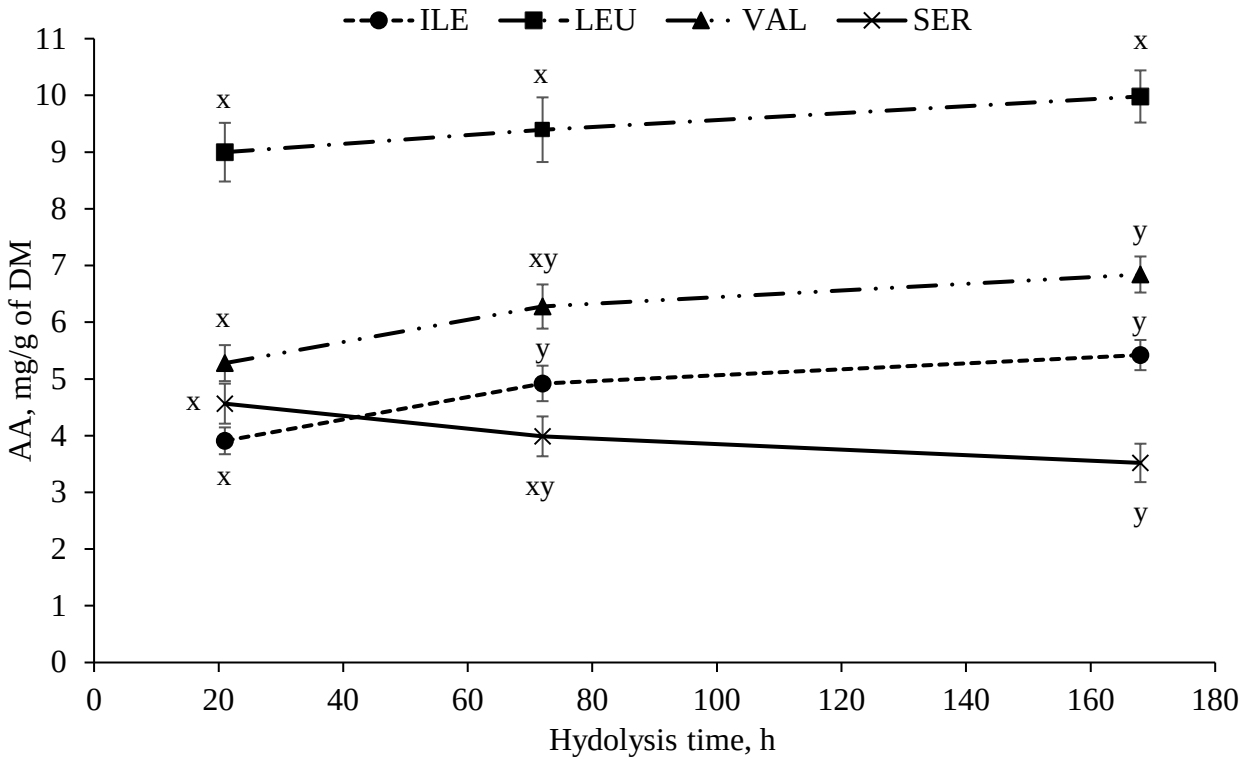


**Figure 2.4.** Effect of hydrolysis time (h) on concentration of the branched-chain amino acids (Ile, Leu, and Val) and Ser (mg AA/g of DM). Data are mean ± SE (error bars), averaged among 7 perennial ryegrass samples.

**Table 2.7.** Comparison of the AA concentration (mg/g DM) of 7 perennial ryegrass samples using a single 21-h hydrolysis time, corrected for recovery based on long hydrolysis time (Lapierre et al. 2019) or the maximum recovery from multiple-hydrolysis times to ensure adequate release and recovery of some of the more acid insoluble amino acids.

| | Method[a] | | | | |
|---|---|---|---|---|---|
| Amino Acid, mg/g DM | 21-h | Cor. Factor | Max | SEM | P-Value |
| EAA | | | | | |
| Arg | 6.7 | 7.0 | 6.8 | 0.39 | 0.88 |
| His | 2.3 | 2.5 | 2.5 | 0.13 | 0.53 |
| Ile | 3.9[x] | 4.3[x] | 5.4[y] | 0.25 | <0.01 |
| Leu | 9.0 | 9.2 | 10.0 | 0.50 | 0.36 |
| Lys | 6.6 | 6.8 | 7.1 | 0.35 | 0.60 |
| Met | 2.6 | 2.6 | 2.8 | 0.14 | 0.59 |
| Phe | 6.8 | 6.9 | 7.5 | 0.35 | 0.32 |
| Thr | 4.8 | 5.1 | 4.9 | 0.29 | 0.81 |
| Trp[b] | 3.5 | 3.7 | 3.5 | 0.22 | 0.78 |
| Val | 5.3[x] | 5.4[x] | 6.8[y] | 0.45 | <0.01 |
| NEAA | | | | | |
| Ala | 7.3 | 7.5 | 7.8 | 0.39 | 0.64 |
| Asp | 10.6 | 10.9 | 11.2 | 1.14 | 0.88 |
| Cys | 1.1 | 1.2 | 1.1 | 0.05 | 0.30 |
| Glu | 12.2 | 12.8 | 12.9 | 0.79 | 0.78 |
| Gly | 5.5 | 6.0 | 6.1 | 0.31 | 0.42 |
| Pro | 3.1 | 3.2 | 3.3 | 0.18 | 0.73 |
| Ser | 4.6 | 5.0 | 4.6 | 0.27 | 0.46 |
| Tyr | 6.1 | 6.4 | 6.1 | 0.31 | 0.73 |

[a]AA concentration (mg/g of DM) determined using a single hydrolysis time (21 h), calculated by applying hydrolysis time correction factors reported by Lapierre et al. (2019) to the AA concentration determined after a 21-h hydrolysis time (Cor. Factor), and the maximum concentration observed after multiple-hydrolysis times (Max).
[b]Tryptophan was determined after 16 h of hydrolysis: see text for detail.
[x-y]Means within row with different superscripts are significantly different ($P < 0.05$).

Finally, the maximum AA concentrations recovered after multiple-hydrolysis times were expressed on a g/kg total N and g/kg AAN basis. On average, the EAA represented 325 g/kg total N, the NEAA represented 239 g/kg total N, and therefore combined, all AA represented 564 g/kg total N. The amount of $NH_3$-N recovered was 105 g/kg total N (Table 2.8). When expressed on a g/kg AAN basis the EAA represented 576 g/kg AAN whereas NEAA represented 424 g/kg AAN.

**Table 2.8.** Maximum AA-N concentrations of 7 perennial ryegrass samples expressed as g/kg total N and g/kg AAN.

| Item | Max[a] N | SE | N, g/kg AAN |
|---|---|---|---|
| Total N, g/kg DM | 27 | 1.3 | |
| EAA-N, g/kg TN | | | |
| Arg | 83 | 1.4 | 146 |
| His | 26 | 0.3 | 46 |
| Ile | 22 | 0.3 | 38 |
| Leu | 40 | 0.5 | 71 |
| Lys | 51 | 0.8 | 90 |
| Met | 10 | 0.2 | 17 |
| Phe | 24 | 0.4 | 42 |
| Thr | 21 | 0.4 | 38 |
| Trp | 18 | 0.6 | 32 |
| Val | 31 | 0.4 | 54 |
| Total EAA | 325 | 4.8 | 576 |
| NEAA-N, g/kg TN | | | |
| Ala | 46 | 0.6 | 82 |
| Asp | 44 | 1.4 | 78 |
| Cys | 5 | 0.1 | 9 |
| Glu | 46 | 0.9 | 82 |
| Gly | 42 | 0.5 | 75 |
| Pro | 15 | 0.3 | 27 |
| Ser | 23 | 0.4 | 40 |
| Tyr | 18 | 0.2 | 31 |
| Total NEAA | 239 | 2.3 | 424 |
| TAA-N, g/kg TN | 564 | 6.1 | |
| NH3-N, g/kg TN | 105 | 2.5 | |

[a]AAN concentration (g/kg total N) determined using the maximum concentration observed after multiple-hydrolysis times.

## 2.5. DISCUSSION

### *2.5.1. Characterization of Insoluble Fibre Concentration and Digestibility*

In the current study, aNDF consistently estimated a greater insoluble fibre concentration compared with aNDFom (Table 2.2). A more pronounced difference between the two approaches occurred during the spring and autumn periods when soil conditions are typically more inclement. Higher Fe concentration is indicative of soil contamination and was elevated in samples obtained during spring and autumn in comparison with summer and drought, suggesting that soil contamination caused aNDF to overestimate the insoluble fibre concentration of the PRG. The majority of ash in soil is insoluble in neutral detergent solution, therefore, forage samples contaminated with soil can have 20 g/kg to 120 g/kg aNDF as ash (Mertens, 2015). This overestimation of the insoluble fibre fraction results in difficult to interpret data and develops uncertainty in the inference of its effect on variables such as DMI, digestibility, passage rate and microbial yield. This can result in biased estimations of the other chemical fractions and potential energy supply of a feed. Hence, aNDFom should be the routine assay for estimating insoluble fibre concentration.

The mathematical description of forage aNDFom digestion is more accurate when fractionated into two digestible and one indigestible fraction (Ellis et al., 2005; Huhtanen et al., 2008). The time points necessary to explain most of the digestion variation in typical northeastern United States temperate forages, are 30, 120, and 240 h (Raffrenato et al., 2019). However, Raffrenato et al. (2019) suggested that additional, earlier time points might be required to capture the rapid early digestion if the forage being analyzed exhibits rates of digestion as observed for PRG (Figure 2.1). In the current study, when time points earlier than 30 h (6 h and 12 h) were excluded from the initial screening of fermentation residues, and the results expressed on the semi-log scale (data not

shown), the linear portion of the aNDFom decay curve was not represented. Thus, the missing information resulted in a biased and skewed representation of the digestion data. Therefore, for all subsequent analysis, a 12-h time point was included to describe the early portion of the aNDFom degradation curve, in combination with 30, 120, and 240-h time points. Including the 12-h time point altered the estimated size of pdNDFom fractions and increased the overall rate of aNDFom digestion (Table 2.3). This demonstrates that the 12-h time point captured the linear portion of the disappearance of the fast pool in the aNDFom degradation curve of PRG. Utilizing the rates of degradation generated with and without a 12-h time point, and an assumed passage rate of 0.016 $h^{-1}$, ruminal digestibility of pdNDFom is predicted to increase when including the 12-h time point compared to without (0.82 vs 0.80 pdNDFom digestibility, respectively). The higher prediction of ruminal pdNDFom digestibility is in agreement with observations from an omasal flow study investigating pasture-fed dairy cows (Sairanen et al., 2005). Furthermore, the aNDFom characteristics of the spring samples in the current study resembled that of the pasture fed by Sairanen et al. (2005), as similar uNDFom concentrations were reported (98 and 96 g/kg aNDFom, respectively). Notably, there was close agreement between the aNDFom degradation rate predicted by the in vitro methodology for the spring samples in the current study and the measured in vivo rate of cows consuming the grass-only diet of Sairanen et al. (2005; 0.098 vs. 0.092 $h^{-1}$, respectively).

In comparison with the forages investigated by Raffrenato et al. (2018), which included brown mid-rib corn silages, the spring and summer samples in the current experiment had a much higher digestion rate of the $pdNDFom_1$ and lower concentrations of $uNDFom_{240h}$, further demonstrating the rapid digestion characteristics exhibited by PRG. In the current study, autumn pasture had higher $uNDFom_{240h}$ concentration than either spring or summer herbage (Table 2.4), which is in

agreement with previously reported organic matter digestibility (**OMD**; O'Donovan and Kennedy, 2007; McEvoy et al., 2010), estimated by an in vitro enzymatic neutral detergent cellulase method (Morgan et al., 1989). As described by Van Soest (1994), neutral detergent divides forage DM into soluble matter that is 98% digestible and an insoluble residue. Therefore, OMD is essentially a function of the digestibility of NDF (Rinne et al., 2006). In a recent update and evaluation of the neutral detergent cellulase method, the $R^2$ between the method and in vivo OMD was satisfactory (0.745; Garry et al., 2018). In a similar evaluation to predict in vivo OMD, Nousiainen (2004) reported much higher $R^2$ at the forage specific level (range 0.885 – 0.996) highlighting potential limitations of the neutral detergent cellulase method (Mertens and Grant, 2007). In the evaluation of Nousiainen (2004), iNDF concentration measured after 12 d of in situ fermentation (Huhtanen et al., 2006b), was regressed against in vivo OMD. Garry (2016) also found a strong relationship between in situ iNDF and in vivo OMD for 27 PRG samples ($R^2 = 0.921$). However, the in situ procedure is quite laborious involving multiple handling of cannulated cows. The in vitro procedure conducted in this study (Raffrenato et al., 2018) is capable of being conducted in a commercial laboratory setting and is repeatable and adaptable for routine feed evaluation. Raffrenato et al. (2018) also showed close agreement between the in vitro digestibility method and that of the in situ procedure for determination of iNDF concentrations ($R^2 = 0.89$). This emphasizes the potential of the $uNDFom_{240h}$ assay to accurately predict in vivo variables in PRG-based systems. Furthermore, in a recent investigation with sheep, the in vivo aNDF digestibility of PRG was a better predictor of DMI than in vivo OMD (Beecher et al., 2018) emphasizing the utility and enhanced understanding of mechanisms governing intake of PRG, which can be achieved, from an assay capable of describing the non-uniform aNDFom fraction.

Although the methodology of Raffrenato et al. (2018) is less intensive than the in situ procedures, long-term in vitro fermentation can be expensive and laborious. Therefore, we evaluated if the extent of fermentation in PRG samples could be reached by 120 h, as observed in non-forage fibre sources (Zontini, 2016). A lower determination of uNDFom was observed at 240 h in comparison with 120 h for summer, autumn and drought samples indicating that in order to reach the extent of digestion for PRG, 240 h of fermentation is required. However, a high Pearson correlation coefficient between the 120-h and 240-h time points (0.98) was observed, suggesting the potential to predict the uNDFom at 240 h from the 120-h time point for this data set. Additional evaluation with a larger data set is required to confirm this relationship and construct a robust equation to predict the 240-h outcome.

Several other attempts have been made to predict the extent of aNDFom digestion from the lignin (sa) concentration of the plant on either a DM or aNDF basis (Chandler et al., 1980; Traxler et al., 1998). In the past, the CNCPS used a factor of 2.4 × lignin (sa; g/kg aNDFom) to predict uNDFom of forages (Van Soest et al., 2005). In the current study, when $uNDFom_{240h}$ was regressed on lignin (sa) the overall slope was 2.92 with unsatisfactory accuracy ($R^2$= 0.68; RMSE = 27 g/kg aNDFom; Figure 2.2). This is in agreement with previous findings (Krämer et al., 2012; Raffrenato et al., 2018) and further supports that while lignin (sa) is involved in the extent of cell wall degradation, its relationship with uNDFom is highly variable and therefore is not robust at determining uNDFom or consequently pdNDFom.

As the additional 12-h time point, recommended by the current study for PRG, is already being performed by commercial laboratories to describe non-forage fibre samples (12, 72, 120 h; Zontini, 2016), its implementation to describe PRG should be convenient. Further, near infrared reflectance spectroscopy has been shown to successfully predict iNDF concentration (Nousiainen et al., 2004;

Krizsan et al., 2014). It is essential that the near infrared reflectance spectroscopy be calibrated properly with accurate wet-chemistry analysis as biological measurements such as in vitro- or in situ- aNDFom digestibility can be difficult to predict if the error in reference methods is high relative to the range of data (Mentink et al., 2006). Altogether, this 3-fraction scheme of dividing aNDFom into fast, slow and undigested fractions, with respective rates, should provide improved knowledge about the heterogeneity and digestibility of the aNDFom that might influence DMI, passage rates and microbial yield of dairy cows grazing PRG. By combining this type of precise chemistry with mathematical modelling an enhanced understanding of the nutritional value of PRG can be achieved. Further work is required, however, to confirm the relationships between the estimated in vitro aNDFom digestibility described here and the in vivo aNDFom digestibility of cows consuming PRG.

### *2.5.2. Nitrogen Concentration, Fractionation, and its Relationship with Soluble Carbohydrates in PRG*

The two main procedures to determine total N concentration are combustion analysis or Kjeldahl procedures. For the samples compared in the current study, the Kjeldahl procedure determined N concentrations slightly lower than that of the combustion analysis (Table 2.6) which is in agreement with the findings of Etheridge et al. (1998). McGeehan (1988) reported that in material containing nitrates, N concentration is lower using the Kjeldahl procedure compared with combustion analysis. A number of other studies were in agreement with this (Buckee, 1994; Simonne et al., 1995); however, Etheridge et al. (1998) suggested that the difference in N content was related to the higher temperature (1300ºC) used in the combustion analyses rather than the presence of nitrates. Regardless of the cause, the current study indicates that care must taken when

comparing total N concentration measured by combustion analysis versus Kjeldahl procedures for PRG samples.

Tungstic acid was used as a precipitating agent to define peptides of three AA or more as true protein (Pichard and Van Soest, 1977). Based on this classification, 783 g/kg total N was true protein (Table 2.6) or conversely, 217 g/kg total N was NPN, for the samples investigated in this study. Variables such as N fertilization (Reid and Strachan, 1974), age of regrowth (Peyraud and Astigarraga, 1998), and climatic conditions (Bowerman and Goodman, 1971) have been shown to affect the proportion of NPN in the plant. Large NPN fractions such as those described in the current experiment have previously been reported (Hoekstra et al., 2008; Bryant et al., 2012). In previous versions of the CNCPS, this NPN would have been considered as the PA1 fraction (Tylutki et al., 2008). However, as described by Higgs et al. (2015) the updated PA1 fraction now comprises solely of ammonia-N. This was suggested by Van Amburgh et al. (2010) as small peptides and free AA present in the NPN fraction have been reported to escape rumen degradation and contribute to metabolisable AA supply (Choi et al., 2002; Reynal et al., 2007). The NPN fraction of fresh PRG contains very small quantities of ammonia-N and thus, the present fractionation scheme combines the majority of the NPN with the soluble true protein in the PA2 fraction (Table 2.6). However, non-AAN compounds such as nitrate-N have been shown to contribute substantially to the NPN fraction of fresh PRG (Reid and Strachan, 1974). Further work is required to evaluate the updated PA1 fraction in the CNCPS for fresh PRG and consequently the impact on predicted metabolisable AA supply.

Total PRG N was further fractionated into the PB1 fraction, which is calculated by difference, as the rumen buffer insoluble true protein minus NDIN. This fraction has been typically regarded as having an intermediate degradation rate (Loaiza et al., 2017). The current study found over half

of the feed N to reside in the PB1 fraction (541 g/kg total N) which is in agreement with previous findings (Hoekstra et al., 2008; Loaiza et al., 2017). In a model sensitivity evaluation, the feed N escaping ruminal digestion was most sensitive to the rate of degradation of the PB1 fraction (Higgs et al., 2015). In vitro procedures, to quantify the PB1 rate of degradation, are extremely difficult to perform due to difficulty in accounting for microbial contamination and non-uniform behavior of the feed fraction. The current datasets, utilized by the CNCPS to describe PB1 degradation rates contain very few PRG samples (NRC, 2001; Lanzas et al., 2007). Therefore, a feasible laboratory assay is urgently required to more accurately describe the impact of ruminal degradation rates of PRG N on metabolisable AA flow in vivo (Higgs et al., 2015).

The remaining protein fractions include that which is insoluble in neutral-detergent solution (**NDIN**) but soluble in acid-detergent solution (PB2), and that which is insoluble in acid-detergent solution (**ADIN**; PC). The fraction of N insoluble in neutral detergent is typically cited as being slowly degradable and therefore contributes substantially to rumen escapable N. However, as discussed earlier, a large proportion of the material insoluble in neutral detergent is rapidly degraded in the rumen of cows consuming PRG due to the high digestibility and low-crosslinking of the carbohydrate fraction. Using the data generated in the current study, 140 g/kg total N was NDIN, which had approximately a 0.72 rumen digestibility, resulting in only 39 g/kg total N escaping the rumen as NDIN. The contribution of this N, which is bound to the cell wall fraction, to the overall metabolisable N supply is probably minimal. Additionally, Chaudhry and Webster (1993), utilizing rodent models, reported that the N insoluble in ADIN seems to be essentially indigestible. This demonstrates a critical contribution from microbial N as the main source of N to PRG fed ruminants as rumen escape of feed N is likely to be low (Beever et al., 1986). Useful methodology to describe protein quality should accurately quantify the degree to which a protein

contributes to microbial N and feed N that escapes rumen degradation (Broderick, 1994). The methodology outlined in this study provides the capability to fractionate PRG N to provide more robust information on the nutritional characteristics of the forage. Indeed, combined with in vitro assays capable of predicting fraction specific ruminal degradation rates, an enhanced understanding of the amount and source of AA passing from the reticulorumen of PRG-fed ruminants can be achieved.

A strong negative correlation between WSC and N concentration of grazed pastures has previously been reported (McGrath, 1992; Loaiza et al., 2017). Reid and Strachan (1974) showed that for every 10 g/kg DM decrease in sward CP, sward WSC concentration increased by 10 g/kg DM. The increased WSC concentration has been attributed to reduced incorporation of carbon chains into protein backbones, reduced energy demand for protein synthesis and reduced energy demand for nitrate reduction (Peyraud and Astigarraga, 1998). In the present study, a similar negative correlation was observed between WSC and N concentration of the PRG samples (Figure 2.3); however, the relationship was not as severe (for every 8 g/kg DM decrease in sward CP, sward WSC concentration increased by 10 g/kg DM) as that reported by Reid and Strachan, (1974). This relationship between WSC and N concentration of swards has been suggested as a useful indicator to improve N use efficiency under grazing conditions (da Silva et al., 2013, 2014). When ESC concentration was regressed on N concentration no relationship was obtained (Figure 2.3). The WSC assay seems to describe a more uniform fraction, based on microbial action, as the fructans and lower molecular weight carbohydrates are more wholly extracted in the WSC assay in comparison with the ESC assay (Hall, 2014). This could also explain the loss of a relationship between ESC and N as there is a larger fluctuation in fructosan concentration, in comparison with the more constant simple carbohydrates with altering levels of N concentration/fertilization

(Nowakowski, 1962). Furthermore, within the CNCPS the choice of soluble carbohydrate assay impacts the calculation of soluble fibre (i.e. 100 – (CP + Organic Acids + WSC/ESC + Starch + aNDFom + Ash + Ether Extract). In the present study, soluble fibre concentration was considerably lower when utilizing the WSC assay in comparison with utilizing the ESC assay. As fermentation of soluble fibre by rumen microbes cannot yield lactate, it again seems more appropriate to utilize the WSC assay to quantify the soluble fibre fraction. In the literature pertaining to the nutritive value of pasture-based forages, there is inconsistent reporting of the soluble carbohydrates fraction (i.e. WSC, ESC, or no measure). For the reasons outlined above, the inclusion of WSC assay over ESC to estimate the nutritive value characteristics of PRG samples is more appropriate, especially with application for the CNCPS.

### *2.5.3. Amino Acid Determination*

Multiple-hydrolysis time AA analysis, that can simultaneously quantify the release of an AA from the protein and the degradation of said AA, is required to obtain the true AA composition of a protein (Robel and Crane, 1972; Rutherfurd et al., 2008). Branched-chain AA in particular have been reported to contain peptide bonds that are difficult to cleave (Blackburn, 1968). In the current study, a longer time was required to fully release Ile and Val, but not Leu, from the protein matrix (Figure 2.4) which is in agreement with the recommendations of the Swine NRC (2012). Furthermore, the concentration of Ser decreased after a 168-h hydrolysis when compared with the concentration after a 21-h hydrolysis (Figure 2.4). Previous findings have reported decreased concentration of Ser with extended hydrolysis times as Ser is an acid-labile AA that can be partially destroyed after its release from the protein matrix (Rutherford, 2009). In the current study, all other measured AA were statistically unaffected by hydrolysis time.

Extended hydrolysis times are both extremely laborious and expensive. Therefore, the correction factors proposed by Lapierre et al. (2019) were compared with the AA concentrations determined using a single 21-h hydrolysis time and the maximum AA concentrations recovered after multiple-hydrolysis times (Table 2.7). Overall, the majority of AA concentrations measured were not affected by determination method. However, the AA concentrations of Ile and Val were significantly lower when calculated using the correction factors compared with the maximum AA concentrations recovered after multiple-hydrolysis times. This suggests that further refinement of the correction factors to more accurately predict the release rate of Ile and Val from the protein matrix of PRG is required. Future investigations into the effect of hydrolysis time on the true AA concentration of PRG should incorporate more frequent and earlier hydrolysis time points combined with least-squares non-linear regression techniques as described by (Robel and Crane, 1972).

The maximum AA concentrations recovered after multiple-hydrolysis times (mg/g DM) were recalculated, accounting for the differing N concentration of each AA, and expressed relative to total feed N (AAN, g/kg total N; Table 2.8). This provided the ability to assess how much each AA contributed to the total N. As groups, the EAA represented 325 g/kg, the NEAA represented 239 g/kg and therefore combined all AA represented 564 g/kg total N. This seems low considering that 783 g/kg total N was determined to be true protein (> 3 AA), utilizing the tungstic acid assay as discussed earlier. There was a relatively high amount of $NH_3$-N recovered (105 g/kg total N; Table 2.8) which can indicate loss of certain AA during acid hydrolysis (Reid and Strachan, 1974). In an experiment to further understand the NPN fraction, Ferguson and Terry (1954) reported that after hydrolysis, amino-N contributed 640 g/kg and 520 g/kg total N in two grasses. Their findings also highlighted the many contributors to NPN in fresh forages such as amino-, amide-, ammonia-

, nitrate-, purine-, betaine-, and choline-N. Wilson and Tilley (1965) reported a mean AAN recovery of 660 g/kg total N for whole fresh lucerne herbage. When the same forage was processed to remove the 'water-soluble non-protein' creating a 'purified protein' fraction, AAN recovery increased to 840 g/kg total protein N. In the study of Wilson and Tilley (1965), aminobutyric acid was identified as an additional contributor to the NPN fraction along with humin-N which was removed from the hydrolysates by filtration and quantified. Reid and Strachan (1974) also reported low recoveries of AAN, with an average of 600 g/kg total nitrate-free N, for fresh PRG. If the recoveries of Reid and Strachan (1974) were corrected to a total N basis, average recovery would reduce further. Clearly, the proportion of AAN is extremely dynamic in fresh PRG and thus, AAN recovery as a percent of total N will vary. However, a consistent theme among all studies was that if the AAN was recalculated on a common basis (g/kg AAN recovered), no large differences in AA composition were noted (Reid and Strachan, 1974). Finally, the results for the current study expressed as AAN, g/kg AAN recovered, are shown in Table 2.8. There is general agreement with previous studies (Wilson and Tilley, 1965; Reid and Strachan, 1974); however, further comparison with more recent literature is limited as there is a lack of this type of data for PRG. If an accurate assessment of the non-AAN can be determined, the AA profile of the remaining N should be consistent from which intake of AAN can be predicted for feed formulation purposes.

## 2.6. CONCLUSIONS

This study evaluated the nutritive value of PRG using new and updated feed chemistry methods. The data generated demonstrated that immature PRG comprises of a large potentially digestible pool with the majority of the plant fibre material degrading prior to 30 h of fermentation. This resulted in rapid rates of aNDFom degradation when calculated utilizing composite decay models.

In addition, low uNDFom$_{240h}$ concentrations, particularly for spring and summer PRG, were observed in comparison with other forage ingredients previously evaluated using the same methodology. Our results suggest that the new in vitro digestibility methodology, combined with a 12-h time point, can be utilized to characterize the aNDFom degradation of PRG. Based on the N-fractionation scheme employed by the CNCPS, a large proportion of the feed N in PRG is soluble with the remainder highly degradable in the rumen thus, contributing poorly to metabolisable AA supply. An in vitro procedure, after accounting for microbial contamination, is required to more precisely quantify the ruminal degradation rate of PRG N. Using a single 21-h HCl hydrolysis to determine AA concentrations underestimated the concentration of Ile and Val indicating that multiple-hydrolysis time procedures are required to predict the true AA concentration of PRG. In conclusion, the new feed chemistry procedures conducted in this study, allowed a more accurate characterization of the nutritive value of PRG. These new methodologies, in combination with the CNCPS, can provide greater understanding of the nutrition of grazing dairy cows.

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## 2.8. REFERENCES


Beecher, M., R. Baumont, M. O'Donovan, T. Boland, J. Aufrere, C. Fleming, N. Galvin, and E. Lewis. 2018. Effects of harvesting perennial ryegrass at different levels of herbage mass on voluntary intake and in vivo digestibility in sheep. Grass Forage Sci. 73:553-561. https://doi.org/10.1111/gfs.12319

Beecher, M., D. Hennessy, T. Boland, M. McEvoy, M. O'Donovan, and E. Lewis. 2015. The variation in morphology of perennial ryegrass cultivars throughout the grazing season and effects on organic matter digestibility. Grass Forage Sci. 70:19-29. https://doi.org/10.1111/gfs.12081

Beever, D. E., H. R. Losada, S. B. Cammell, R. T. Evans, and M. J. Haines. 1986. Effect of forage species and season on nutrient digestion and supply in grazing cattle. Br. J. Nutr. 56:209-225. https://doi.org/10.1079/bjn19860101

Blackburn, S. 1968. Amino acid determination: Methods and techniques. M. Dekker, New York, NY.

Bowerman, A. and P. Goodman. 1971. Variation in nitrate reductase activity in Lolium. Ann. Bot. 35:353-366. https://doi.org/10.1093/oxfordjournals.aob.a084483

Broderick, G. A. 1994. Quantifying forage protein quality. Pages 200–228 in Forage Quality, Evaluation, and Utilization. G. C. Fahey Jr., D. R. Collins, D. R. Mertens, and L. E. Moser, ed. Am. Soc. Agron. Inc., Crop Sci. Soc. Am. Inc., and Soil Sci. Soc. Am. Inc., Madison, WI. https://doi.org/10.2134/1994.foragequality.c5

Bryant, R., P. Gregorini, and G. Edwards. 2012. Effects of N fertilisation, leaf appearance and time of day on N fractionation and chemical composition of Lolium perenne cultivars in spring. Anim. Feed Sci. Technol. 173:210-219. https://doi.org/10.1016/j.anifeedsci.2012.02.003

Buckee, G. 1994. Determination of total nitrogen in barley, malt and beer by kjeldahl procedures and the dumas combustion methodcollaborative trial. J. Inst. Brew. 100:57-64. https://doi.org/10.1002/jib.1994.100.2.57

Chandler, J. A., W. J. Jewell, J. M. Gossett, P. J. Van Soest, and J. B. Robertson. 1980. Predicting methane fermentation biodegradability. Biotechnol. Bioeng. Symp. 10:93–107.

Chaudhry, A. and A. Webster. 1993. The true digestibility and biological value for rats of undegraded dietary nitrogen in feeds for ruminants. Anim. Feed Sci. Technol. 42:209-221. https://doi.org/10.1016/0377-8401(93)90099-6

Choi, C. W., S. Ahvenjarvi, A. Vanhatalo, V. Toivonen, and P. Huhtanen. 2002. Quantitation of the flow of soluble non-ammonia nitrogen entering the omasal canal of dairy cows fed grass silage based diets. Anim. Feed Sci. Technol. 96:203-220. https://doi.org/10.1016/S0377-8401(01)00348-0

Cotanch, K. W., R. J. Grant, M. E. Van Amburgh, A. Zontini, M. Fustini, A. Palmonari, and A. Formigoni. 2014. Applications of uNDF in ration modeling and formulation. Pages 114-131 in Proc. Cornell Nutrition Conference, Syracuse, NY. Cornell Univ., Ithaca, NY.

da Silva, M. S., G. F. Tremblay, G. Bélanger, J. Lajeunesse, Y. A. Papadopoulos, S. A. Fillmore, and C. C. Jobim. 2014. Forage energy to protein ratio of several legume–grass complex mixtures. Anim. Feed Sci. Technol. 188:17-27. https://doi.org/10.1016/j.anifeedsci.2013.11.006

da Silva, M. S., G. F. Tremblay, G. Bélanger, J. Lajeunesse, Y. A. Papadopoulos, S. A. Fillmore, and C. C. Jobim. 2013. Energy to protein ratio of grass–legume binary mixtures under frequent clipping. Agron. J. 105:482-492. https://doi.org/10.2134/agronj2012.0281

Dillon, P., T. Hennessy, L. Shalloo, F. Thorne, and B. Horan. 2008. Future outlook for the Irish dairy industry: a study of international competitiveness, influence of international trade reform and requirement for change. Int. J. Dairy Technol. 61:16-29. https://doi.org/10.1111/j.1471-0307.2008.00374.x

Dubois, M., K. A. Gilles, J. K. Hamilton, P. T. Rebers, and F. Smith. 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28:350-356.

Elkin, R. and J. Griffith. 1984. Hydrolysate preparation for analysis of amino acids in sorghum grains: effect of oxidative pretreatment. J. AOAC 68:1117-1121. https://doi.org/10.1093/jaoac/68.6.1117

Ellis, W. C., M. Mahlooji, and J. H. Matis. 2005. Models for estimating parameters of neutral detergent fiber digestion by ruminal microorganisms. J Anim Sci 83:1591-1601. https://doi.org/10.2527/2005.8371591x

Etheridge, R., G. Pesti, and E. Foster. 1998. A comparison of nitrogen values obtained utilizing the Kjeldahl nitrogen and Dumas combustion methodologies (Leco CNS 2000) on samples

typical of an animal nutrition analytical laboratory. Anim. Feed Sci. Technol. 73:21-28. https://doi.org/10.1016/S0377-8401(98)00136-9

Ferguson, W. and R. Terry. 1954. The fractionation of the non-protein nitrogen of grassland herbage. J. Sci. Food Agric 5:515-524. https://doi.org/10.1002/jsfa.2740051103

Fessenden, S. W., T. J. Hackmann, D. A. Ross, A. Foskolos, and M. E. Van Amburgh. 2017. Ruminal bacteria and protozoa composition, digestibility, and amino acid profile determined by multiple hydrolysis times. J. Dairy Sci. 100:7211-7226. https://doi.org/10.3168/jds.2016-12531

Garry B, M. O'Donovan, M. Beecher, L. Delaby, C. Fleming, R. Baumont, and E. Lewis. 2018. Predicting in vivo digestibility of perennial ryegrass using the neutral detergent cellulase method: updating the equation. Pages 194–196 in Sustainable Meat and Milk Production from Grasslands. Proceedings of the 27th General Meeting of the European Grassland Federation, Cork, Ireland, 17–21 June, 2018. Wageningen, The Netherlands: Wageningen. Academic Publishers.

Garry, B. 2016. The effect of animal and sward factors on the in vivo organic matter digestibility of perennial ryegrass. PhD dissertation. Sch. Agri. Food Sci., Univ. College Dublin, Belfield, Dublin 4, Ireland.

Goering, H. K. and P. J. Van Soest. 1970. Forage fiber analyses (apparatus, reagents, procedures, and some applications). Vol. 379. US Agricultural Research Service Washington.

Hall, M. B. 2014. Selection of an empirical detection method for determination of water-soluble carbohydrates in feedstuffs for application in ruminant nutrition. Anim. Feed Sci. Technol. 198:28-37. https://doi.org/10.1016/j.anifeedsci.2014.08.009

Higgs, R. and M. E. Van Amburgh. 2016. Evolution of the CNCPS-Development of V7. Pages 125-146 in Proc. Cornell Nutrition Conference, Syracuse, NY. Cornell Univ., Ithaca, NY.

Higgs, R., L. Chase, D. Ross, and M. E. Van Amburgh. 2015. Updating the Cornell Net Carbohydrate and Protein System feed library and analyzing model sensitivity to feed inputs. J. Dairy Sci. 98:6340-6360. https://doi.org/10.3168/jds.2015-9379

Hoekstra, N., P. Struik, E. Lantinga, M. E. Van Amburgh, and R. Schulte. 2008. Can herbage nitrogen fractionation in Lolium perenne be improved by herbage management? NJAS-Wagen. J. Life. Sci. 55:167-180. https://doi.org/10.1016/S1573-5214(08)80035-8

Huhtanen, P., A. Seppälä, M. Ots, S. Ahvenjärvi, and M. Rinne. 2008. In vitro gas production profiles to estimate extent and effective first-order rate of neutral detergent fiber digestion in the rumen. J. Anim. Sci. 86:651-659. https://doi.org/10.2527/jas.2007-0246

Huhtanen, P., J. Nousiainen, and M. Rinne. 2006a. Recent developments in forage evaluation with special reference to practical applications. Agric. Food Sci. 15:293-323.

Huhtanen, P., S. Ahvenjarvi, M. R. Weisbjerg, and P. Norgaard. 2006b. Digestion and passage of fibre in ruminants. Pages 87-135 in Ruminant Physiology: Digestion, Metabolism and Impact of Nutrition on Gene Expression, Immunology and Stress. K. Sejrsen, T. Hvelplund, and M. O. Nielsen, ed. Wageningen Academic Publisher, Wageningen, Netherlands.

Krämer, M., M. R. Weisbjerg, P. Lund, C. Jensen, and M. Pedersen. 2012. Estimation of indigestible NDF in forages and concentrates from cell wall composition. Anim. Feed Sci. Technol. 177:40–51. https://doi.org/10.1016/j.anifeedsci.2012.07.027

Krizsan, S., Z. Mussadiq, M. Hetta, M. Ramin, L. Nyholm, and P. Huhtanen. 2014. Predicting feeding value of forage maize hybrids harvested at different maturities and sites. J. Anim. Feed Sci. 23:269-278. https://doi.org/10.22358/jafs/65690/2014

Lanzas, C., C. J. Sniffen, S. Seo, L.O. Tedeschi, and D. G. Fox. 2007. A feed carbohydrate fractionation scheme for formulating rations for ruminants. Anim. Feed Sci. Technol. 136: 167–190. https://doi.org/10.1016/j.anifeedsci.2006.08.025

Lapierre, H., S. Binggeli, M. Sok, D. Pellerin, and D. Ouellet. 2019. Estimation of correction factors to determine the true amino acid concentration of protein after a 24-hour hydrolysis. J. Dairy Sci. 102:1205-1212. https://doi.org/10.3168/jds.2018-15392 .

Licitra, G., T. M. Hernandez, and P. J. Van Soest. 1996. Standardization of procedures for nitrogen fractionation of ruminant feeds. Anim. Feed Sci. Technol. 57:347-358. https://doi.org/10.1016/0377-8401(95)00837-3

Loaiza, P., O. Balocchi, and A. Bertrand. 2017. Carbohydrate and crude protein fractions in perennial ryegrass as affected by defoliation frequency and nitrogen application rate. Grass Forage Sci. 72:556-567. https://doi.org/10.1111/gfs.12258

Lorenz, H., T. Reinsch, S. Hess, and F. Taube. 2019. Is low-input dairy farming more climate friendly? A meta-analysis of the carbon footprints of different production systems. J. Clean. Prod. 211:161-170. https://doi.org/10.1016/j.jclepro.2018.11.113

McCarthy, B., L. Delaby, K. Pierce, A. Brennan, and B. Horan. 2013. The effect of stocking rate and calving date on milk production of Holstein–Friesian dairy cows. Livest. Sci.153:123-134. https://doi.org/10.1016/j.livsci.2013.01.013

McClearn B., Gilliland T.J., Galvin N., Guy C., Dineen M., Coughlan F., and B. McCarthy. 2018. The effect of grass ploidy and white clover inclusion on dry-matter intake of grazing dairy cows. Pages 298–300 in Sustainable Meat and Milk Production from Grasslands. Proceedings of the 27th General Meeting of the European Grassland Federation, Cork, Ireland, 17–21 June, 2018. Wageningen, The Netherlands: Wageningen. Academic Publishers.

McEvoy, M., L. Delaby, J. Murphy, T. Boland, and M. O'Donovan. 2010. Effect of herbage mass and allowance on sward characteristics, milk production, intake and rumen volatile fatty acid concentration. Grass Forage Sci. 65:335-347. https://doi.org/10.1111/j.1365-2494.2010.00752.x

McGeehan, S. and D. Naylor. 1988. Automated instrumental analysis of carbon and nitrogen in plant and soil samples. Commun. Soil Sci. Plant Anal. 19:493-505. https://doi.org/10.1080/00103628809367953

McGrath, D. 1992. A note on the influence of nitrogen application and time of cutting on water-soluble carbohydrate production by Italian ryegrass. Irish J. Agr. Food Res. 31:189-192. https://www.jstor.org/stable/25562191

Mentink, R., P. Hoffman, and L. Bauman. 2006. Utility of near-infrared reflectance spectroscopy to predict nutrient composition and in vitro digestibility of total mixed rations. J. Dairy Sci. 89:2320-2326. https://doi.org/10.3168/jds.S0022-0302(06)72303-7

Mertens, D. R. 2015. Underlying Fiber Concepts and Definitions. Pages 125-136 in Proc. Cornell Nutrition Conference, Syracuse, NY. Cornell Univ., Ithaca, NY

Mertens, D. R. and R. J. Grant. 2007. Digestibility and intake. Pages 487–507 in Forages Volume II: The Science of Grassland Agriculture, 6th ed. R. F. Barnes, C. J. Nelson, K. J. Moore, M. Collins, ed. Iowa State Univ. Press: Ames, IA.

Mertens, D. R. 2002. Gravimetric determination of amylase-treated neutral detergent fiber in feeds with refluxing in beakers or crucibles: collaborative study. J. AOAC Int. 85:1217-1240.

Met Éireann, 2018. Warm Dry Weather of June and July 2018. Dublin, Ireland: Met Éireann. Available at https://www.met.ie/cms/assets/uploads/2018/08/DryWarmWx06072018_SS.pdf (Accessed 14 March 2020).

Morgan, D., G. Stakelum, and J. Dwyer. 1989. Modified neutral detergent cellulase digestibility procedure for use with the'Fibertec'system. Irish J. Agr. Food Res. 91-92.

National Research Council. 2012. Nutrient requirements of Swine. 11th ed.; Natl. Acad. Press, Washington, DC, USA.

National Research Council. 2001. Nutrient Requirements of Dairy Cattle. 7th rev. ed. Natl. Acad. Press, Washington, DC, USA.

Nousiainen, J. 2004. Development of tools for the nutritional management of dairy cows on silage-based diets. PhD dissertation. Helsinki University, Helsinki, Finland.

Nousiainen, J., S. Ahvenjärvi, M. Rinne, M. Hellämäki, and P. Huhtanen. 2004. Prediction of indigestible cell wall fraction of grass silage by near infrared reflectance spectroscopy. Anim. Feed Sci. Technol. 115:295-311. https://doi.org/10.1016/j.anifeedsci.2004.03.004

Nowakowski, T. 1962. Effects of nitrogen fertilizers on total nitrogen, soluble nitrogen and soluble carbohydrate contents of grass. J. Agri. Sci. 59:387-392. https://doi.org/10.1017/S0021859600015471

O'Donovan, M., and E. Kennedy. 2007. Using grass to reduce feed costs. Pages 63–80 in Exploiting the Freedom to Milk, Teagasc National Dairy Conference 2007. Teagasc, Carlow, Ireland.

Peyraud, J.-L. and R. Delagarde. 2013. Managing variations in dairy cow nutrient supply under grazing. Anim. 7:57-67. https://doi.org/10.1017/S1751731111002394

Peyraud, J. and L. Astigarraga. 1998. Review of the effect of nitrogen fertilization on the chemical composition, intake, digestion and nutritive value of fresh herbage: consequences on animal nutrition and N balance. Anim. Feed Sci. Technol. 72:235-259. https://doi.org/10.1016/S0377-8401(97)00191-0

Pichard, G. and P. Van Soest. 1977. Protein solubility of ruminant feeds. Pages 91-98 in Proc. Cornell Nutr. Conf. Cornell Univ Ithaca, NY.

Raffrenato, E., C. Nicholson, and M. E. Van Amburgh. 2019. Development of a mathematical model to predict pool sizes and rates of digestion of 2 pools of digestible neutral detergent

fiber and an undigested neutral detergent fiber fraction within various forages. J. Dairy Sci. 102:351-364. https://doi.org/10.3168/jds.2018-15102

Raffrenato, E., D. Ross, and M. E. Van Amburgh. 2018. Development of an in vitro method to determine rumen undigested aNDFom for use in feed evaluation. J. Dairy Sci. 101:9888-9900. https://doi.org/10.3168/jds.2018-15101

Raffrenato, E. and M. E. Van Amburgh. 2011. Technical note: Improved methodology for analyses of acid detergent fiber and acid detergent lignin. J. Dairy Sci. 94:3613-3617. https://doi.org/10.3168/jds.2010-3701

Reid, D. and N. Strachan. 1974. The effects of a wide range of nitrogen rates on some chemical constituents of the herbage from perennial ryegrass swards with and without white clover. J. Agri. Sci. 83:393-401. https://doi.org/10.1017/S002185960002685X

Reynal, S., I. Ipharraguerre, M. Lineiro, A. Brito, G. Broderick, and J. Clark. 2007. Omasal flow of soluble proteins, peptides, and free amino acids in dairy cows fed diets supplemented with proteins of varying ruminal degradabilities. J. Dairy Sci. 90:1887-1903. https://doi.org/10.3168/jds.2006-158

Rinne, M., A. Olt, J. Nousiainen, A. Seppälä, M. Tuori, C. Paul, M. D. Fraser, and P. Huhtanen. 2006. Prediction of legume silage digestibility from various laboratory methods. Grass Forage Sci. 61:354-362. https://doi.org/10.1111/j.1365-2494.2006.00542.x

Robel, E. J. and A. B. Crane. 1972. An accurate method for correcting unknown amino acid losses from protein hydrolyzates. Anal. Biochem. 48:233-246. https://doi.org/10.1016/0003-2697(72)90186-8

Ross, D. A. 2004. Amino acid composition of ruminant feeds and feed fractions and evaluation of the methods used to obtain the insoluble and true precipitate protein fractions of feedstuffs. MS Thesis. Dep. Ani. Sci., Cornell Univ., Ithaca, NY.

Rutherfurd, S. M. 2009. Accurate determination of the amino acid content of selected feedstuffs. Int. J. Food Sci. Nutr. 60:53-62. https://doi.org/10.1080/09637480802269957 .

Rutherfurd, S., P. Moughan, D. Lowry, and C. Prosser. 2008. Amino acid composition determined using multiple hydrolysis times for three goat milk formulations. Int. J. Food Sci. Nutr. 59:679-690. https://doi.org/10.1080/09637480701705424

Sairanen, A., H. Khalili, J. I. Nousiainen, S. Ahvenjärvi, and P. Huhtanen. 2005. The effect of concentrate supplementation on nutrient flow to the omasum in dairy cows receiving

freshly cut grass. J. Dairy Sci. 88:1443-1453. https://doi.org/10.3168/jds.S0022-925 0302(05)72812-5

Simonne, E. H., J. N. McCrimmon, H. L. Scoggins-Mantero, H. A. Mills, and C. P. Cresman III. 1995. Adjustments of sufficiency ranges of selected ornamentals and turfgrasses for assessing nitrogen status with Dumas-N data. Commun. Soil Sci. Plant Anal. 26:2243-2251. https://doi.org/10.1080/00103629509369443

Traxler, M., D. Fox, P. Van Soest, A. Pell, C. Lascano, D. Lanna, J. Moore, R. Lana, M. Vélez, and A. Flores. 1998. Predicting forage indigestible NDF from lignin concentration. J. Anim. Sci. 76:1469-1480. https://doi.org/10.2527/1998.7651469x

Tylutki, T. P., D. G. Fox, V. M. Durbal, L. O. Tedeschi, J. B. Russell, M. E. Van Amburgh, T. R. Overton, L. E. Chase, and A. N. Pell. 2008. Cornell Net Carbohydrate and Protein System: A model for precision feeding of dairy cattle. Anim. Feed Sci. Technol. 143:174-202. https://doi.org/10.1016/j.anifeedsci.2007.05.010

Van Amburgh, M. E., E. Collao-Saenz, R. Higgs, D. Ross, E. Recktenwald, E. Raffrenato, L. Chase, T. Overton, J. Mills, and A. Foskolos. 2015. The Cornell Net Carbohydrate and Protein System: Updates to the model and evaluation of version 6.5. J. Dairy Sci. 98:6361-6380. https://doi.org/10.3168/jds.2015-9378

Van Amburgh, M. E., L. E. Chase, T. R. Overton, D. A. Ross, E. B. Recktenwald, R. J. Higgs, and T. P. Tylutki. 2010. Updates to the Cornell Net Carbohydrate and Protein System v6.1 and implications for ration formulation. Pages 144-159 in Proc. Cornell Nutrition Conference, Syracuse, NY. Cornell Univ., Ithaca, NY.

Van Soest, P., M. E. Van Amburgh, J. Robertson, and W. Knaus. 2005. Validation of the 2.4 times lignin factor for ultimate extent of NDF digestion, and curve peeling rate of fermentation curves into pools. Pages 139–149 in Proc. Cornell Nutrition Conference, Syracuse, NY. Cornell Univ., Ithaca, NY.

Van Soest, P. J. 1994. Nutritional ecology of the ruminant. 2nd Ed. Cornell Univ. Press, Ithaca, NY.

Vieira, R. A., L. O. Tedeschi, and A. Cannas. 2008. A generalized compartmental model to estimate the fibre mass in the reticulorumen: 2. Integrating digestion and passage. J. Theor. Biol. 255:357–368. https://doi.org/10.1016/j.jtbi.2008.08.013

Wilson, R. and J. Tilley. 1965. Amino-acid composition of lucerne and of lucerne and grass protein preparations. J. Sci. Food Agri. 16:173-178. https://doi.org/10.1002/jsfa.2740160401

Zontini, A. M. 2016. Characterization of fiber fractions in plant by-products and modeling fiber digestion with application in CNCPS v. 7.0. PhD Dissertation. Department of Animal Science, Cornell Univ., Ithaca, NY.

Zontini, A., A. Foskolos, D. A. Ross, J. Metcalf, P. H. Doane, and M. E. Van Amburgh. 2015. Research Update: Formulating diets for lactating cattle using multiple pools of NDF digestibility. Pages 189–196 in Proc. Cornell Nutrition Conference, Syracuse, NY. Cornell Univ., Ithaca, NY.

# CHAPTER 3: RUMEN METABOLISM, OMASAL FLOW OF NUTRIENTS, AND MICROBIAL DYNAMICS IN LACTATING DAIRY COWS FED FRESH PERENNIAL RYEGRASS (*LOLIUM PERENNE* L.) NON-SUPPLEMENTED OR SUPPLEMENTED WITH ROLLED BARLEY GRAIN

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## 3.1. ABSTRACT

The objective of this study was to evaluate the effect of rolled barley grain supplementation on rumen metabolism, omasal flow of nutrients, and microbial dynamics in lactating dairy cows fed fresh perennial ryegrass (*Lolium perenne* L.; **PRG**) based diets. Ten ruminally cannulated Holstein cows averaging (mean ± standard deviation) 49 ± 23 d in milk and 513 ± 36 kg of body weight were assigned to one of two treatments in a switchback design. The treatment diets were PRG-only (**G**) or PRG plus 3.5 kg dry matter rolled barley grain (**G+RB**). The study consisted of three 29-d periods where each period consisted of 21 d of diet adaptation and 8 d of data and sample collection. A double marker system was used to quantify nutrient flow entering the omasal canal along with labeled $^{15}$N-ammonium sulfate to measure bacterial, protozoal and non-microbial N

flow. Rumen evacuation techniques were used to determine nutrient and microbial pool size, allowing the calculation of fractional rates of digestion and microbial growth. There was no difference in daily milk yield or energy corrected milk yield between treatments. Milk fat concentration and milk urea N decreased while milk protein concentration increased in cows fed the G+RB diet. During the omasal sampling phase, dry matter intake was higher in cows fed the G+RB diet. Ruminal and total tract neutral detergent fiber digestibility was lower in G+RB cows; however, no difference was observed in reticulorumen pH. The rumen pool size of fermentable carbohydrate was increased in cows fed the G+RB diet; however, the fractional rate of digestion was decreased. Flow of non-ammonia N and bacterial N at the omasal canal increased in cows fed the G+RB diet compared with the G diet. Protozoa N flow was not different between diets; however, protozoa appeared to supply a much larger amount of microbial N and exhibited shorter generation time than previously considered. Feed N ruminal digestibility, corrected for microbial contribution, was similar for both treatments (88.4% and 89.0% for G and G+RB, respectively). In conclusion, rolled barley grain supplementation did not benefit overall animal performance; however, it reduced ruminal neutral detergent fiber digestibility and increased bacterial N flow. The results demonstrate the large dependence of cows consuming PRG-based diets on microbial N as the main source of non-ammonia N supply. Additional quantitative research is required, to further describe the supply of nutrients and microbial dynamics in cows consuming PRG-based diets in an effort to determine most limiting nutrients.

**Key Words:** pasture, fermentable carbohydrate, omasal flow, protozoa

## 3.2. INTRODUCTION

In pasture-based systems, there is potential to increase the efficiency and productivity on a per cow basis by incorporating more nutrients (i.e. nitrogen and carbon) into milk and meat products. In a review of supplementation of pasture-based diets, individual milk production increased linearly as the amount of supplemental concentrate increased (Bargo et al., 2003). However, wide variation in milk yield response exists among studies with little mechanistic explanation of how or why this variation occurs (Penno, 2002). In temperate regions, where a large proportion of the pastures are primarily perennial ryegrass (*Lolium perenne* L.; **PRG**), modern grazing management practices aim to maintain the plant at an immature stage (O'Donovan et al., 2002) resulting in PRG forage with OM digestibility in excess of 85% (Smit et al., 2005; Wims et al., 2013). Despite well-managed PRG being highly digestible, ME supply is considered first limiting for milk production of grazing cows (Kolver and Muller, 1998; Nicol and Brookes, 2007). There is a breadth of reports investigating the effects of supplementing cereal grains to cows consuming PRG-based diets (Bargo et al., 2003; Baudracco et al., 2010). However, the majority of these reports examine the effects at a whole animal level which prevents the determination of the mechanistic nutritional physiology involved at the rumen or post-ruminal level. As a result, there is a limited understanding of the in vivo ruminal digestion and passage of nutrients in cows consuming PRG-based diets.

The supply of AA to the small intestine is also considered co-limiting milk production in cows consuming PRG-based diets (Delaby et al., 1995; O'Mara et al., 2000). In some cases, this can be attributed to extensive rumen proteolysis and substantial pre-duodenal losses of the PRG N (Beever et al., 1986, 1987). Consequently, microbial protein contributes a large proportion of the NAN flow in cows consuming pasture-based diets (Younge et al., 2004; Sairanen et al., 2005).

This contribution, however, is likely underestimated in pasture-fed cows as protozoal N has not been accounted for. Ahvenjärvi et al. (2002) and Fessenden et al. (2019a) have reported protozoal N to contribute 15% to 20% of the microbial protein flow in silage-based diets.

While it is evident that protozoa have a significant impact on ruminant physiology (Newbold et al., 2015) an incomplete understanding of their metabolism currently exists (Firkins et al., 2020). Direct measurement of protozoa rumen pool size and omasal flow simultaneously are rarely conducted even though this is the most correct way to represent protozoal generation time in vivo (Karnati et al., 2007). Cows consuming PRG-based diets exhibit rapid rumen turnover, which is suggested as a principal factor associated with protozoal generation time (Potter and Dehority, 1973; Sylvester et al., 2009). Furthermore, the rumen of a grazing cow seems optimal for efficient protozoal growth due to an ample supply of sugars, soluble true protein and moderate pH levels across the day (Clarke, 1965; Williams and Coleman, 1988). Thus, cows fed PRG-based diets, could be an effective model to enhance our current understanding of protozoal metabolism.

Therefore, the objective of this study was to evaluate the effect of rolled barley grain (**RB**) supplementation on rumen metabolism, omasal flow of nutrients, and microbial dynamics in lactating dairy cows fed fresh PRG-based diets. To determine the effect of RB supplementation on nutrient and microbial growth and passage, the omasal sampling technique (Huhtanen et al., 1997; Reynal and Broderick, 2005) was utilized in combination with rumen evacuation and microbial isolation procedures. The hypothesis of this experiment was that the inclusion of RB would increase the fermentable carbohydrate (**CHO**) supply for rumen microbes, and thereby, increase the microbial N (**MicN**) flow at the omasal canal compared with a PRG-only diet.

## 3.3. MATERIALS AND METHODS

### *3.3.1. Experimental Site*

The experiment was conducted from April to July 2017 at the Teagasc, Animal and Grassland Research and Innovation Centre, Moorepark, Fermoy, Co. Cork, Ireland (52°16′N; 8°25′W; 49 m a.s.l). All procedures described in this experiment were approved by the Teagasc Animal Ethics Committee and conducted under experimental license (AE19132-P054) from the Health Products Regulatory Authority under European directive 2010/63/EU and S.I. no. 543 of 2012.

### *3.3.2. Sward Management and Measurements*

An area of ground dedicated to the study (5.5 ha) was divided into six sub-plots and managed as described by O'Donovan et al. (2002) to ensure high pasture quality. Nitrogen fertilizer was applied to the sub-plots in two applications of 17 kg N/ha approximately 21 and 14 d prior to each harvest in the form of Calcium Ammonium Nitrate with added S (Goulding Chemicals Limited, Centre Park Road, Marina, Cork, Ireland).

### *3.3.3 Animals, Experimental Design, and Treatment Administration*

Ten ruminally cannulated multiparous Holstein cows averaging (mean ± SD) 49 ± 23 DIM and 513 ± 36 kg of BW were enrolled in a 3-wk pre-study acclimation period where all animals were managed and housed in a free-stall barn. The pre-study diet consisted of fresh PRG, offered indoors to all cows ad libitum to allow a 10% refusal rate, and 3.5 kg DM of rolled barley grain. At the end of the 3-wk period, cows were stratified by pre-study milk yield and randomly assigned to one of two treatment sequences in a switchback design. The study consisted of three 29-d periods, where each period contained 21 d for diet adaptation and 8 d of data and sample collection. For

the first 21 d of each period, the cows were housed in a free-stall barn. For each 8-d period of sample collection, the cows were housed in individual tie-stalls with free access to water. Cows were milked twice daily (0730 and 1530 h) through a parlor except during the 8-d sampling phases, when they were milked in the tie-stalls. Milk yield was recorded and milk samples taken at each milking on d 21, 22, and 23 of each period and analyzed for fat, CP, lactose, SCC and MUN using mid-infrared spectroscopy analysis (Milkoscan 203, DK-3400; Foss Electric, Hillerød, Denmark). Body weights were measured weekly after the 0730 h milking using an electronic portable weighing scale and Winweigh software package (Tru-test Limited, Auckland, New Zealand). Body condition score was recorded weekly as the average of two trained scorers using a 1–5 scale (where 1 = emaciated and 5 = extremely fat) with 0.25 increments (Edmonson et al., 1989). The changes in BW and BCS were calculated as the difference between measurements taken on d 29 of each period.

Treatment diets were 1) PRG-only (**G**) or 2) PRG plus 3.5 kg DM RB (**G+RB**; Table 3.1). In addition, cows assigned to both treatments were fed 40 g/d of magnesium (120 g/d of Sweetened Cal Mag; Nutribio Ltd, Tivoli, Cork, Ireland) to reduce the risk of hypomagnesaemia. The swards of PRG were mechanically harvested twice daily (0800 and 1500 h) with a GrassTech Grazer GT80 (Future Grass Technology, Borris Business Park, Clonegoose, Borris, Co. Carlow, Ireland, R95 E032). Swards were cut at 4 cm above ground level with no additional processing to minimize disruption of the cellular structure of the plant, which resulted in particle sizes ranging from 26 to 37 cm. The cut forage was collected, weighed, and a sub-sample taken to determine DM concentration in an oven at 60°C for 48 h. During the 21-d adaptation period the cows were offered PRG twice daily at the time of harvesting. During the 8 d of data and sample collection, the cows were offered PRG 6 times daily at 0630, 0830, 1230, 1530, 1930 and 2130 h with the PRG

refrigerated at 4°C between feedings to minimize respiration and nutrient loss. The quantity offered to each animal was recorded and refusals were collected the following morning at 0730 h and weights recorded. The feeding rates were adjusted daily to yield refusals of 5% to 10% of intake. The RB was offered to the respective cows at the time of milking (0730 and 1530 h) as two equal meals. Daily, samples of PRG and RB were dried at 105°C for 15 h and analyzed for DM. Additional samples were either freeze dried (LS40+chamber, MechaTech Systems Ltd., Bristol, U.K.) at -55°C for 120 h or oven dried at 60°C for 48 h. Dried samples were ground through a 1-mm screen using a Cyclotech 1093 Sample Mill (Foss, DK-3400 Hillerød, Denmark) and stored for subsequent nutrient composition analysis.

### *3.3.4. Sampling Procedures*

Each experimental period contained an infusion, omasal sampling and rumen evacuation phase, which occurred in the final 8 d of the period. The double marker method utilizing CoEDTA (Udén et al., 1980) and undigested amylase- and sodium sulfite-treated NDF corrected for ash residue after 240 h of in vitro fermentation (**uNDFom**; Raffrenato et al., 2018) were used to quantify liquid and particle flow entering the omasal canal, respectively. From d 21 at 1400 h until the end of the period, CoEDTA was dissolved in distilled water and continuously infused into the rumen at a rate of 2.7 g/d Co in 2.5 L of solution/d via peristaltic pump (Masterflex, Cole-Parmer Instrument Company, LLC, Vernon Hills, IL). All cows received a 3-L priming dose of CoEDTA (3.3 g of Co) into the rumen via the rumen cannula immediately prior to starting infusion. To enrich microbial N with $^{15}N$, 8.8 g/d of ammonium sulfate (Cambridge Isotope Laboratories Inc., Andover, MA, USA) with a 10% enrichment of $^{15}N$ (187 mg/d of $^{15}N$) was added to CoEDTA

infusate. Prior to starting the infusion, samples of ruminal contents were taken randomly from several regions of the rumen for later determination of $^{15}N$ background.

Spot samples of omasal digesta were obtained using the omasal sampling technique developed by Huhtanen et al. (1997) and adapted by Reynal and Broderick (2005). Omasal sampling began approximately 74 h after the beginning of marker infusion to allow uniform marker and isotope distribution. Samples of omasal contents were collected from the omasal canal during three 8-h intervals: at 1600, 1800, 2000, and 2200 h on d 24; at 0000, 0200, 0400, and 0600 h on d 26; and at 0800, 1000, 1200, and 1400 h on d 27. This sampling schedule encompassed every 2 h of the 24-h cycle. The sampling device was placed in position at the beginning of each interval and before each time point within the interval; the location of the sampling device was confirmed and repositioned if necessary. At the end of each interval, the sampling device was removed. A 425-mL spot sample was obtained during the first 3 sampling time points, and a 675-mL spot sample was obtained during the last sampling time points of each interval. Each spot sample was split into subsamples of 50-mL (x 2), 125-mL, and 200-mL; with an additional 250-mL subsample at the last time point. One of the 50-mL samples (omasal fluid; **OF**) was filtered through a single layer of a large pore polyethylene cheesecloth (Graytec, GD Textile, Manchester, UK), acidified with 50% $H_2SO_4$, and stored at -20°C for subsequent $NH_3$-N and VFA analysis, and the other was processed and stored for a separate investigation. The 125-mL subsample was placed on ice and combined within interval, yielding a 500-mL sample for bacterial isolation. Bacteria were isolated using a modification of the procedure of Whitehouse et al. (1994). The omasal contents were squeezed through a single layer of cheesecloth, and the retained solids were washed once with saline solution and squeezed again through a single layer of cheesecloth. The resulting filtrate (A) was stored at 4°C for further centrifugation. The solids retained were placed in a shaking incubator

for 1 h at 39°C in a 0.1% methylcellulose solution, to detach solid-associated bacteria, and transferred to a 4°C cooler for 24 h. After 24 h, the sample was blended for 1 minute. The blended sample was squeezed through a single layer of cheesecloth and the retained solids were washed once with saline solution and squeezed again through cheesecloth. The resulting filtrate (B) was stored at 4°C for further centrifugation. Filtrates A and B were centrifuged at 1,000 x *g* for 5 min at 4°C to remove small feed particles and protozoa. The supernatant was centrifuged at 15,000 x *g* for 20 min at 4°C and the bacterial pellet was collected and stored at −20°C until lyophilization and later analysis. The bacterial pellets recovered from filtrates A and B represented the omasal liquid- (**OLAB**) and omasal particle-associated bacteria (**OPAB**), respectively. The bacterial isolations from each 8-h interval were subsequently combined within period to generate an OLAB and an OPAB sample per cow per period. The 200-mL subsamples were combined within period and stored at - 20°C, generating a 2.4-L composite. This omasal composite was subsequently thawed and separated into omasal large particle (**LP**), small particle (**SP**) and liquid phase (**LQ**) as described in Reynal and Broderick (2005), and these were stored at -20°C until freeze-dried. The additional 250-mL subsample obtained on the final time point of each interval was processed immediately to isolate omasal protozoa (**OP**) as described by Denton et al. (2015).

In parallel with the omasal sampling, fecal and rumen fluid samples were also obtained. Fecal samples were composited by period and stored at - 20°C while rumen fluid (**RF**) was acidified with 50% $H_2SO_4$ and stored at - 20°C. Blood samples were harvested at the second time point of each interval via coccygeal vein puncture. Blood samples were collected into tubes containing sodium heparin, centrifuged (3,000 × *g* for 20 min at 4°C), and plasma was harvested and stored at - 20°C. On d 28 and d 29 of each period, rumen contents were evacuated, two hours before (0630 h) and two hours after (1030 h) the main meal, respectively. The rumen contents were

weighed, mixed, and a representative sample was obtained and stored at - 20°C. Rumen contents were returned to the cow via the rumen cannula. Prior to beginning rumen evacuations, random, composite samples of rumen contents from multiple sites in the rumen were removed for isolation of rumen liquid-associated bacteria (**RLAB**), rumen particle-associated bacteria (**RPAB**) and rumen protozoa (**RP**) as described above for the omasal isolations.

### *3.3.5. Laboratory Analysis*

Feed samples were analyzed for chemical composition using wet chemistry methods (CPM Plus Package) by Cumberland Valley Analytical Services (Waynesboro, PA). In addition to this analysis, feed samples were analyzed at our laboratory for amylase- and sodium sulfite-treated NDF corrected for ash residue (**aNDFom**; Mertens, 2002), and uNDFom after in vitro incubation with rumen fluid, according to Raffrenato et al. (2018). The time points selected for PRG were 12, 30, 120 and 240 h with the 12-h time point included to capture the rate of digestion in the linear phase of digestion as the rate of degradation of the immature PRG was quite high (Chapter 2). For the RB, time points of 12, 72 and 120 h were selected as described by Zontini et al. (2016). Water-soluble carbohydrates (**WSC**) were determined according to the procedures of Hall (2014). Finally, NPN, NDIN, and ADIN of the feed samples were determined according to Licitra et al. (1996). The chemical composition of the PRG by period, RB and treatment diets are presented in Table 3.1.

**Table 3.1.** Nutrient composition (mean ± SD)[1] of feeds, selected supplement and experimental diets used in the experiment

| | Period[2] | | | | Treatment[3] | |
|---|---|---|---|---|---|---|
| Nutrient composition | GP1 | GP2 | GP3 | RB[4] | G | G+RB |
| DM, % | 19.5 ± 2.3 | 18.4 ± 1.5 | 22.6 ± 1.0 | 86.9 ± 0.8 | 20.2 | 34.2 |
| CP, % of DM | 12.5 ± 1.1 | 18.1 ± 1.5 | 18.3 ± 1.6 | 11.6 ± 0.4 | 16.3 | 15.3 |
| NPN, % of N | 25.7 ± 2.5 | 24.2 ± 0.1 | 20 ± 1.9 | - | 23.3 | - |
| Soluble N, % of N | 37.6 ± 2.5 | 36.1 ± 2.5 | 32.2 ± 1.8 | 17.1 ± 1.9 | 35.3 | 31.7 |
| NDIN, % of N | 13.8 ± 0.2 | 14.1 ± 0.4 | 16.8 ± 0.9 | 8.0 ± 1.0 | 14.9 | 13.5 |
| ADIN, % of N | 2.8 ± 0.1 | 2.3 ± 0.2 | 2.3 ± 0.2 | 3.0 ± 0.7 | 2.5 | 2.6 |
| Starch, % of DM | 3.1 ± 1.9 | 2.3 ± 0.7 | 2.2 ± 0.7 | 60.7 ± 0.7 | 2.5 | 14.7 |
| WSC, % of DM | 27.1 ± 2.4 | 20.9 ± 1.8 | 19.2 ± 1.4 | 7.1 ± 0.6 | 22.4 | 19.2 |
| NFC, % of DM | 43.6 ± 2.6 | 35.9 ± 1.5 | 33.0 ± 3.8 | 65.0 ± 0.5 | 37.5 | 43.3 |
| aNDFom[5], % of DM | 35.2 ± 2.1 | 35.6 ± 1.2 | 37.6 ± 1.4 | 19.2 ± 1.0 | 36.1 | 32.6 |
| 12-h uNDFom, % of aNDFom | 56.5 ± 8.2 | 54.6 ± 10.4 | 49.7 ± 7.4 | 71.0 ± 0.3 | 53.6 | - |
| 30-h uNDFom, % of aNDFom | 24.1 ± 6.6 | 25.0 ± 8.4 | 19.0 ± 1.8 | - | 22.7 | - |
| 72-h uNDFom, % of aNDFom | - | - | - | 38.4 ± 1.4 | - | - |
| 120-h uNDFom, % of aNDFom | 11.6 ± 0.4 | 11.9 ± 1.1 | 11.4 ± 0.8 | 32.9 ± 0.6 | 11.6 | - |
| 240-h uNDFom, % of aNDFom | 9.7 ± 0.5 | 9..9 ± 1.0 | 9.9 ± 0.3 | - | 9.8 | - |
| ADF, % of DM | 19.0 ± 0.6 | 22.2 ± 1.2 | 20.5 ± 0.7 | 5.0 ± 0.7 | 20.6 | 17.3 |
| ADL, % of NDF | 3.8 ± 1.1 | 4.3 ± 0.4 | 4.7 ± 0.4 | 11.8 ± 2.7 | 4.2 | 5.8 |
| Ether extract, % of DM | 2.4 ± 0.2 | 3.3 ± 0.5 | 3.7 ± 0.6 | 1.7 ± 0.2 | 3.1 | 2.8 |
| Ash, % of DM | 6.6 ± 0.4 | 7.1 ± 1.0 | 7.4 ± 0.5 | 2.6 ± 0.6 | 7.0 | 6.1 |
| Pre-cutting yield, kg DM/ha | 2,018 ± 389 | 1,383 ± 191 | 1,421 ± 166 | - | 1,608 | |

[1]Analyzed values from 12 samples (4 day x 3 period).
[2]GP1 = Perennial ryegrass period 1; GP2 = Perennial ryegrass period 2; GP3 = Perennial ryegrass period 3.
[3]G = 100% (DM basis) perennial ryegrass; G+RB = 79% perennial ryegrass and 21% rolled barley grain.
[4]RB = rolled barley grain.
[5]aNDFom = amylase- and sodium sulfite-treated NDF corrected for ash residue; uNDFom = undigested amylase- and sodium sulfite treated NDF corrected for ash residue.

All omasal phase samples were freeze-dried and either ground through a 1-mm screen on a Cyclotech mill (LP) or homogenized with a mortar and pestle (SP and LQ) before analysis. The concentration of Co was determined by inductively coupled plasma-mass spectrometry (ICPMS) in all phase samples (Cornell University Nutrient Analysis Laboratory, Ithaca, NY) and the LP and SP phases were analyzed for uNDFom as described above. All omasal samples were analyzed for DM, aNDFom and WSC as described previously for feed samples as well as ash (AOAC, 2005), total N (Leco FP-528 N Analyzer, Leco Corp., St. Joseph, MI) and starch (Hall et al., 2015) to determine ruminal digestion and flow parameters. As digesta was fractionated into three phases, the SP was considered to be part of the particulate matter in the double-marker system. Concentrations of Co and uNDFom were then used to calculate the concentration of each nutrient in a sample theoretically representing omasal true digesta (**OTD**; France and Siddons, 1986). Subsamples of rumen contents obtained from the rumen evacuations were freeze-dried. Composite fecal samples were thawed, thoroughly mixed, and a subsample was placed in a forced air oven at 60°C until completely dried. Both the rumen contents and feces were then ground to pass a 1-mm screen on a Cyclotech mill and analyzed for DM, organic matter (**OM**), total N, WSC, starch, aNDFom and uNDFom as described above and then utilized for both pool size and fecal excretion calculations. Volatile fatty acid (acetic, propionic, butyric, valeric, iso-butyric and iso-valeric) concentrations in RF and OF were determined using a Varian CP-3000 GC analyzer (Varian Inc., Palo Alto, CA, USA) as described by Ranfft (1973). Samples were first thawed and centrifuged, and a 250 ul subsample mixed with 3.75 ml distilled water and 1 ml of a 0.5 g 3-methyl-N-valeric acid in 1 l of 0.15M oxalic acid solution. Ammonia N concentration was also determined in RF and OF using an ABX Horiba Pentra 400 chemistry analyzer (Horiba-ABXDiagnostics, 2, Micano-higashi, Kisshoin, Minami-ku, Kyoto 601-8510, Japan).

Omasal digesta phases, rumen contents, RLAB, RPAB, RP, OLAB, OPAB and OP were analyzed for NAN and $^{15}N$. The concentration of NAN and abundance of $^{15}N$ were determined using a Carlo Erba NC2500 elemental analyzer interfaced with an isotope ratio mass spectrometer (Cornell University Stable Isotope Laboratory, Ithaca, NY). Sample preparation and ammonia volatilization was carried out as described by Fessenden et al. (2019a). Samples of rumen contents taken in each period immediately prior to initiation of marker infusion were prepared and analyzed separately in the same manner as the enriched samples to evaluate natural abundance of $^{15}N$.

Lastly, the plasma samples were analyzed for urea using an enzymatic assay (Kit No. UR3825, Randox Laboratories Ltd., Antrim, Northern Ireland; University College Dublin, Co. Dublin, Ireland).

### *3.3.6. Rumination and Reticulorumen pH*

During the 8 d of data and sample collection for each experimental period, cows were equipped with RumiWatch noseband sensors to record rumination behavior (Nydegger and Bollhalder, 2010; RumiWatchSystem, Itin + Hoch GmbH, Liestal Switzerland). At the end of each recording period, the sensors were removed and data were collated following the procedure of Werner et al. (2018). The RumiWatch Manager 2 (V.2.1.0.0) was used to download the data and the RumiWatch Converter (V.0.7.3.36) was used to convert the raw data into 24-h summaries. At the beginning of the experiment, a wireless telemetry bolus that included a pH sensor (Mottram et al., 2008; eBolus, eCow Ltd., Exeter, Devon, UK) was orally administered to all animals using a balling gun. The boluses were removed via the rumen cannulate immediately after the completion of the first experimental period. The boluses were evaluated with a number of pH standards to ensure no

measurement drift had occurred. After completion of the evaluation, the boluses were returned to the animals for the remainder of the experiment.

### *3.3.7. Calculations*

Ruminal apparent digestibility of OM, aNDFom, WSC, starch, and N were determined by first subtracting the omasal flow of each nutrient from their respective intake and then dividing by the respective intake. Ruminal true digestibility of OM and N were determined by correcting apparent digestibility for microbial nutrient flow with an additional correction applied to OM for VFA flow (Ahvenjärvi et al., 2002). For aNDFom the apparent digestibility was assumed to be the true digestibility, as there is no metabolic loss associated with these nutrients (Van Soest, 1994). To calculate fecal output and apparent total-tract digestibility of OM, aNDFom, WSC, starch and N, the fecal concentration of uNDFom was used as an internal marker.

Rumen turnover of OM, potentially digestible aNDFom (**pdNDFom;** aNDFom – uNDFom), and uNDFom were calculated according to Van Soest et al. (1992) using the following equation:

$$\text{Turnover (h)} = \text{(rumen pool size, kg)} / [1/24 \times \text{(intake, kg/d)}]$$

Turnover of OM is the apparent turnover due the presence of metabolic matter. For pdNDFom and uNDFom the rate of intake and rate of passage over a 24-h period were calculated as follows:

$$\text{Rate of intake (ki)} = 1/24 \times \text{(intake, kg/d)} / \text{(rumen pool size, kg)}$$

$$\text{Rate of passage (kp)} = 1/24 \times \text{(omasal flow, kg/d)} / \text{(rumen pool size, kg)}$$

From this, the rate of pdNDFom digestion was calculated by difference:

$$\text{Rate of digestion (kd)} = \text{ki} - \text{kp}$$

The concentration of ammonia N in the OF sample in combination with the flow of liquid determined by the double marker system was used to calculate omasal flow of ammonia N. This

was subtracted from the total N flow to determine NAN flow. The NAN flow was partitioned into four fractions that consisted of particle-associated bacteria N, liquid- associated bacteria N, protozoa N, and non-microbial N. This non-ammonia non-microbial N (**NANMN**) was assumed to contain primarily undigested feed N and a smaller contribution of endogenous N. To determine microbial NAN flow, $^{15}$N atom percent excess (**APE**) for the OTD, OLAB, OPAB and OP samples was calculated as:

$$^{15}\text{N APE} = \text{enriched } ^{15}\text{N atom \%} - \text{mean natural } ^{15}\text{N atom \%}$$

The mean natural abundance of $^{15}$N in rumen contents was 0.3686 (SD ± 0.0002) and the natural abundance of $^{15}$N in rumen contents was assumed to be representative of OLAB, OPAB, OP, and OTD, respectively (Ahvenjärvi et al., 2002). Omasal protozoa OM (g/L) was calculated using gravimetric determinations in a known quantity of omasal liquid, as described by Fessenden et al. (2019b), assuming that protozoa only leave the rumen in the liquid phase (Ahvenjärvi et al., 2002; Karnati et al., 2007). To calculate OP OM flow (g/d), the quantity of OP OM (g/L) was multiplied by the daily volume of liquid flow (L/day) at the omasal canal. To calculate OP NAN flow (g/d), the OP OM flow (g/d) was multiplied by OP NAN concentration (g/g of OM). Accounting for $^{15}$N APE in omasal protozoa, the OLAB NAN (g/d) flow was calculated as;

$$\begin{aligned}\text{Omasal LAB NAN flow (g/d)} &= \{[\text{Liquid NAN flow (g/d) x Liquid } ^{15}\text{N APE (g/g of NAN)}] \\ &- [\text{OP NAN flow (g/d) x OP } ^{15}\text{N APE (g/g of NAN)}]\} / \text{OLAB } ^{15}\text{N APE (g /g of NAN)}\end{aligned}$$

Omasal particle-associated bacteria NAN flow was calculated as:

$$\text{Omasal PAB NAN flow (g/d)} = [\text{Particle NAN flow (g/d)} \times \text{Particle } {}^{15}\text{N APE (g/g of NAN)}] / \text{OPAB } {}^{15}\text{N APE (g/g of NAN)}$$

From this, total bacteria and total MicN flow were calculated as:

$$\text{Omasal bacteria NAN flow (g/d)} = \text{OLAB NAN flow (g/d)} + \text{OPAB NAN flow (g/d)}$$

$$\text{Omasal microbial NAN flow (g/d)} = \text{OP NAN flow (g/d)} + \text{Omasal bacteria NAN flow (g/d)}$$

The isolated OLAB and OPAB were assumed to be representative of the bacterial biomass flowing with the liquid and particulate phases, respectively (Reynal and Broderick, 2005). The NAN concentration (g/g of OM) of the OLAB, OPAB and OP samples was used to calculate the flow of total microbial biomass. The flow of NANMN was calculated as the difference between total NAN flow and microbial NAN flow.

The rumen pool size of digestible OM and total fermentable CHO were calculated as described by Fessenden et al. (2019b). The rumen protozoa OM (g/L) was calculated using gravimetric determinations of protozoa OM in rumen liquid (g/L). To calculate RP OM pool size (g), the quantity of RP OM (g/L) was multiplied by the rumen liquid pool size (L). To calculate RP NAN pool size (g), RP OM (g) was multiplied by RP NAN concentration (g/g of OM). Accounting for $^{15}$N APE in rumen protozoa, the rumen bacteria (**RB**) and microbial NAN pool size were calculated as follows:

$$\text{Rumen bacteria NAN (g)} = \{[\text{Rumen contents NAN (g)} \times \text{Rumen contents } {}^{15}\text{N APE (g/g of NAN)}] - [\text{RP NAN (g)} \times \text{RP } {}^{15}\text{N APE (g/g of NAN)}]\} / \text{RB } {}^{15}\text{N APE (g/g of NAN)}$$

$$\text{Rumen microbial NAN (g)} = \text{RP NAN (g)} + \text{RB NAN (g)}$$

The fractional growth rate of total microbial, bacterial, and protozoal fractions were then calculated as follows:

$$\text{Fractional growth rate } (h^{-1}) = \text{flow of microbial, bacterial, or protozoal N (g/h)} / \text{rumen pool size of microbial, bacterial, or protozoal N (g)}$$

The ruminal true digestion rate (g/h) and fractional rates of digestion ($h^{-1}$) of OM and CHO were calculated as described by Fessenden et al. (2019b). These results in combination with the microbial fractional growth rates were used to calculate Yg (g of cell DM/g of CHO degraded):

$$\text{Yg} = \text{fractional rate of microbial growth} / \text{fractional rate of CHO degradation}$$

### *3.3.8. Statistical Analysis*

Data were analyzed using the MIXED procedure of SAS version 9.4 (SAS Institute Inc. Cary, NC) by the following model:

$$Y_{ijkl} = \mu + S_i + C_{j:i} + P_k + T_l + PT_{kl} + \varepsilon_{ijkl}$$

where $Y_{ijkl}$ = dependent variable, $\mu$ = overall mean, $S_i$ = fixed effect of sequence i, $C_{j:i}$ = random effect of cow within sequence, $P_k$ = fixed effect of period k, $T_l$ = fixed effect of treatment l, $PT_{kl}$ = fixed interaction effect of period k and treatment l, and $\varepsilon_{ijkl}$ = residual error. Sequence effects and the interaction term including period and treatment were removed from the model when $P > 0.1$. Degrees of freedom were determined using the Kenward-Roger option and means were determined using the least squares means statement. Data describing pH and rumination measurements were

analyzed in a repeated measurements model using SAS MIXED procedures. The model included tests for the fixed effects of sequence, period, treatment, time, the interaction of period and treatment, and the interaction of time and treatment. Repeated measures (time) and random effects (cow within sequence) were also included in the model. The cow was considered the experimental unit. Effects were removed from the model when $P > 0.1$. Using the Akaike's information criterion, an autoregressive of order 1 covariance structure provided the best fit to the data. Statistical significance was considered at $P \leq 0.05$ and trends were considered at $0.05 < P \leq 0.10$.

## 3.4. RESULTS AND DISCUSSION

### *3.4.1. Diet Nutrient Composition*

The CP concentration of the harvested PRG was slightly lower than anticipated, averaging 16.3% across the three experimental periods due to the lower than anticipated CP concentration in Period 1 (Table 3.1). During April 2017, directly prior to the Period 1 sampling phase, the monthly rainfall was 19.3 mm, an 85%-reduction of the April average. This reduction in rainfall affected plant N concentration (He and Dijkstra, 2014) which we hypothesize is the likely reason for reduced CP observed in Period 1. As a result, there were a number of significant period by treatment interactions detected, which are discussed throughout the paper. The predictions from the rate and pool size calculations, as described by Raffrenato et al. (2019), partitioned 76.3%, 13.8% and 9.9% of the aNDFom into the fast, slow and indigestible pools with rates of 12.9%/h, 2.1%/h and 0.0%/h, respectively. The pre-cutting yield across the experiment was 1,608 kg DM/ha (above 4 cm horizon) which was close to the optimal target (O'Donovan et al., 2002). The CP, WSC, and aNDFom concentrations were all lower in the G+RB diet compared with the G diet

(Table 3.1). The starch concentration, as was intended in diet formulation, was greater for the G+RB diet, which resulted in an increase of NFC.

### *3.4.2. Animal Performance*

During the milk sampling phase (d 21-23; Table 3.2), total DMI was numerically higher in cows fed the G+RB diet compared with the G diet; however, a large substitution rate of 0.88 kg of pasture DMI per kg of RB DMI was observed. This high substitution rate is in accordance with other studies when a starch-based supplement was offered to cows consuming fresh pasture (Delagarde and Peyraud, 1995; Sheahan et al., 2013).

The inclusion of RB had no effect on daily milk yield, ECM or milk solids (kg fat + protein; Table 3.2). This is inconsistent with previous studies supplementing RB to pasture-based diets (Stakelum, 1986; Khalili and Sairanen, 2000). However, the PRG swards used in the current study were considerably higher in WSC and lower NDF concentrations in comparison with previous studies. This indicates a greater ME supply from the forage in the current experiment, and that the difference in ME supply might explain the lack of milk response to additional energy dense supplements. The milk fat concentration decreased while milk protein concentration increased in cows fed the G+RB diet, which are similar to the results discussed in reviews of studies providing energy dense supplements to pasture-based diets (Peyraud and Delaby, 2001; Bargo et al., 2003). In cows fed the G+RB diet, the MUN and plasma urea N (**PUN**) were lower compared with the cows fed the G diet (Table 3.2; 12.7 vs. 16.5 mg/dL; $P < 0.01$ for MUN; 7.6 vs. 9.2 mg/dL: $P < 0.01$ for PUN). This was likely due to the lower rumen ammonia pool size and concentration in G+RB fed cows (Table 3.3). There was a significant period x treatment interaction effect detected for MUN and PUN. In Period 1, no effect was observed for MUN and PUN, whereas there was a

significant treatment effect in Period 2 and Period 3. This was likely due to the lower N concentration of the PRG forage during Period 1. Feed efficiency (ECM/DMI) was reduced in cows fed the G+RB diet compared with the G diet ($P < 0.05$) and this was unexpected given the added fermentable CHO. Sairanen et al. (2005) reported increased milk yield from cows fed pasture-based diets supplemented with concentrates suggesting that energy supply was the limiting factor for the non-supplemented diet. The pasture-only treatment described by Sairanen et al. (2005) included the pasture species timothy (*Phleum pratense* L.) and meadow fescue (*Festuca pratensis* L.) and the NDF concentration was higher in comparison with the G treatment in this experiment (509 v 360 g/kg, respectively) likely diluting the energy availability of the diet.

**Table 3.2.** Effect of rolled barley inclusion on DMI, milk production, and animal performance of pasture-fed lactating dairy cows

| | Treatment[2] | | | |
|---|---|---|---|---|
| Item[1] | G | G+RB | SEM | *P*-Value |
| DMI, kg/d | 17.2 | 17.6 | 0.3 | 0.11 |
| Milk yield, kg/d | 21.2 | 21.4 | 1.0 | 0.81 |
| ECM[3], kg/d | 24.6 | 24.1 | 0.8 | 0.41 |
| Milk solids[4], kg/d | 1.68 | 1.65 | 0.05 | 0.43 |
| Milk fat, % | 4.52 | 4.29 | 0.16 | <0.05 |
| Milk fat, kg/d | 0.96 | 0.90 | 0.03 | 0.09 |
| Milk crude protein, % | 3.44 | 3.54 | 0.07 | <0.05 |
| Milk crude protein, kg/d | 0.73 | 0.75 | 0.02 | 0.19 |
| MUN[5], mg/dL | 16.5 | 12.7 | 0.9 | <0.01 |
| Plasma urea N, mg/dL | 9.2 | 7.6 | 0.3 | <0.01 |
| Feed efficiency[6] | 1.45 | 1.36 | 0.05 | <0.05 |
| BW change, kg/d | 0.27 | 0.23 | 0.15 | 0.85 |

[1]Values calculated from data collected on d 21 to 23 of each experimental period.
[2]G = 100% (DM basis) perennial ryegrass; G+RB = 80% perennial ryegrass and 20% rolled barley grain.
[3]Energy corrected milk, estimated according to Tyrrell and Reid, (1965).
[4]Milk solids = kg fat + protein
[5]MUN = milk urea nitrogen.
[6]ECM/DMI.

### *3.4.3. Rumen Characteristics and Rumination*

The inclusion of RB reduced ruminal $NH_3$ pool sizes and concentration (Table 3.3). This was likely due to the increased incorporation of feed N into MicN in G+RB cows as indicated by a higher MicN flow. There was a significant period x treatment interaction effect detected for ruminal $NH_3$, similar to that described above for MUN and PUN. Throughout the experiment, the reticulorumen pH was not different among treatments averaging 6.35 (Table 3.3). The mean pH was slightly higher than the ruminal mean reported by Kolver and deVeth (2002) of 6.15 for a number of pasture-based treatments. However, Falk et al. (2016) concluded that reticulorumen pH recordings are on average 0.24 pH units higher than in the rumen. Applying this correction to the current study would bring the calculated ruminal pH into agreement with that reported for cows consuming high quality PRG forage (Delagarde et al., 1997; Rius et al., 2012). The daily pattern of reticulorumen pH was affected by treatment (Figure 3.1); however, the numerical differences were small with possibly low biological relevance. In another study, de Veth and Kolver (2001) reported that in vivo digestibility of pasture is not compromised unless the ruminal pH is < 6.0 for dairy cows fed highly digestible pasture.

The reduction in ruminal pH of pasture-based cows when supplemented with starch-based concentrates is inconsistent (Bargo et al., 2003). The results of this study are consistent with a number of reports where offering a supplement high in starch at a moderate level to pasture-based diets does not affect mean ruminal pH (Van Vuuren et al, 1993; Khalili and Sairanen, 2000). Overall, the mean rumen pH for the cows fed the G diet in this study was higher than those previously reported (Stakelum and Dillon, 2003; McEvoy et al., 2010). Other variables such as timing of rumen sample collection and method of detection might also influence these results.

The concentration of total VFA in the rumen was increased in cows fed the G+RB diet; however, the pool size determined from rumen evacuations was not affected by treatment (Table 3.3). In cows fed the G+RB diet, the concentration and pool size of propionate were increased in comparison with the G diet and this is consistent with previous reports of barley supplementation to cows being fed fresh pasture (Garcia et al., 2000). The rumen pool size of acetate tended to be reduced with supplementation of RB. This combined with the effect on propionate resulted in a lower ruminal acetate:propionate ratio in cows fed the G+RB diet in comparison with the G diet. The increased proportion of propionate, when cows consumed the G+RB diet, can help explain the increased milk protein concentration observed through the mechanism of increased glucose supply and potentially the influence on the insulin mTOR pathway (Rius et al., 2010). Overall, changes in the concentration of ruminal VFA of pasture-based cows in response to supplementation is inconsistent. This might be due to the considerable diversity of pasture chemical composition and digestibility among studies (Bargo et al., 2003). Further, the level of supplementation will influence the absolute change in supply of rumen-fermentable CHO ultimately dictating VFA concentrations and pool sizes (Sairanen et al., 2005).

**Table 3.3.** Effect of rolled barley inclusion on rumen pool size[1] and concentration of ammonia N, VFA and reticulorumen pH of pasture-fed lactating dairy cows

| | Treatment[2] | | | |
|---|---|---|---|---|
| Item | G | G+RB | SEM | *P*-Value |
| Ammonia N pool size, g | 6.4 | 3.9 | 0.5 | <0.01 |
| Ammonia N concentration, mg/dL | 9.0 | 5.9 | 0.5 | <0.01 |
| VFA[3] pool size, mol | | | | |
| Total VFA | 8.26 | 8.27 | 0.51 | 0.96 |
| Acetate (A) | 5.14 | 4.87 | 0.29 | 0.10 |
| Propionate (P) | 1.74 | 1.99 | 0.13 | <0.01 |
| Butyrate | 1.11 | 1.05 | 0.07 | 0.25 |
| Isobutyrate | 0.06 | 0.05 | 0.01 | 0.11 |
| Valerate | 0.12 | 0.16 | 0.02 | <0.01 |
| Isovalerate | 0.12 | 0.12 | 0.01 | 0.89 |
| Branched-chain VFA | 0.18 | 0.17 | 0.02 | 0.75 |
| A:P ratio, mol/mol | 2.95 | 2.50 | 0.07 | <0.01 |
| VFA[3] concentration, mM | | | | |
| Total VFA | 121.8 | 126.0 | 2.0 | <0.05 |
| Acetate | 75.8 | 74.6 | 1.1 | 0.32 |
| Propionate | 25.7 | 30.2 | 0.8 | <0.01 |
| Butyrate | 16.0 | 16.2 | 0.3 | 0.67 |
| Isobutyrate | 0.9 | 0.8 | 0.1 | 0.43 |
| Valerate | 1.7 | 2.4 | 0.2 | <0.01 |
| Isovalerate | 1.6 | 1.8 | 0.1 | <0.05 |
| Branched-chain VFA | 2.5 | 2.7 | 0.1 | 0.09 |
| Reticulorumen pH | 6.34 | 6.35 | 0.02 | 0.51 |

[1]Nutrient concentration × rumen liquid volume measured from total rumen evacuation.
[2]G = 100% (DM basis) perennial ryegrass; G+RB = 80% perennial ryegrass and 20% rolled barley grain.
[3]VFA = volatile fatty acid.

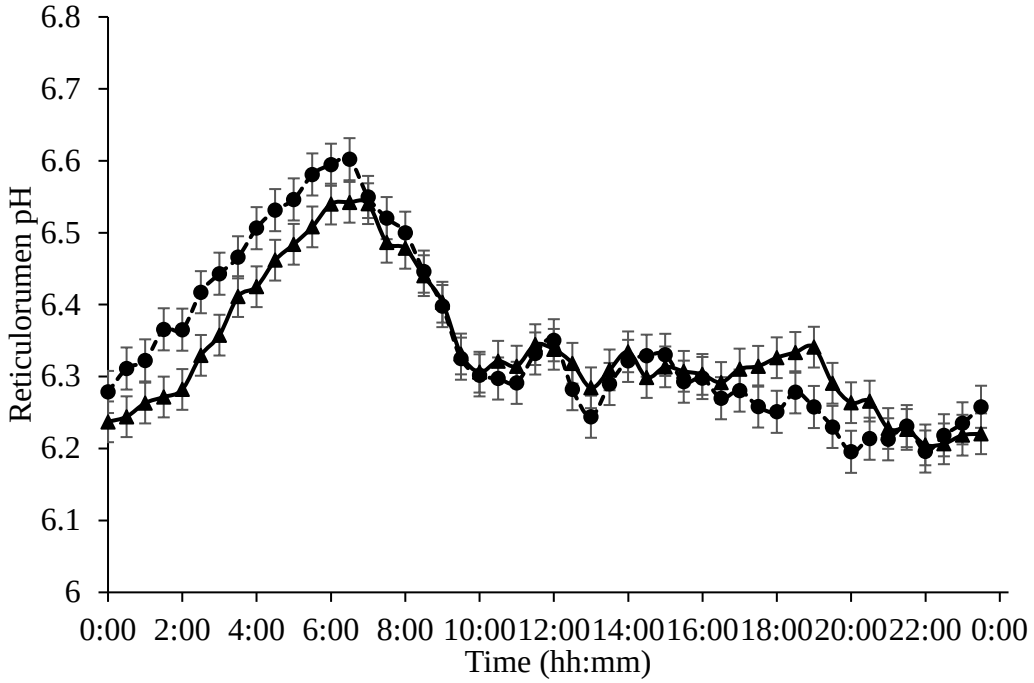


**Figure 3.1.** Reticulorumen pH (lsmean ± SEM) across the day for cows fed the perennial ryegrass-only diet (▲) and the perennial ryegrass supplemented with rolled barley diet (●).

There was no difference in the majority of rumination characteristics between the G and G+RB diets (Table 3.4). Total ruminating time for both treatments was similar to that previously reported for cows consuming PRG-based diets (Gregorini et al., 2013; O'Sullivan et al., 2019). Ruminating time per kg of pdNDFom was increased in cows fed the G+RB diet while ruminating time per kg of uNDFom was decreased in comparison with G (Table 3.4). To the author's knowledge, this is the first time results for these variables have been reported in the literature for cows fed pasture based-diets.

**Table 3.4.** Effect of rolled barley inclusion on rumination parameters of pasture-fed lactating dairy cows

| | Treatment[1] | | | |
|---|---|---|---|---|
| Item | G | G+RB | SEM | *P*-Value |
| Ruminating time, min/d | 493 | 492 | 10 | 0.91 |
| Ruminating bouts, no./d | 19.2 | 19.0 | 0.6 | 0.76 |
| Ruminating bout duration, min/bout | 26 | 27 | 1 | 0.58 |
| Ruminating mastications, no./d | 34,118 | 34,650 | 910 | 0.48 |
| Ruminating mastication rate, no. mastications/min | 69 | 70 | 1 | 0.12 |
| Ruminating boli, no./ruminating bout | 30.8 | 30.6 | 1.0 | 0.72 |
| Ruminating time, min/bolus | 0.9 | 0.9 | 0.1 | 0.37 |
| Ruminating time, min/kg aNDFom per d | 87 | 91 | 3 | 0.1 |
| Ruminating time, min/kg pdNDFom per d | 97 | 104 | 3 | <0.05 |
| Ruminating time, min/kg uNDFom per d | 844 | 725 | 22 | <0.01 |
| Ruminating mastication, no./kg aNDFom per d | 1,720 | 1,694 | 189 | 0.85 |

[1]G = 100% (DM basis) perennial ryegrass; G+RB = 80% perennial ryegrass and 20% rolled barley grain.

### *3.4.4. Digestion of OM, aNDFom, WSC, and Starch*

During the omasal sampling portion of the study, cows were exposed to increased human contact and this might have modestly reduced their DMI, therefore separate intakes are reported for the milk yield data versus the omasal sampling data (Table 3.2 and Table 3.5, respectively). During the omasal sampling phase (d 24-28), the inclusion of RB increased DM and OM intake, and the flow of OM at the omasal canal ($P < 0.01$; Table 3.5). The amount of OM truly degraded in the rumen was greater in cows fed the G+RB diet ($P < 0.01$). However, on a percent of OM intake basis, the ruminal and total tract digestibility (**TTD**) of OM was reduced in cows fed the G+RB diet ($P < 0.01$). The TTD of OM was calculated utilizing uNDFom as an internal marker and values are similar to those previously reported for PRG-diets utilizing total fecal collection (Rius et al., 2012; Beecher et al., 2014).

The intake of aNDFom was reduced in cows fed the G+RB diet; however, aNDFom flow at the omasal canal was increased relative to cows fed the G diet (Table 3.5). Accordingly, aNDFom digestibility decreased, both ruminally and in the total tract, in cows fed the G+RB diet. These results are in agreement with Sairanen et al. (2005) and a number of reviews investigating the response to concentrate supplementation in dairy cows (Huhtanen, 1998; Bargo, 2003). This reduction in aNDFom digestibility explains the majority of the reduction in digestibility observed in the OM fraction. A reduction in rumen pH due to the addition of rapidly fermentable supplements to pasture-based diets is a mechanism commonly cited to explain this reduction in feed digestibility (Dixon and Stockdale, 1999). In the current study, reticulorumen pH was not affected by treatment suggesting this mechanism was not responsible for the negative effect on feed digestion observed. Reduced aNDFom digestibility can be a multifaceted issue. The concentration of uNDFom was higher in the RB grain compared with PRG due to the barley grain

containing hull material (Table 3.1; Firkins et al., 2001). This might have contributed to some of the reduction in aNDFom digestibility, as reported in other studies (Van Vuuren et al., 1993; Sairanen et al., 2005). In a review, Hoover (1986) suggested that the rumen ammonia N concentration required to optimize nutrient digestion was 6.2 mg/dL while microbial growth was optimized at a lower ammonia N concentration of 3.3 mg/dL. Others have suggested that the rumen ammonia N concentration required by the particulate-associated microbes digesting fiber might be greater than that of the liquid-associated microbes (Allison, 1980, McAllan and Smith, 1983). Further, Satter and Slyter (1974) demonstrated that a rumen ammonia level of 5 mg/dL was the minimum required to maintain adequate microbial growth. In the current experiment, rumen ammonia N concentration was close to the threshold of 5 mg/dL in cows fed the G+RB diet, potentially explaining a portion of the reduced aNDFom digestibility. This suggests that on a dynamic basis, with variable rumen ammonia levels throughout the day, there might have been periods where the NFC bacteria could likely outcompete the fiber bacteria for ammonia, decreasing aNDFom digestion. The results of the current experiment suggest that pH was not the main driver of reduced feed digestion in cows fed the G+RB diet. Further work is required to better describe the variables and mechanisms involved in the reduction of pasture digestibility when supplements are provided.

Feed intake is closely related to rumen pool size and rumen turnover (Van Soest, 1994; Forbes, 1995). In pasture-based systems, physical capacity of the reticulorumen, due to the relatively high aNDFom concentration of the diet, has been suggested to limit DMI (Allen, 1996; Baudracco et al., 2010). In the current experiment, the average aNDFom concentration of PRG was 36% of DM resulting in a rumen aNDFom pool size of 1% BW. The inclusion of RB increased rumen OM, aNDFom, and uNDFom pool size ($P < 0.05$; Table 3.5), which suggests that DMI intake of the G

diet was not limited by physical fill capacity of the rumen. The rumen aNDFom pool size in cows fed the G+RB in the current study (1.1% BW) was similar to that previously reported for cows consuming pasture-based diets (Sairanen et al., 2005; Taweel et al., 2005). Sairanen et al. (2005) remarked that the rumen NDF pools observed when cows consumed highly digestible pasture was considerably lower compared with that of cows fed grass silage or red clover-grass silage (Khalili and Huhtanen, 2002; Rinne et al., 2002). This suggests that in the current study, the G+RB cows had the potential for greater rumen fill capacity if other environmental conditions allowed.

**Table 3.5.** Effect of rolled barley inclusion on rumen pool size, nutrient flow to the omasum, and digestibility of DM, OM, aNDFom, and uNDFom of pasture-fed lactating dairy cows

| | Treatment[2] | | | |
|---|---|---|---|---|
| Item[1] | G | G+RB | SEM | *P*-Value |
| DM | | | | |
| Intake, kg/d | 16.1 | 17.1 | 0.4 | <0.01 |
| OM | | | | |
| Intake, kg/d | 15.1 | 16.1 | 0.4 | <0.01 |
| Rumen pool, kg/d | 8.3 | 9.1 | 0.5 | <0.05 |
| Flow at omasal canal, kg/d | 6.9 | 7.7 | 0.3 | <0.01 |
| Apparently digested in the rumen, kg/d | 8.2 | 8.4 | 0.2 | 0.28 |
| Truly digested in the rumen, kg/d | 13.2 | 13.9 | 0.3 | <0.01 |
| % of OM intake | 87.9 | 86.1 | 0.6 | <0.01 |
| Total-tract apparent digestibility, % | 85.2 | 82.0 | 0.3 | <0.01 |
| aNDFom[4] | | | | |
| Intake, kg/d | 5.8 | 5.6 | 0.2 | <0.05 |
| Rumen pool, kg/d | 4.8 | 5.5 | 0.3 | <0.01 |
| Flow at omasal canal, kg/d | 1.6 | 2.0 | 0.1 | <0.01 |
| Apparently digested in the rumen, kg/d | 4.2 | 3.6 | 0.1 | <0.01 |
| % of aNDFom intake | 72.3 | 63.1 | 0.9 | <0.01 |
| % of pdNDFom[5] intake | 80.4 | 72.3 | 1.0 | <0.01 |
| Total-tract apparent digestibility, % | | | | |
| % of aNDFom intake | 83.2 | 74.5 | 0.6 | <0.01 |
| % of pdNDFom intake | 92.5 | 85.4 | 0.7 | <0.01 |
| uNDFom[6] | | | | |
| Intake, kg/d | 0.58 | 0.71 | 0.02 | <0.01 |
| Rumen pool, kg/d | 1.55 | 1.75 | 0.10 | <0.01 |

[1]Values calculated from data collected on d 24 to 28 of each experimental period.
[2]G = 100% (DM basis) perennial ryegrass; G+RB = 80% perennial ryegrass and 20% rolled barley grain.
[3]Corrected for microbial and VFA contribution to flows.
[4]aNDFom = amylase- and sodium sulfite-treated NDF corrected for ash residue.
[5]pdNDFom = potentially digestible aNDFom.
[6]uNDFom = undigested amylase- and sodium sulfite treated NDF corrected for ash residue.

The average WSC concentration of the PRG in this experiment was comparable to that of grass cultivars selected for high sugar concentration (Taweel et al., 2005; Moorby et al., 2006). Water-soluble carbohydrate intake was reduced in cows fed the G+RB diet (Table 3.6) due to the high substitution rate and a lower WSC concentration in the RB. Further, both the amount and proportion of WSC digested in the rumen and in the total-tract were decreased with the supplementation of RB. This might indicate lower digestibility of WSC in G+RB fed cows in comparison with the G; however, for cows fed the G+RB diet, intermediates of starch degradation might have been detected as, and contributed to, WSC omasal flow. In cows fed the G diet, over 90% of the WSC was degraded in the rumen, which has important implications for both the cow's energy supply via VFA production and the synthesis of bacterial and protozoal N. To the authors' knowledge no other data are available quantifying ruminal digestion of WSC in pasture-based diets. The ruminal digestibility of starch in cows fed the G+RB diet was 89%, similar to the results suggested in a review by Nocek and Tamminga (1991). In the current experiment, the apparent TTD of starch was 98%, which was comparable and slightly higher than that reported by Firkins et al. (2001) and Overton et al. (1995).

**Table 3.6.** Effect of rolled barley inclusion on nutrient flow to the omasum and digestibility of WSC and starch of pasture-fed lactating dairy cows

| | Treatment[2] | | | |
|---|---|---|---|---|
| Item[1] | G | G+RB | SEM | *P*-Value |
| WSC[3] | | | | |
| Intake, kg/d | 3.55 | 3.25 | 0.10 | <0.01 |
| Flow at omasal canal, kg/d | 0.24 | 0.37 | 0.03 | <0.01 |
| Apparently digested in the rumen, kg/d | 3.32 | 2.87 | 0.08 | <0.01 |
| % of WSC intake | 93.2 | 88.7 | 0.5 | <0.01 |
| Total-tract apparent digestibility, % | 99.4 | 98.6 | 0.1 | <0.01 |
| Starch | | | | |
| Intake, kg/d | 0.38 | 2.42 | 0.01 | <0.01 |
| Flow at omasal canal, kg/d | - | 0.26 | - | - |
| Apparently digested in the rumen, kg/d | - | 2.16 | - | - |
| % of starch intake | - | 89.2 | - | - |
| Total-tract apparent digestibility, % | - | 97.9 | - | - |

[1]Values calculated from data collected on d 24 to 28 of each experimental period.
[2]G = 100% (DM basis) perennial ryegrass; G+RB = 80% perennial ryegrass and 20% rolled barley grain.
[3]WSC= Water Soluble Carbohydrate.

### *3.4.5. Rumen Digestion Kinetics*

There was no difference in the apparent turnover of OM between treatments (Table 3.7). Compared with the G diet, the inclusion of RB increased the turnover time required for pdNDFom in the rumen and this is consistent with the decreased rumen ammonia concentration of those cows and previous studies that increased the starch concentration of the diet (Van Vuuren et al., 1993). Conversely, when cows consumed the G+RB diet, the turnover time for uNDFom was reduced due to the inclusion of RB in the diet, and the rate of passage increased for pdNDFom and uNDFom. The rate of digestion of pdNDFom was decreased with the inclusion of RB, which is similar to that previously discussed for ruminal pdNDFom digestibility with fermentable carbohydrate supplementation (Sairanen et al., 2005). The liquid flow tended to decrease when cows consumed the G+RB diet; however, the liquid rate of passage was not different between treatments (Table 3.7). The liquid rate of passage measured in the current experiment (approximately 0.20 $h^{-1}$) was similar to that previously estimated for cows consuming pasture-based diets (Van Vuuren et al., 1992; Rius et al., 2012).

**Table 3.7.** Effect of rolled barley inclusion on rumen turnover, rate of passage, and rate of digestion of OM, pdNDFom, uNDFom, and liquid flow of pasture-fed lactating dairy cows

| | Treatment[1] | | | |
|---|---|---|---|---|
| Item | G | G+RB | SEM | *P*-Value |
| OM | | | | |
| Apparent turnover, h | 13.3 | 13.6 | 0.6 | 0.44 |
| pdNDFom[3] | | | | |
| Turnover, h | 15.4 | 18.9 | 1.0 | <0.01 |
| Rate of passage, %/h | 1.3 | 1.5 | 0.1 | <0.01 |
| Rate of digestion, %/h | 5.6 | 4.2 | 0.3 | <0.01 |
| uNDFom[4] | | | | |
| Turnover, h | 64.0 | 59.0 | 2.6 | <0.05 |
| Rate of passage, %/h | 1.6 | 1.8 | 0.1 | <0.01 |
| Liquid | | | | |
| Liquid flow, L/d | 336 | 309 | 18 | 0.08 |
| Rate of passage, %/h | 20.9 | 19.9 | 0.9 | 0.39 |

[1]G = 100% (DM basis) perennial ryegrass; G+RB = 80% perennial ryegrass and 20% rolled barley grain.
[2]aNDFom = amylase- and sodium sulfite-treated NDF corrected for ash residue.
[3]pdNDFom = potentially digestible aNDFom.
[4]uNDFom = undigested amylase- and sodium sulfite treated NDF corrected for ash residue.

### *3.4.6. Digestion and Omasal Flow of Nitrogen*

During the sampling phase, N intake was similar between treatments due to the lower N concentration but higher DMI of cows consuming the G+RB diet (Table 3.8). Inclusion of RB increased the flow of NAN at the omasal canal ($P < 0.01$) compared with the G diet. This is consistent with the results observed by Van Vuuren et al. (1993) that offered a starch supplement and Sairanen et al. (2005) that offered a low CP pelleted supplement. In the current experiment, the increase in NAN can be attributed to an increased flow of MicN in cows fed the G+RB diet compared with the G diet ($P < 0.01$). This increase in MicN flow, when cows consumed the G+RB diet, can help explain the increased milk protein concentration observed (Table 3.2) through the mechanism of increased AA supply and greater propionate concentration. There was no difference in NANMN flow between the treatments; however, the contribution of NANMN to the total NAN flow was relatively low compared with previous studies (O'Mara et al., 1997; Younge et al., 2004). The NANMN flow is typically estimated by difference (i.e. NAN flow – MicN flow) therefore, any error in either of these estimations will be partitioned into the NANMN flow. Key differences between the current study and previous studies were that in both Younge et al. (2004) and O'Mara et al. (1997) protozoal N flow was not determined and purine derivatives were used to determine MicN. Estimates utilizing purine derivatives as microbial markers have been shown to have lower precision and accuracy compared with techniques using $^{15}N$ (Firkins and Reynolds, 2005; Del Valle et al., 2019). These inaccuracies have further implications in regards to the determination of ruminal digestible feed N, as an underestimated MicN flow will underestimate digestibility. In the present study, the average feed N ruminal digestibility, corrected for microbial contributions, was 89%. This was not different between treatments and was comparable to that reported by Sairanen et al. (2005; 85%) and Kolver and Muller, (1998; 84%). The results of the current experiment,

utilizing the omasal sampling technique and $^{15}N$ isotope labelling, confirms that extensive rumen proteolysis of ingested N occurs when PRG is consumed (Beever et al., 1986; Delagarde et al., 1997; Table 3.8). This highlights the significant dependence of cows grazing such swards, on MicN as their main NAN supply and source of metabolizable AA.

Of the total MicN flow, protozoal N contributed on average 22% and was not different between treatments. There are few quantitative data describing protozoal N flow in pasture-fed cows. The levels reported in this study are within the range proposed by Dijkstra et al. (1998; 10.7% – 26.1%) in computer simulations of cows consuming similar amounts of DMI. Supplementation with RB did not increase protozoal N flow in the current experiment, which is in contrast to previous reports for cows consuming grass- (Khalili and Sairanen, 2000) and grass silage-based diets (Ahvenjärvi et al., 2002). It is difficult to ascertain the reason for this; however, the high WSC concentration of the fresh PRG might have provided ample sugar to sustain high protozoal growth (Clarke, 1965; Williams and Coleman, 1988). Denton et al. (2015) recently demonstrated that mixed protozoa could sequester sugar away from bacteria, giving protozoa a competitive advantage under such ruminal conditions.

**Table 3.8.** Effect of rolled barley inclusion on the flow of N of pasture-fed lactating dairy cows

| | Treatment[2] | | | |
|---|---|---|---|---|
| Item[1] | G | G+RB | SEM | *P*-Value |
| N intake, g/d | 429 | 424 | 11 | 0.53 |
| Flow at omasal canal | | | | |
| Total N, g/d | 394 | 436 | 18 | <0.01 |
| Ammonia N, g/d | 21 | 14 | 1 | <0.01 |
| NAN[3] | | | | |
| g/d | 373 | 422 | 18 | <0.01 |
| % of N intake | 90.9 | 99.3 | 2.8 | <0.05 |
| NANMN[4] | | | | |
| g/d | 49.1 | 47.7 | 4.1 | 0.78 |
| % of N intake | 11.6 | 11.0 | 0.9 | 0.65 |
| Microbial NAN | | | | |
| g/d | 324 | 374 | 15 | <0.01 |
| % of total NAN | 87.1 | 88.8 | 0.8 | 0.17 |
| Bacteria NAN | | | | |
| g/d | 248 | 298 | 18 | <0.01 |
| % of microbial NAN flow | 76.5 | 80.1 | 3.2 | 0.24 |
| Protozoa NAN | | | | |
| g/d | 79 | 73 | 11 | 0.55 |
| % of microbial NAN flow | 23.5 | 20.0 | 3.2 | 0.24 |
| g of microbial N/kg of OTDR[5] | 24.4 | 26.6 | 0.7 | <0.05 |
| True ruminal N digestibility, % | 88.4 | 89.0 | 0.9 | 0.65 |

[1]Values calculated from data collected on d 24 to 28 of each experimental period.
[2]G = 100% (DM basis) perennial ryegrass; G+RB = 80% perennial ryegrass and 20% rolled barley grain.
[3]NAN = non-ammonia nitrogen.
[4]NANMN = non-ammonia non-microbial nitrogen.
[5]OTDR = organic matter truly digested in the rumen.

### *3.4.7. Microbial Dynamics*

Compared with the G diet, the inclusion of RB increased the rumen pool size and true ruminal digestion rate of both digestible OM and fermentable carbohydrates (Table 3.9). The rumen bacterial pool size was not affected by treatment; however, cows fed the G diet had a greater microbial OM contribution to the total OM rumen pool than cows fed the G+RB diet (29.3% vs. 26.5% for G vs. G+RB, respectively; $P < 0.01$). Consistent with the observed increase in bacterial N flow (Table 3.8), fractional growth rate of bacteria increased in cows fed the G+RB diet, with a number of studies reporting similar effects (Nocek and Russell, 1988). The Yg (i.e. yield of microbial DM per g of CHO degraded) increased in cows fed the G+RB diet compared with G (0.56 vs. 0.43, respectively). Variable Yg values, in vitro, have previously been reported due to differing CHO sources (Nocek, 1988); however, values greater than 0.5, the theoretical maximum (Isaacson et al., 1975) are rare. Using a biochemical approach, Stouthamer (1973) reported a maximal Yg of approximately 0.8 g/g of glucose, indicating the potential for higher yields to be achieved in vivo.

Protozoa are expected to sequester in the rumen and contribute about 50% of the microbial biomass (Jouany, 1996). However, these inferences are primarily based on in vitro data or data that was often generated from procedures with low accuracy (Sylvester et al., 2005; Karnati et al., 2007). Until recently, no measurements of in vivo protozoal generation time existed for high-producing dairy cows (Karnati et al., 2007). In the current study, protozoal rumen pool size, fractional growth rate, and generation time were not affected by treatment (Table 3.9). For both treatments, however, protozoa N contributed considerably less to the total MicN pool in the rumen (5%) in comparison with at the omasal canal (22%). This resulted in an average protozoa generation time of 4.1 h, which is extremely short in comparison with current expectations

(Jouany, 1996). Using protozoa cell count procedures, Karnati et al. (2007) determined a protozoa generation time of 16.4 h. The authors noted, however, that low ruminal pH appeared to reduce protozoal diversity and this low rumen pH might have also influenced protozoal metabolism (Franzolin and Dehority, 2010). Firkins et al. (2007) suggested that a higher rumen passage rate could allow less lysis of protozoa in vivo than in vitro, lower ruminal pool size of protozoa relative to bacteria, and improve efficiency of protozoal cell growth (i.e. faster cell division). In agreement, Sylvester et al. (2009) demonstrated that rumen ciliated protozoa could decrease generation time in response to increasing dilution rate; with other researchers reporting similar effects (Harrison et al., 1976; Dehority, 2004). In the current study, the liquid passage rate averaged 0.20 $h^{-1}$ (Table 3.7), a 27% increase compared with Karnati et al. (2007). This might provide a mechanism to help explain the high protozoal growth efficiency and low rumen pool size relative to bacteria observed in the current study. However, based on any published information, an in vivo generation time as short as the current study has only once been previously reported (Warner, 1962).

To maintain viable populations, protozoa must have a generation time that is shorter than the retention time of the phase in which they leave the rumen (Dehority, 2003). Hence, passage of protozoa from the rumen has previously been estimated by comparing the calculated protozoal generation time with the retention time of the liquid or particle rumen phases (Sylvester et al., 2005; Karnati et al., 2007). In the current experiment, liquid retention time and protozoa generation time averaged 5 h and 4.1 h, respectively, which demonstrates the protozoa's ability to leave the rumen in the liquid phase. Others have demonstrated that the mean generation time of protozoa seems to approximate the mean retention time of particulate matter in the rumen (Sylvester et al., 2005; Karnati et al., 2007) which has been interpreted to reflect chemotaxis of protozoa for feed particles (Diaz et al., 2014). In the current study, the fractional rate of CHO digestion was quite

high, averaging 0.13 $h^{-1}$, as fresh, immature PRG can have a high digestion rate. This implies that the CHO in the rumen turned over every 8 h. Thus, even if a portion of the protozoa exhibit a chemotaxis toward digestible particulates, they still need to have a generation time that is shorter than previously characterized other than that reported by Warner (1962).

Calculation of protozoa generation time involves both rumen pool size and omasal flow of protozoa, therefore, error in either measurement can influence the result obtained. Using a real-time polymerase chain reaction assay, Sylvester et al. (2005) reported low protozoal proportions in the rumen (9%), similar to the current study. However, rumen protozoa, especially isotrichids, have been reported to follow a diurnal cycle (Potter and Dehority, 1973; Dehority, 2003). As rumen protozoa were sampled at a lower frequency in the current study compared with Karnati et al. (2007), this might have contributed to an underestimation of the rumen pool size and hence, an underestimation of protozoal generation time. Further studies investigating both the rumen pool size and omasal flow of protozoa are required to confirm the dynamics observed in the current study.

**Table 3.9.** Effect of rolled barley inclusion on rumen pool sizes, fractional rates of microbial growth and nutrient digestion, and generation time of pasture-fed lactating dairy cows

| | Treatment[1] | | | |
|---|---|---|---|---|
| Item | G | G+RB | SEM | *P*-Value |
| Rumen pool size | | | | |
| Digestible OM[2], kg | 4.35 | 4.98 | 0.33 | <0.05 |
| Total fermentable CHO[3], kg | 3.43 | 4.09 | 0.29 | <0.01 |
| Total NAN, g | 333 | 325 | 15 | 0.45 |
| Microbial NAN[4], g | 239 | 233 | 11 | 0.54 |
| Microbial OM proportion of rumen OM pool, % | 29.3 | 26.5 | 0.8 | <0.01 |
| Bacteria NAN[5], g | 226 | 221 | 12 | 0.60 |
| Protozoa NAN, g | 13 | 12 | 3 | 0.73 |
| Protozoa NAN pool, % total microbial NAN pool | 5.3 | 5.2 | 1.1 | 0.92 |
| Rumen kinetics | | | | |
| Fractional growth rate of bacteria[6], $h^{-1}$ | 0.046 | 0.057 | 0.003 | <0.05 |
| Fractional growth rate of protozoa[6], $h^{-1}$ | 0.301 | 0.299 | 0.035 | 0.98 |
| Fractional growth rate of all microbes, $h^{-1}$ | 0.058 | 0.067 | 0.003 | <0.01 |
| Ruminal true OM digestion rate, g/h | 551 | 580 | 13 | <0.01 |
| Ruminal true CHO digestion rate, g/h | 453 | 479 | 11 | <0.01 |
| Fractional rate of OM digestion[7], $h^{-1}$ | 0.133 | 0.122 | 0.007 | <0.05 |
| Fractional rate of CHO digestion[7], $h^{-1}$ | 0.141 | 0.124 | 0.008 | <0.01 |
| Observed Yg[8], g of cells / g of CHO degraded | 0.43 | 0.56 | 0.03 | <0.01 |
| Generation time of bacteria[9], h | 22.6 | 18.5 | 1.2 | <0.05 |
| Generation time of protozoa[9], h | 4.0 | 4.1 | 0.5 | 0.93 |
| Generation time of microbes[9], h | 18.0 | 15.4 | 0.8 | <0.05 |
| Liquid retention time[10], h | 5.0 | 5.1 | 0.2 | 0.71 |

[1]G = 100% (DM basis) perennial ryegrass; G+RB = 80% perennial ryegrass and 20% rolled barley grain.
[2]Measured OM from rumen evacuation, corrected for microbial OM and undigested NDF after 240 h of in vitro digestion and analyzed with amylase, sodium sulfite and ash corrected (Raffrenato et al., 2018).
[3]Rumen OM pool – (rumen CP pool – microbial CP pool) – (rumen DM pool × diet fat concentration).
[4]NAN = non-ammonia nitrogen.
[5]Microbial NAN pool – protozoal NAN pool
[6]Bacterial or protozoal daily flow (g/h)/bacterial or protozoal pool size (g)
[7]Organic matter or carbohydrate degraded (g/h)/ organic matter or carbohydrate rumen pool size (g)
[8]Fractional microbial growth rate/fractional rate of CHO digestion.
[9]Reciprocal of fractional growth rate of bacteria, protozoa or all microbes
[10]Reciprocal of liquid passage rate

## 3.5. CONCLUSIONS

In this study, RB supplementation did not benefit overall performance and reduced ruminal aNDFom digestibility; however, this was not mediated through a reduction in reticulorumen pH. Rumen pool size and fractional digestion rate of digestible OM and fermentable CHO were increased in cows fed the G+RB diet. It seems likely that this increased fermentable CHO supply mediated an increased fractional growth rate and omasal flow of bacteria in cows fed G+RB diet. For both diets, the contribution of MicN to the total flow of NAN, together with high ruminal digestibility of feed N, underlines the large dependence of cows consuming fresh PRG-based diets on MicN. Further quantification of the specific AA contributing to this NAN flow is required. Protozoa N flow was not different between diets, although, protozoa appear to supply a much larger amount of MicN and exhibit shorter generation time than previously considered. This was most likely due to the rapid rumen turnover and high sugar concentration of PRG-based diets. The data generated in this study can be used to evaluate predictions from nutrition models and modify predictions of bacterial and protozoal growth and passage under conditions of high quality pasture intake.

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## 3.7. REFERENCES


Ahvenjärvi, S., A. Vanhatalo, and P. Huhtanen. 2002. Supplementing barley or rapeseed meal to dairy cows fed grass-red clover silage: I. Rumen degradability and microbial flow. J. Anim. Sci. 80:2176– 2187. https://doi.org/10.1093/ansci/80.8.2176.

Allen, M. S. 1996. Physical constraints on voluntary intake of forages by ruminants. J. Anim. Sci. 74**:**3063-3075. https://doi.org/10.2527/1996.74123063x.

Allison, M. 1980. Nitrogen requirements of ruminal bacteria. Protein requirements for cattle. Stillwater: Oklahoma State University**,** 128-132.

AOAC International. 2005. Official Methods of Analysis. 18th ed. AOAC International, Gaithersburg, MD

Bargo, F., L. D. Muller, E. S. Kolver, and J. E. Delahoy. 2003. Invited review: Production and digestion of supplemented dairy cows on pasture. J. Dairy Sci. 86:1–42. https://doi.org/10.3168/jds.S0022-0302(03)73581-4.

Baudracco, J., N. Lopez-Villalobos, C. Holmes, and K. Macdonald. 2010. Effects of stocking rate, supplementation, genotype and their interactions on grazing dairy systems: a review. N.Z. J. Agric. Res. 53**:**109-133. https://doi.org/10.1080/00288231003777665.

Beecher, M., F. Buckley, S. M. Waters, T. Boland, D. Enriquez-Hidalgo, M. Deighton, M. O'Donovan, and E. Lewis. 2014. Gastrointestinal tract size, total-tract digestibility, and rumen microflora in different dairy cow genotypes. J. Dairy Sci. 97**:**3906-3917. https://doi.org/10.3168/jds.2013-7708.

Beever, D., H. Losada, D. Gale, M. Spooner, and M. Dhanoa. 1987. The use of monensin or formaldehyde to control the digestion of the nitrogenous constituents of perennial ryegrass (Lolium perenne cv. Melle) and white clover (Trifolium repens cv. Blanca) in the rumen of cattle. Br. J. Nutr. 57**:**57-67. https://doi.org/10.1079/BJN19870009.

Beever, D. E., H.R. Losada, S.B. Cammell, R. T. Evans, and M. J. Haines. 1986. Effect of forage species and season on nutrient digestion and supply in grazing cattle. Br. J. Nutr 56**:**209-225. https://doi.org/10.1079/BJN19860101.

Clarke, R. 1965. Diurnal variation in the numbers of rumen ciliate protozoa in cattle. N.Z. J. Agric. Res. 8**:**1-9. https://doi.org/10.1080/00288233.1965.10420018.

Diaz, H., S. Karnati, M. Lyons, B. Dehority, and J. L. Firkins. 2014. Chemotaxis toward carbohydrates and peptides by mixed ruminal protozoa when fed, fasted, or incubated with

polyunsaturated fatty acids. J. Dairy Sci. 97:2231-2243. https://doi.org/10.3168/jds.2013-7428.

de Veth, M. J., and E. S. Kolver. 2001. Digestion of ryegrass pasture in response to change in pH in continuous culture. J. Dairy Sci. 84:1449–1457. https://doi.org/10.3168/jds.S0022-0302(01)70178-6.

Dehority, B. A. 2004. In vitro determination of generation times for Entodinium exiguum, Ophryoscolex purkynjei, and Eudiplodinium maggii. J. Eukaryot. Microbiol. 51:333–338. https://doi.org/10.1111/j.1550-7408.2004.tb00575.x.

Dehority, B. A. 2003. Rumen microbiology, Nottingham: Nottingham University Press.

Dehority, B. A. 1998. Generation times of Epidinium caudatum and Entodinium caudatum, determined in vitro by transferring at various time intervals. J. Anim. Sci. 76**:**1189-1196. https://doi.org/10.2527/1998.7641189x.

Del Valle, T. A., L. G. Ghizzi, E. M. D. C. Zilio, J. Marques, M. Dias, T. Silva, L. S. Gheller, G. Silva, N. Sconamiglio, and A. Nunes. 2019. Evaluation of $^{15}N$ and purine bases as microbial markers to estimate ruminal bacterial nitrogen outflow in dairy cows. Anim. Feed Sci. Technol. 258**:**114297. https://doi.org/10.1016/j.anifeedsci.2019.114297.

Delaby, L., Peyraud, J., Vérité, R. and Marquis, B. 1995. Effet de la complémentation protéique sur les performances des vaches laitières au pâturage conduit à 2 niveaux de fertilisation. Ann. Zootech. 44:173-188. https://hal.archives-ouvertes.fr/hal-00889176.

Delagarde, R., J. Peyraud, and L. Delaby. 1997. The effect of nitrogen fertilization level and protein supplementation on herbage intake, feeding behaviour and digestion in grazing dairy cows. Anim. Feed Sci. Technol. 66**:**165-180.

Delagarde, R., and J. Peyraud. 1995. Influence of wheat supplementation on intake and digestibility in dairy cows fed autumn grass ad libitum indoors. Ann. Zootech. 44:210-210. https://hal.archives-ouvertes.fr/hal-00889372.

Denton, B. L., L. E. Diese, J. L. Firkins, and T. J. Hackmann. 2015. Accumulation of reserve carbohydrate by rumen protozoa and bacteria in competition for glucose. Appl. Environ. Microbiol. 81**:**1832-1838. https://doi.org/10.1128/AEM.03736-14.

Dijkstra, J., J. France, and S. Tamminga. 1998. Quantification of the recycling of microbial nitrogen in the rumen using a mechanistic model of rumen fermentation processes. J. Agric. Sci. 130**:**81-94. https://doi.org/10.1017/S0021859697004929.

Dineen, M., B. McCarthy, P. Dillon, S. Fessenden, P. LaPierre, and M. E. Van Amburgh. 2019. Evaluation and development of the Cornell Net Carbohydrate and Protein System v. 7 using a unique pasture-based data set. Pages 197-215 in Proc. Cornell Nutrition Conference, Syracuse, NY. Cornell University, Ithaca, NY. https://ecommons.cornell.edu/handle/1813/67045.

Dixon, R. M., and C. R. Stockdale. 1999. Associative effects between forages and grains: Consequences for feed utilization. Aust. J. Agric. Res. 50:757–773. https://doi.org/10.1071/AR98165.

Edmonson, A., I. Lean, L. Weaver, T. Farver, and G. Webster. 1989. A body condition scoring chart for Holstein dairy cows. J. Dairy Sci. 72**:**68-78. https://doi.org/10.3168/jds.S0022-0302(89)79081-0.

Falk, M., A. Münger, and F. Dohme-Meier. 2016. A comparison of reticular and ruminal pH monitored continuously with 2 measurement systems at different weeks of early lactation. J. Dairy Sci. 99**:**1951-1955. https://doi.org/10.3168/jds.2015-9725.

Fessenden, S. W., A. Foskolos, T. J. Hackmann, D. A. Ross, E. Block, and M. E. Van Amburgh. 2019a. Effects of a commercial fermentation byproduct or urea on milk production, rumen metabolism, and omasal flow of nutrients in lactating dairy cattle. J. Dairy Sci. 102:3023–3035. https://doi.org/10.3168/jds.2018-15447.

Fessenden, S. W., T. J. Hackmann, D. A. Ross, E. Block, A. Foskolos, and M. E. Van Amburgh. 2019b. Rumen digestion kinetics, microbial yield, and omasal flows of nonmicrobial, bacterial, and protozoal amino acids in lactating dairy cattle fed fermentation by-products or urea as a soluble nitrogen source. J. Dairy Sci. 102:3036–3052. https://doi.org/10.3168/jds.2018-15448.

Firkins, J. L., Z. Yu, T. Park, and J. E. Plank. 2020. Extending Burk Dehority's perspectives on the role of ciliate protozoa in the rumen. Front Microbiol 11:123. https://doi.org/10.3389/fmicb.2020.00123.

Firkins, J. L., Z. Yu, and M. Morrison. 2007. Ruminal nitrogen metabolism: perspectives for integration of microbiology and nutrition for dairy. J. Dairy Sci. 90(Suppl. 1), E1–E16. https://doi.org/10.3168/jds.2006-518.

Firkins, J. and Z. Yu. 2006. Characterisation and quantification of the microbial populations in the rumen. Pages 19-54 in Ruminant physiology, digestion, metabolism and impact of nutrition

on gene expression, immunology and stress. K. Sejrsen, T. Hvelplund, and M. O. Nielsen, ed. Wageningen Academic Publishers, Netherlands.

Firkins, J. L., and C. K. Reynolds. 2005. Whole animal nitrogen balance in cattle. Pages 167–185 in Nitrogen and Phosphorus Nutrition of Cattle: Reducing the Environmental Impact of Cattle Operations. E. Pfeffer and A. N. Hristov, ed. CAB International, Wallingford, UK.

Firkins, J. L., M. L. Eastridge, N. R. St-Pierre, and S.M. Noftsger. 2001. Effects of grain variability and processing on starch utilization by lactating dairy cattle. J. Anim. Sci. 79**:**218-238. https://doi.org/10.2527/jas2001.79E-SupplE218x.

Forbes, J. M. 1995. Voluntary food intake and diet selection in farm animals. CAB International, Wallingford, UK.

France, J., and R. Siddons. 1986. Determination of digesta flow by continuous market infusion. J. Theor. Biol. 121:105–119. https://doi.org/10.1016/S0022-5193(86)80031-5.

Franzolin, R., and B. A. Dehority. 2010. The role of pH on the survival of rumen protozoa in steers. R Bras Zootec 39:2262–2267. https://doi.org/10.1590/S1516-35982010001000023.

Garcia, S. C., C. J. Santini, and J. C. Elizalde. 2000. Sites of digestion and bacterial protein synthesis in dairy heifers fed fresh oats with or without corn or barley grain. J. Dairy Sci. 83:746–755. https://doi.org/10.3168/jds.S0022-0302(00)74937-X.

Gregorini, P., E. Minnee, W. Griffiths, and J. Lee. 2013. Dairy cows increase ingestive mastication and reduce ruminative chewing when grazing chicory and plantain. J. Dairy Sci. 96:7798-7805. https://doi.org/10.3168/jds.2013-6953.

Hall, M. B. 2015. Determination of dietary starch in animal feeds and pet food by an enzymatic-colorimetric method: Collaborative study. J. AOAC International, 98**:**397-409. https://doi.org/10.5740/jaoacint.15-012.

Hall, M. B. 2014. Selection of an empirical detection method for determination of water-soluble carbohydrates in feedstuffs for application in ruminant nutrition. Anim. Feed Sci. Technol. 198**:**28-37. https://doi.org/10.1016/j.anifeedsci.2014.08.009.

Harrison, D. G., D. E. Beever, D. J. Thomson, and D.F. Osbourn. 1976. Manipulation of fermentation in the rumen. J. Sci. Food Agri. 27**:**617-620. https://doi.org/10.1002/jsfa.2740270706.

He, M., and F. A. Dijkstra. 2014. Drought effect on plant nitrogen and phosphorus: a meta-analysis. New Phytologist. 204**:**924-931. https://doi.org/10.1111/nph.12952.

Hoover, W. 1986. Chemical factors involved in ruminal fiber digestion. J. Dairy Sci. 69**:**2755-2766. https://doi.org/10.3168/jds.S0022-0302(86)80724-X.

Huhtanen, P., H. Khalili, J. I. Nousiainen, M. Rinne, S. Jaakkola, T. Heikkilä, and J. Nousiainen. 2002. Prediction of the relative intake potential of grass silage by dairy cows. Livest. Prod. Sci. 73**:**111-130. https://doi.org/10.1016/S0301-6226(01)00279-2.

Huhtanen, P. 1998. Supply of nutrients and productive responses in dairy cows given diets based on restrictively fermented silage. Agric. Food Sci. Finl. 7:219–250. https://doi.org/10.23986/afsci.72861.

Huhtanen, P., P. G. Brotz, and L. D. Satter. 1997. Omasal sampling technique for assessing fermentative digestion in the forestomach of dairy cows. J. Anim. Sci. 75:1380–1392. https://doi.org/10.2527/1997.7551380x.

Hungate, R. E. 1966. The rumen and its microbes. Academic Press, New York.

Isaacson, H. R., F. C. Hinds, M. P. Bryant, and F. N. Owens. 1975. Efficiency of energy utilization by mixed rumen bacteria in continuous culture. J. Dairy Sci. 58:1645–1659. https://doi.org/10.3168/jds.S0022-0302(75)84763-1.

Jouany, J. P. 1996. Effect of rumen protozoa on nitrogen utilization by ruminants. J. Nutr. 126: 1335S–1346S. https://doi.org/10.1093/jn/126.suppl_4.1335S.

Karnati, S. K. R., J. T. Sylvester, S. M. Noftsger, Z. Yu, N. R. St-Pierre, and J. L. Firkins. 2007. Assessment ofruminal bacterial populations and protozoal generation time in cows fed different methionine sources. J. Dairy Sci. 90:798–809. https://doi.org/10.3168/jds.S0022-0302(07)71564-3

Khalili, H., and A. Sairanen. 2000. Effect of concentrate type on rumen fermentation and milk production of cows at pasture. Anim. Feed Sci. Technol. 84:199–212. https://doi.org/10.1016/S0377-8401(00)00130-9.

Kolver, E. S., and M. J. de Veth. 2002. Prediction of ruminal pH on pasture-based diets. J. Dairy Sci. 85:1255–1266. https://doi.org/10.3168/jds.S0022-0302(02)74190-8.

Kolver, E. S., and L. D. Muller. 1998. Performance and nutrient intake of high producing Holstein cows consuming pasture or total mixed ration. J. Dairy Sci. 81:1403–1411. https://doi.org/10.3168/jds.S0022-0302(98)75704-2.

Licitra, G., T. M. Hernandez, and P. J. Van Soest. 1996. Standardization of procedures for nitrogen fractionation of ruminant feeds. Anim. Feed Sci. Technol. 57:347-358. https://doi.org/10.1016/0377-8401(95)00837-3.

McAllan, A., and R. Smith. 1983. Factors influencing the digestion of dietary carbohydrates between the mouth and abomasum of steers. Br. J. Nutr 50:445-454. https://doi.org/10.1079/BJN19830112.

McEvoy, M., L. Delaby, J. Murphy, T. Boland, and M. O'Donovan. 2010. Effect of herbage mass and allowance on sward characteristics, milk production, intake and rumen volatile fatty acid concentration. Grass Forage Sci. 65:335-347. https://doi.org/10.1111/j.1365-2494.2010.00752.x.

Mertens, D. R. 2002. Gravimetric determination of amylase-treated neutral detergent fiber in feeds with refluxing in beakers or crucibles: Collaborative study. J. AOAC Int. 85:1217–1240.

Moorby, J. M., R. T. Evans, N. D. Scollan, J. C. MacRae, and M. K. Theodorou. 2006. Increased concentration of water-soluble carbohydrate in perennial ryegrass (Lolium perenne L.). Evaluation in dairy cows in early lactation. Grass Forage Sci. 61:52-59. https://doi.org/10.1111/j.1365-2494.2006.00507.x.

Mottram, T., J. Lowe, M. Mcgowan, and N. Phillips. 2008. A wireless telemetric method of monitoring clinical acidosis in dairy cows. Comp. Elec. Agric. 64:45-48. https://doi.org/10.1016/j.compag.2008.05.015.

Newbold, C. J., G. de la Fuente, A. Belanche, E. Ramos-Morales, and N. R. McEwan. 2015. The role of ciliate protozoa in the rumen. Front. Microbiol. 6:1313. https://doi.org/10.3389/fmicb.2015.01313

Nicol, A., and I. Brookes. 2007. The metabolisable energy requirements of grazing livestock. Pages 151-172 in Pasture and supplements for grazing animals. N.Z. Soc. Anim. Prod. Occ. Pub. No 14. Hamilton, New Zealand.

Nocek, J. E., and S. Tamminga. 1991. Site of digestion of starch in the gastrointestinal tract of dairy cows and its effect on milk yield and composition. J. Dairy Sci. 74:3598-3629. https://doi.org/10.3168/jds.S0022-0302(91)78552-4.

Nocek, J. E. 1988. In situ and other methods to estimate ruminal protein and energy digestibility: a review. J. Dairy Sci. 71:2051-2069. https://doi.org/10.3168/jds.S0022-0302(88)79781-7.

Nocek, J. E. and J. B. Russell. 1988. Protein and energy as an integrated system. Relationship of ruminal protein and carbohydrate availability to microbial synthesis and milk production. J. Dairy Sci. 71:2070-2107. https://doi.org/10.3168/jds.S0022-0302(88)79782-9.

Nydegger, F., and H. Bollhalder. 2010. Vorrichtung zum Erfassen der Kauaktivität. Swiss Patent, 494.

O'Donovan, M., P. Dillon, M. Rath, and G. Stakelum. 2002. A comparison of four methods of herbage mass estimation. Ir. J. Agric. Food Res. 17-27. https://www.jstor.org/stable/25562447.

O'Mara, F. P., J. J. Murphy, and M. Rath. 2000. The effect of concentrate supplements differing in ruminal protein degradability on milk production and blood metabolite concentrations of dairy cows grazing perennial ryegrass pasture. Livest. Prod. Sci. 64**:**183-191. https://doi.org/10.1016/S0301-6226(99)00150-5.

O'Mara, F. P., G. K. Stakelum, P. Dillon, J. J. Murphy, and M. Rath. 1997. Rumen fermentation and nutrient flows for cows fed grass and grass supplemented with molassed beet pulp pellets. J. Dairy Sci. 80**:**2466-2474. https://doi.org/10.3168/jds.S0022-0302(97)76199-X.

O'Sullivan, M., P. Dillon, K. O'Sullivan, K. Pierce, N. Galvin, M. Egan, and F. Buckley. 2019. Intake, efficiency, and feeding behavior characteristics of Holstein-Friesian cows of divergent Economic Breeding Index evaluated under contrasting pasture-based feeding treatments. J. Dairy Sci. 102**:**8234-8246. https://doi.org/10.3168/jds.2019-16371.

Overton, T. R., M. R. Cameron, J. P. Elliottt, J. H. Clark, and D. R. Nelson, 1995. Ruminal fermentation and passage of nutrients to the duodenum of lactating cows fed mixture of corn and barley. J. Dairy Sci. 78**:**1981-1998. https://doi.org/10.3168/jds.S0022-0302(95)76824-2.

Penno, J. W. 2002. The response by grazing dairy cows to supplementary feeds. PhD. Animal Science, Institute of Veterinary, Animal and Biomedical Sciences, Massey Univ., Palmerston North, N.Z. http://hdl.handle.net/10179/2070.

Peyraud, J., and L. Delaby. 2001. Ideal concentrate feeds for grazing dairy cows – response to concentrates in interaction with grazing management and grass quality. Pages 203-220 in Recent Advances in Animal Nutrition. P. G. Garnsworthy and J. Wiseman, ed. University of Nottingham University Press, Nottingham, UK.

Peyraud, J., L. Astigarraga, and P. Faverdin. 1997. Digestion of fresh perennial ryegrass fertilized at two levels of nitrogen by lactating dairy cows. Anim. Feed Sci. Technol. 64**:**155-171. https://doi.org/10.1016/S0377-8401(96)01056-5.

Potter, E. L., and B. A. Dehority. 1973. Effects of changes in feed level, starvation, and level of feed after starvation upon the concentration of rumen protozoa in the ovine. Appl. Microbiol. 26:692–698. https://doi.org/10.1128/aem.26.5.692-698.1973.

Raffrenato, E., C. Nicholson, and M. E. Van Amburgh. 2019. Development of a mathematical model to predict pool sizes and rates of digestion of 2 pools of digestible neutral detergent fiber and an undigested neutral detergent fiber fraction within various forages. J. Dairy Sci. 102**:** 351-364. https://doi.org/10.3168/jds.2018-15102.

Raffrenato, E., D. A. Ross, and M. E. Van Amburgh. 2018. Development of an in vitro method to determine rumen undigested aNDFom for use in feed evaluation. J. Dairy Sci. 101:9888–9900. https://doi.org/10.3168/jds.2018-15101.

Ranfft, K. 1973. Gas-chromatography determination of short-chain volatile fatty acids in the ruminal fluid. Archiv fur Tierernahrung. 23**:**343. https://doi.org/10.1080/17450397309420984.

Reynal, S. M., and G. A. Broderick. 2005. Effect of dietary level of rumen-degraded protein on production and nitrogen metabolism in lactating dairy cows. J. Dairy Sci. 88:4045–4064. https://doi.org/10.3168/jds.S0022-0302(05)73090-3.

Rinne, M., P. Huhtanen, and S. Jaakkola. 2002. Digestive processes of dairy cows fed silages harvested at four stages of grass maturity. J. Anim. Sci. 80:1986–1998. https://doi.org/10.2527/2002.8071986x.

Rius, A. G., J. Appuhamy, J. Cyriac, D. Kirovski, O. Becvar, J. Escobar, M. L. Mcgilliard, B. J. Bequette, R. M. Akers, and M. D. Hanigan. 2010. Regulation of protein synthesis in mammary glands of lactating dairy cows by starch and amino acids. J. Dairy Sci. 93**:**3114-3127. https://doi.org/10.3168/jds.2009-2743.

Rius, A., S. Kittelmann, K. Macdonald, G. Waghorn, P. Janssen, and E. Sikkema. 2012. Nitrogen metabolism and rumen microbial enumeration in lactating cows with divergent residual feed intake fed high-digestibility pasture. J. Dairy Sci. 95**:**5024-5034. https://doi.org/10.3168/jds.2012-5392.

Sairanen, A., H. Khalili, J. I. Nousiainen, S. Ahvenjärvi, and P. Huhtanen. 2005. The effect of concentrate supplementation on nutrient flow to the omasum in dairy cows receiving freshly cut grass. J. Dairy Sci. 88**:**1443-1453. https://doi.org/10.3168/jds.S0022-0302(05)72812-5.

Satter, L. D., and L. L. Slyter. 1974. Effect of ammonia concentration on rumen microbial protein production in vitro. Br. J. Nutr. 32:199–208. https://doi.org/10.1079/BJN19740073.

Sheahan, A., J. Kay, and J. Roche. 2013. Carbohydrate supplements and their effects on pasture dry matter intake, feeding behavior, and blood factors associated with intake regulation. J. Dairy Sci. 96**:**7818-7829. https://doi.org/10.3168/jds.2013-6981.

Smit, H., B. Tas, H. Taweel, S. Tamminga, and A. Elgersma. 2005. Effects of perennial ryegrass (Lolium perenne L.) cultivars on herbage production, nutritional quality and herbage intake of grazing dairy cows. Grass Forage Sci. 60**:**297-309. https://doi.org/10.1111/j.1365-2494.2005.00480.x.

Stakelum, G., and P. Dillon. 2003. The effect of supplement type on the rumen fermentation pattern of cows fed fresh grass and the in sacco disappearance of grass in the rumen. Ir J. Agric. Food Res. 213-228. https://www.jstor.org/stable/25562491.

Stakelum, G. 1986. Herbage intake of grazing dairy cows: 3. Effects of herbage mass, herbage allowance and concentrate feeding on the herbage intake of dairy cows grazing on mid-summer pasture. Ir. J. Agric. Res. 179-189. https://www.jstor.org/stable/25556147.

Stouthamer, A. H. 1973. A theoretical study on the amount of ATP required for synthesis of microbial cell material. Antonie van Leeuwenhoek 39:545–565.

Sylvester, J. T., S. K. R. Karnati, B. A. Dehority, M. Morrison, G. L. Smith, N. R. St-Pierre, and J. L. Firkins. 2009. Rumen ciliated protozoa decrease generation time and adjust 18S ribosomal DNA copies to adapt to decreased transfer interval, starvation, and monensin. J. Dairy Sci. 92**:**256-269. https://doi.org/10.3168/jds.2008-1417.

Sylvester, J. T., S. K. R. Karnati, Z. Yu, C. J. Newbold, and J. L. Firkins. 2005. Evaluation of a real-time PCR assay quantifying the ruminal pool size and duodenal flow of protozoal nitrogen. J. Dairy Sci. 88:2083–2095. https://doi.org/10.3168/jds.S0022-0302(05)72885-X.

Taweel, H., B. Tas, H. Smit, A. Elgersma, J. Dijkstra, and S. Tamminga. 2005. Effects of feeding perennial ryegrass with an elevated concentration of water-soluble carbohydrates on intake,

rumen function and performance of dairy cows. Anim. Feed Sci. Technol. 121:243-256. https://doi.org/10.1016/j.anifeedsci.2005.02.024.

Tyrrell, H. F., and J. T. Reid. 1965. Prediction of the energy value of cow's milk. J. Dairy Sci. 48:1215–1223. https://doi.org/10.3168/jds.S0022-0302(65)88430-2.

Udén, P., P. E. Colucci, and P. J. Van Soest. 1980. Investigation of chromium, cerium and cobalt as markers in digesta. Rate of passage studies. J. Sci. Food Agric. 31:625–632. https://doi.org/10.1002/jsfa.2740310702.

Van Soest, P. J. 1994. Nutritional ecology of the ruminant. 2nd Ed. Cornell Univ. Press, Ithaca, NY.

Van Soest, P., J. France, and R. Siddons. 1992. On the steady-state turnover of compartments in the ruminant gastrointestinal tract. J. Theor. Biol. 159:135-145. https://doi.org/10.1016/S0022-5193(05)80698-8.

Van Vuuren, A., C. Van Der Koelen, and J. Vroons-De Bruin. 1993. Ryegrass versus corn starch or beet pulp fiber diet effects on digestion and intestinal amino acids in dairy cows. J. Dairy Sci. 76:2692-2700. https://doi.org/10.3168/jds.S0022-0302(93)77605-5.

Van Vuuren, A., F. Krol-Kramer, R. A. Van der Lee, and H. Corbijn. 1992. Protein digestion and intestinal amino acids in dairy cows fed fresh lolium perenne with different nitrogen contents. J. Dairy Sci. 75:2215-2225. https://doi.org/10.3168/jds.S0022-0302(92)77982-X.

Warner, A. 1962. Some factors influencing the rumen microbial population. Microbiology, 28: 129-146. https://doi.org/10.1099/00221287-28-1-129.

Werner, J., L. Leso, C. Umstatter, J. Niederhauser, E. Kennedy, A. Geoghegan, L. Shalloo, M. Schick, and B. O'Brien. 2018. Evaluation of the RumiWatchSystem for measuring grazing behaviour of cows. J. Neuro. Meth. 300:138-146. https://doi.org/10.1016/j.jneumeth.2017.08.022.

Whitehouse, N. L., V. Olson, C. Schwab, W. Chesbrot, K. Cunningham, and T. Lykos. 1994. Improved techniques for dissociating particle-associated mixed ruminal microorganisms from ruminal digesta solids. J. Anim. Sci. 72:1335–1343. https://doi.org/10.2527/1994.7251335x.

Williams, A. G., and G. S. Coleman. 1988. The Rumen Protozoa. Springer-Verlag, NY.

Wims, C., M. McEvoy, L. Delaby, T. Boland, and M. O'Donovan. 2013. Effect of perennial ryegrass (Lolium perenne L.) cultivars on the milk yield of grazing dairy cows. Animal 7**:**410-421. https://doi.org/10.1017/S1751731112001814.

Younge, B., J. Murphy, and M. Rath. 2004. Nutrient metabolism in the rumen and milk production in cows fed on grass-silage and fresh grass based diets. Livest. Prod. Sci. 88**:**43-54. https://doi.org/10.1016/j.livprodsci.2003.11.003.

Zontini, A., M,. 2016. Characterization of fiber fractions in plant by-products and modeling fiber digestion with application in CNCPS 7.0. PhD. Department of Animal Science, Cornell Univ., Ithaca, NY

# CHAPTER 4: MICROBIAL COMPOSITION AND OMASAL FLOWS OF BACTERIAL, PROTOZOAL, AND NON-MICROBIAL AMINO ACIDS IN LACTATING DAIRY COWS FED FRESH PERENNIAL RYEGRASS (*LOLIUM PERENNE* L.) NON-SUPPLEMENTED OR SUPPLEMENTED WITH ROLLED BARLEY

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## 4.1. ABSTRACT

The objective of this study was to evaluate the effect of rolled barley supplementation on microbial composition and omasal flows of bacterial, protozoal, and non-microbial amino acids in cows fed fresh perennial ryegrass (*Lolium perenne* L.; **PRG**). Ten ruminally cannulated Holstein cows averaging (mean ± standard deviation) 49 ± 23 d in milk and 513 ± 36 kg of body weight were assigned to one of two treatments in a switchback design. The treatment diets were PRG-only (**G**) or PRG plus 3.5 kg dry matter rolled barley (**G+RB**). The study consisted of three 29-d periods where each period consisted of 21 d of diet adaptation and 8 d of data and sample collection. A double marker system was used to quantify nutrient flow entering the omasal canal along with $^{15}$N-ammonium sulfate to label and measure the bacterial, protozoal, and non-microbial omasal flow of AA. Overall, rolled barley supplementation had no effect on the AA composition

of the omasal liquid-associated and particle-associated bacteria. Rolled barley supplementation marginally increased some, but not all, protozoa AA concentrations. Particle-associated bacteria AA flow was increased for all AA, except for Trp and Pro, in cows fed the G+RB diet. Rolled barley supplementation had no effect on protozoal AA flow. However, on average, protozoa accounted for 23% of the microbial essential AA flow, which ranged from 17% to 28% for Thr and Lys, respectively. The flow of all AA in omasal true digesta increased in cows fed the G+RB diet compared with the G diet, resulting in a 228 g/d increase in total AA flow in cows fed the G+RB diet. This increase in total AA flow in cows fed the G+RB diet was due to an increase in microbial AA flow. Rolled barley supplementation had no effect on non-microbial AA flow. The non-microbial AA flow modestly contributed to total AA flow, on average accounting for 15.6%. These results indicate that extensive rumen degradation of PRG AA occurs (83.5%) and demonstrates that cows consuming PRG-based diets exhibit a large dependence on microbial AA to support metabolizable AA supply. In conclusion, rolled barley supplementation can increase the omasal flow of microbial AA in cows consuming PRG-based diets. However, further research is required to elucidate if this increased AA supply can support higher milk production performance under such dietary conditions.

**Key Words:** amino acids, omasal flow, bacteria, protozoa, perennial ryegrass

## 4.2. INTRODUCTION

In many parts of Europe, perennial ryegrass (*Lolium perenne* L.; **PRG**) is one of the most predominant grass species, contributing significantly to the diet of the lactating dairy cow (Humphreys et al., 2010; van den Pol-van Dasselaar et al., 2020). Although immature PRG-swards are energy dense (2.75-2.9 Mcals ME kg/DM), the ME supply to cows consuming such swards is typically cited as the primary limiting factor for milk production (Kolver et al., 1998; Keim et al.,

2011). This assumption is often justified by the observation that the CP concentration of PRG exceeds the CP requirements of the lactating dairy cow (NRC, 2001; Pacheco and Waghorn, 2008). However, PRG can contain high levels of non-protein N, soluble N, and rumen-degradable N, potentially limiting the ability of the forage to meet the AA requirement of the lactating dairy cow (Hoekstra et al., 2008; Chapter 2).

Beever and Siddons (1986) concluded that PRG escaping ruminal fermentation insignificantly contributed to MP supply (3 to 5% of MP), whereas a much larger contribution was identified when estimated using in sacco techniques (20 to 44% of MP; Van Vuuren et al., 1991; Valk et al., 1996). Few studies have quantified the AA flow in lactating dairy cows consuming fresh PRG (O'Mara et al., 1997; Younge et al., 2004). Furthermore, the existing AA flow data were obtained using duodenal cannulas as sampling sites, which is recognized to contain considerable endogenous N contamination thus, reducing our ability to distinguish the source of AA flows (Ørskov et al. 1986; Ahvenjarvi et al., 2000).

Chapter 3 demonstrated, utilizing the omasal sampling technique developed by Huhtanen et al. (1997), that rolled barley (**RB**) supplementation increased the flow of bacterial N (+ 50 g/day) due to a greater amount of fermentable carbohydrate (**CHO**) being digested in the rumen and greater efficiency of microbial protein synthesis, compared with a PRG-only diet. In that study, extensive rumen degradation of the PRG N occurred resulting in a negligible contribution from non-microbial N to the total flow of non-ammonia N. Altogether, these observations warranted further investigation into the possible effects of RB supplementation on both the quantity and source of AA supply in cows consuming PRG-based diets.

In a recent literature review, Sok et al. (2017) demonstrated that there are considerable differences among bacteria and protozoa AA composition. Hence, if the differing AA

compositions and estimates regarding the flow of each microbial population could be integrated, the accuracy and precision in predicting AA supply by nutritional models could be improved (Sok et al., 2017). However, the current lack of data describing the relative contribution from microbial populations to the total AA flow limits the ability for model evaluation (Fessenden et al., 2019a). Furthermore, there is contrasting evidence in the literature as to the effect of dietary characteristics on microbial AA concentrations (Hvelplund, 1986; Korhonen et al., 2002). Thus, to improve the estimation of AA flows, there is a need to quantify the AA composition of both bacteria and protozoa and assess their contribution, in combination with non-microbial sources, to the total AA flow at the omasal canal of lactating dairy cows.

The objective of this study was to evaluate omasal flows of bacterial, protozoal, and non-microbial AA in cows consuming fresh PRG non-supplemented or supplemented with RB. A secondary objective was to describe the effect of dietary characteristics on microbial AA concentrations in cows consuming PRG-based diets. The hypothesis of this experiment was that the supplementation of RB would increase the fermentable CHO supply for rumen microbes, and thereby, increase the microbial AA flow at the omasal canal compared with a PRG-only diet. The effects of RB supplementation on rumen metabolism, omasal flow of nutrients, and microbial dynamics have been previously described in Chapter 3.

## 4.3. MATERIALS AND METHODS

The experiment was conducted from April to July 2017 at the Teagasc, Animal and Grassland Research and Innovation Centre, Moorepark, Fermoy, Co. Cork, Ireland (52°16′N; 8°25′W; 49 m a.s.l). All procedures described in this experiment were approved by the Teagasc Animal Ethics

Committee and conducted under experimental license (AE19132-P054) from the Health Products Regulatory Authority under European directive 2010/63/EU and S.I. no. 543 of 2012.

### *4.3.1. Animals, Experimental Design, and Treatment Administration*

Ten ruminally cannulated multiparous Holstein cows averaging (mean ± SD) 49 ± 23 DIM and 513 ± 36 kg of BW were enrolled in a 3-week pre-study acclimation period where all animals were managed and housed in a free-stall barn and fed a common diet. Cows were then stratified by pre-study milk yield and randomly assigned to one of two treatment sequences in a switchback design. The study consisted of three 29-d experimental periods, where each period contained 21 d for diet adaptation and 8 d of data and sample collection. Treatment diets were 1) PRG-only (**G**) or 2) PRG plus 3.5 kg DM RB (**G+RB**). In addition, both treatments were fed 40 g/d of magnesium (120 g/d of Sweetened Cal-Mag; Nutribio Ltd, Tivoli, Cork, Ireland) to reduce the risk of hypomagnesemia. Full details of the cow and sward management are described in Chapter 3. The nutrient and AA composition of the RB supplement and experimental diets are presented in Table 4.1.

**Table 4.1.** Nutrient composition of rolled barley, perennial ryegrass, and the rolled barley supplemented, perennial ryegrass diet

| | | Diet[1] | |
|---|---|---|---|
| Item[2] | RB[3] | G | G+RB |
| DM, % | 86.9 | 20.2 | 34.2 |
| N, % of DM | 1.9 | 2.6 | 2.5 |
| Soluble N, % of N | 17.1 | 35.3 | 31.7 |
| NDIN, % of N | 8.0 | 14.9 | 13.5 |
| ADIN, % of N | 3.0 | 2.5 | 2.6 |
| Starch, % of DM | 60.7 | 2.5 | 14.7 |
| WSC, % of DM | 7.1 | 22.4 | 19.2 |
| aNDFom, % of DM | 19.2 | 36.1 | 32.6 |
| uNDFom, % of aNDFom | 32.9 | 9.8 | 14.7 |
| Ether extract, % of DM | 1.7 | 3.1 | 2.8 |
| Ash, % of DM | 2.6 | 7.0 | 6.1 |
| EAA N, % of TN | | | |
| Arg | 9.4 | 11.1 | -- |
| His | 3.3 | 3.0 | -- |
| Ile | 2.3 | 3.0 | -- |
| Leu | 4.2 | 5.4 | -- |
| Lys | 3.5 | 5.5 | -- |
| Met | 0.9 | 1.2 | -- |
| Phe | 2.3 | 2.8 | -- |
| Thr | 2.3 | 3.4 | -- |
| Trp | 1.1 | 1.7 | -- |
| Val | 3.3 | 4.0 | -- |
| Total EAA | 32.6 | 41.2 | -- |
| NEAA N, % of TN | | | |
| Ala | 3.7 | 6.6 | -- |
| Asp | 3.6 | 6.4 | -- |
| Cys | 1.2 | 0.7 | -- |
| Glu | 14.8 | 7.2 | -- |
| Gly | 4.5 | 6.5 | -- |
| Pro | 6.7 | 3.2 | -- |
| Ser | 3.5 | 3.7 | -- |
| Tyr | 1.4 | 1.6 | -- |
| Total NEAA | 39.4 | 36.0 | -- |
| TAA N, % of TN | 72.0 | 77.2 | -- |

[1]G = 100% (DM basis) perennial ryegrass; G+RB = 79% perennial ryegrass and 21% rolled barley.
[2]aNDFom = amylase- and sodium sulfite-treated NDF corrected for ash residue; uNDFom = undigested amylase- and sodium sulfite treated NDF corrected for ash residue; TN = total N; TAA = total AA.
[3]RB = rolled barley.

### *4.3.2. Sample Collection and Processing*

Spot samples of omasal digesta were obtained using the omasal sampling technique developed by Huhtanen et al. (1997) and adapted by Reynal and Broderick (2005). The double marker method utilizing CoEDTA (Udén et al., 1980) and undigested amylase- and sodium sulfite-treated NDF corrected for ash residue after 240 h of in vitro fermentation (**uNDFom**; Raffrenato et al., 2018) were used to quantify liquid and particle flow entering the omasal canal, respectively. To estimate microbial flow at the omasal canal, 8.8 g/d of ammonium sulfate (Cambridge Isotope Laboratories Inc., Andover, MA, USA) with a 10% enrichment of $^{15}N$ (187 mg/d of $^{15}N$) was added to the CoEDTA infusate. Prior to starting the infusion, samples of ruminal contents (liquid and solids) were taken for later determination of $^{15}N$ background. Omasal sampling began approximately 74 h after the beginning of marker infusion to allow uniform marker distribution. Further details on marker preparation and infusion are reported in Chapter 3.

Samples of omasal contents were collected from the omasal canal during three 8-h intervals: at 1600, 1800, 2000, and 2200 h on d 24; at 0000, 0200, 0400, and 0600 h on d 26; and at 0800, 1000, 1200, and 1400 h on d 27. A 425-mL spot sample was obtained during the first 3 sampling time points, and a 675-mL spot sample was obtained during the last sampling time points of each interval. Each spot sample was split into subsamples of 50-mL (x 2), 125-mL, and 200-mL; with an additional 250-mL subsample at the last time point. The 50-mL subsamples were used for a separate study of nutrient flows in Chapter 3. The 125-mL subsample was placed on ice and combined within the interval, yielding a 500-mL sample for bacterial isolation. The 200-mL subsamples were combined within the period and stored at - 20°C, generating a 2.4-L composite for digestion phase separation. The additional 250-mL subsample obtained on the final time point of each interval was used to isolate omasal protozoa.

The 2.4-L pooled omasal composite was subsequently thawed and separated into the omasal large particle, small particle, and liquid phase, as described in Chapter 3. All omasal phase samples were freeze-dried and either ground through a 1 mm screen on a Cyclotech mill (large particle) or homogenized with a mortar and pestle (small particle and liquid phase) before analysis. As the digesta was fractionated into three phases, the small particle was considered to be part of the particulate matter in the double-marker system. Concentrations of Co and uNDFom were then used to calculate the concentration of each nutrient in a sample theoretically representing omasal true digesta (**OTD**; France and Siddons, 1986).

Bacterial isolation was performed according to Whitehouse et al. (1994) with modifications. Briefly, omasal samples were squeezed through a single layer of large pore polyethylene cheesecloth (Graytec, GD Textile, Manchester, UK), and the retained solids were washed once with saline solution and squeezed again through single layer cheesecloth. The resulting filtrate (A) was stored at 4°C for further centrifugation. The solids retained were placed in a shaking incubator for 1 h at 39°C in a 0.1% methylcellulose solution, to detach particle-associated bacteria, and transferred to a 4°C cooler for 24 h. After 24 h, the sample was blended for 1 minute. The blended sample was squeezed through single layer cheesecloth and the retained solids were washed once with saline solution and squeezed again through cheesecloth. The resulting filtrate (B) was stored at 4°C for further centrifugation. Filtrates A and B were centrifuged at 1,000 x g for 5 min at 4°C to remove small feed particles and protozoa. The supernatant was centrifuged at 15,000 x g for 20 min at 4°C and the bacterial pellet was collected and stored at −20°C until lyophilization and later analysis. The bacterial pellets recovered from filtrates A and B represented the omasal liquid-associated bacteria (**LAB**) and omasal particle-associated bacteria (**PAB**), respectively. The

bacterial isolations from each 8-h interval were subsequently combined within period to generate a LAB and a PAB sample per cow per period.

The 250-mL subsample obtained on the final time point of each interval was immediately processed to isolate omasal protozoa. The subsample was squeezed through a single layer of polyethylene cheesecloth and protozoa were subsequently isolated as described by Denton et al. (2015) using flocculation and filtration techniques. After isolation, protozoa were stored at − 20°C, followed by lyophilization and measurement of OM to calculate the yield of protozoal OM per liter of omasal liquid (Ahvenjärvi et al., 2002).

### *4.3.3. Laboratory Analysis*

Freeze-dried samples of bacteria, protozoa, and omasal phases were analyzed for residual DM after 16 h at 105°C and ash according to AOAC International (2005). Total N was determined using a combustion assay (Leco FP-528 N Analyzer, Leco Corp., St. Joseph, MI). Samples were analyzed for NAN and $^{15}N$ using a Carlo Erba NC2500 elemental analyzer interfaced with an isotope ratio mass spectrometer (Cornell University Stable Isotope Laboratory, Ithaca, NY). Sample preparation and ammonia volatilization were carried out as described by Fessenden et al. (2019b).

Amino acid concentrations in feed, omasal phases, bacteria, and protozoa were analyzed by HPLC. For all AA, except Trp, 2 mg of N with 50 μL of 125 m*M* norleucine, as an internal standard, were hydrolyzed at 110°C for 21 h in a block heater (Gehrke et al., 1985) with high-purity 6 *M* HCl (5 mL) after flushing with $N_2$ gas. For Met and Cys, prior to acid hydrolysis as described above, another 2 mg of N and internal standard were preoxidized with 1 mL of performic acid (0.9 mL of 88% formic acid, 0.1 mL of 30% $H_2O_2$, and 5 mg of phenol) for 16 h at 4°C

(Mason et al., 1980; Elkin and Griffith, 1985). After hydrolysis, tube contents were filtered through Whatman 541 filter paper (GE Healthcare UK Ltd., Buckinghamshire, UK) and the filtrate was diluted to 50 mL in a volumetric flask with HPLC-grade $H_2O$. Aliquots (0.3 mL) were evaporated at 65°C under constant $N_2$ flushing, with 3 rinses and re-evaporations with HPLC-grade $H_2O$ to remove acid residues. After final evaporation, the hydrolysate was dissolved in 0.6 mL of Na diluent (Na220, Pickering Laboratories, Mountain View, CA).

Individual AA hydrolysates were separated using an Agilent 1100 series HPLC (Agilent Technologies, Santa Clara, CA) fitted with a sodium cation exchange column (Cat. no 1154110T, Pickering Laboratories, Mountain View, CA) using a 4-buffer step gradient and column temperature gradient. Detection of separated AA was performed at 560 nm following post-column ninhydrin derivation. Standards (250 nM/ml) for the individual AA were prepared by diluting a pure standard in sample buffer. The volume of sample and standards loaded onto the column was 10 μL. To determine concentrations of Trp, a separate aliquot of the sample containing 2 mg of N was hydrolyzed with 1.2 g of $Ba(OH)_2$ at 110°C for 16 h on a block heater (Landry and Delhaye, 1992). Included in the hydrolysis was 125 μL of 5-methyl-Trp (5 m*M*) as an internal standard. After cooling to precipitate barium ions, an aliquot (3 μL) of the hydrolysate was added to 1 mL of acetate buffer (0.07 M sodium acetate) and analyzed using fluorescence detection (excitation = 285 nm, emission = 345 nm) after HPLC separation.

### *4.3.4. Calculations*

Total N omasal flow and the partitioning of total N flow are described in Chapter 3. Briefly, the concentration of ammonia N in an omasal fluid sample, in combination with the flow of liquid determined by the double marker system, were used to calculate the omasal flow of ammonia N.

This was subtracted from the total N flow to determine NAN flow. The NAN flow was partitioned into four fractions that consisted of PAB N, LAB N, protozoa N, and non-microbial N. This non-ammonia non-microbial N (**NANMN**) was assumed to contain primarily undigested feed N and a smaller contribution of endogenous N (Ørskov et al., 1986; Lapierre et al., 2008). Microbial NAN flow was determined utilizing $^{15}N$ atom percent excess (**APE**) as described in Chapter 3. The isolated LAB and PAB were assumed to be representative of the bacterial biomass flowing with the liquid and particulate phases, respectively (Reynal and Broderick, 2005). The NAN concentration (g/g of OM) of the LAB, PAB, and protozoa samples was used to calculate the flow of total microbial biomass. The flow of NANMN was calculated as the difference between total NAN flow and microbial NAN flow.

The concentrations of AA in feed, omasal phases, bacteria, and protozoa were corrected for incomplete recovery of AA using correction factors published by Lapierre et al. (2019). Recent data demonstrates that complete recovery of AA is not accomplished by 21 to 24 h of acid hydrolysis (Rutherford, 2009; Lapierre et al., 2019). In the current study, the AA concentrations obtained after 21-h hydrolysis were multiplied by an average correction factor specific to each AA, determined by nonlinear regression ($A_0$) as described by Lapierre et al. (2019). The flows of individual AA in OTD were then calculated using the corrected concentration of AA in each omasal phase and the double marker system described in Chapter 3. Liquid-associated bacteria, PAB, protozoa, total microbial, and non-microbial AA flow were calculated as:

$$\text{Liquid} - \text{associated bacteria AA flow (g/d)} = \text{LAB N flow (g/d)} \times \text{LAB AA (g/g of N)};$$

$$\text{Particle} - \text{associated bacteria AA flow (g/d)} = \text{PAB N flow (g/d)} \times \text{PAB AA (g/g of N)};$$

$$\text{Protozoal AA flow (g/d)} = \text{protozoal N flow (g/d)} \times \text{protozoal AA (g/g of N)};$$

$$\text{Microbial AA flow (g/d)} = \text{LAB AA flow (g/d)} + \text{PAB AA flow (g/d)} +$$

protozoal AA flow (g/d); and

$$\text{Non} - \text{microbial AA flow (g/d)} = \text{OTD AA flow (g/d)} - \text{microbial AA flow (g/d)}.$$

### *4.3.5. Statistical Analysis*

Data were analyzed using the MIXED procedure of SAS version 9.4 (SAS Institute Inc. Cary, NC). The same model as described in Chapter 3 was used:

$$Y_{ijkl} = \mu + S_i + C_{j:i} + P_k + T_l + PT_{kl} + \varepsilon_{ijkl}$$

where $Y_{ijkl}$ = dependent variable, $\mu$ = overall mean, $S_i$ = fixed effect of sequence i, $C_{j:i}$ = random effect of cow within sequence, $P_k$ = fixed effect of period k, $T_l$ = fixed effect of treatment l, $PT_{kl}$ = fixed interaction effect of period k and treatment l, and $\varepsilon_{ijkl}$ = residual error. Sequence effects and the interaction term including period and treatment were removed from the model when $P > 0.1$. Degrees of freedom were determined using the Kenward-Rogers option. Means were determined using the least squares means statement. Statistical significance was considered at $P \leq 0.05$ and trends were considered at $0.05 < P \leq 0.10$.

## 4.4. RESULTS AND DISCUSSION

### *4.4.1. Microbial Composition*

The N concentrations of omasal LAB, PAB, and protozoa were not affected by RB supplementation (Table 4.2). On average, the N concentration of PAB and protozoa were similar (8.9% and 9.0% of OM, respectively) and lower than that of LAB (10.2% of OM). Greater N

concentrations in LAB than in PAB and protozoa were reported (Martin et al., 1994). Reports from the literature on protozoal N concentrations are extremely variable, which is likely due to differing levels of feed particle contamination or washing procedures during isolation (Ahvenjärvi et al., 2002; Reynal et al., 2005). In the current study, $^{15}N$ enrichment was not affected by diet and was similar among all 3 microbial populations. Generally, the $^{15}N$ enrichment of protozoa is reported to be lower than that of bacteria (Hristov and Broderick, 1996; Fessenden et al., 2019a). Lower $^{15}N$ enrichment of protozoa has been attributed to the engulfment of unenriched dietary protein due to the limited capacity of protozoa to utilize ammonia-N for growth (Williams and Coleman, 1997; Reynal et al., 2005). However, when investigating ruminal N metabolism, Ahvenjärvi et al. (2018) noted that the direct incorporation of free AA or peptides to protozoa was negligible and that protozoa selectively ingest bacterial N. Hence, similar $^{15}N$ enrichment among protozoa and bacteria is plausible as the predominant source of N for protozoa is bacteria. In agreement with the current study, similar enrichment levels among protozoa and bacteria were observed when forage was the principal true protein source in the diet (Brito et al., 2006, 2007). Furthermore, the length of $^{15}NH_3$ infusion has been implicated as a factor affecting the relative enrichment of protozoa (Brito et al., 2006). In the current study, $^{15}NH_3$ was infused for 192 h, which might have contributed to the greater relative enrichment of protozoa compared with previous literature (Ahvenjärvi et al., 2002).

Total AA concentration of omasal LAB, PAB, and protozoa were not affected by RB supplementation. The total AA concentration of PAB and protozoa were similar, averaging 72.1% and 72.8% of N, respectively, and were higher than that of LAB (58.6% of N; Table 4.2) and this relationship was previously reported (Volden et al., 1999a). Both Isaacson et al. (1975) and Volden et al. (1999b) suggested that differences in total AA concentration between LAB and PAB could

be attributed to higher RNA content or higher nucleic acid concentration in LAB. The AA composition of omasal LAB and PAB were unaffected by RB supplementation (Table 4.3). Rolled barley supplementation had an effect on some, but not all, protozoal AA concentrations (Table 4.3). Of interest, the concentration of His was greater among all three microbial populations when cows consumed the G+RB diet. Dietary characteristics were shown to affect some AA concentrations of microbes (Martin et al., 1996); however, although some microbial AA concentrations were different in the current study, the biological implications of the differences were small. A lack of data and ambiguous reporting of the AA composition of bacteria limit our current ability to evaluate the true effect of dietary characteristics on microbial AA concentrations (Sok et al., 2017).

The omasal microbial AA composition in the current study agrees with a recent review of the literature (Sok et al., 2017). The Lys concentration of protozoa was higher (+ 28%) compared with bacteria, which is consistent with other findings (Volden et al., 1999; Korhonen et al., 2002), and, similar to Volden et al. (1999) and Sok et al. (2017). For other AA, the concentration of Ala was 56% higher, Thr 16% higher, Val 22% higher, and Ile 13% lower in bacteria compared with protozoa in the current study. Furthermore, the concentration of Met was 33% higher in bacteria compared with protozoa, in agreement with Korhonen et al. (2002). When investigating AA flows in lactating dairy cows, these results demonstrate the importance of quantifying the contribution from both bacteria and protozoa in prediction models.

**Table 4.2.** The N concentration, $^{15}N$ enrichment, and AA concentration of omasal microbes in lactating dairy cows fed fresh PRG[1] non-supplemented or supplemented with rolled barley

| | Treatment[2] | | | |
|---|---|---|---|---|
| Item[3] | G | G+RB | SEM | *P*-Value |
| LAB | | | | |
| N, % of OM | 10.26 | 10.16 | 0.09 | 0.44 |
| $^{15}N$ atom % excess | 0.038 | 0.039 | 0.002 | 0.73 |
| Total AA, % of OM | 44.9 | 44.4 | 0.6 | 0.51 |
| Total AAN, % of N | 58.6 | 58.5 | 0.7 | 0.90 |
| PAB | | | | |
| N, % of OM | 8.98 | 8.86 | 0.11 | 0.42 |
| $^{15}N$ atom % excess | 0.040 | 0.041 | 0.002 | 0.78 |
| Total AA, % of OM | 49.5 | 47.7 | 0.8 | 0.13 |
| Total AAN, % of N | 72.7 | 71.5 | 1.2 | 0.47 |
| Protozoa | | | | |
| N, % of OM | 9.12 | 8.78 | 0.28 | 0.22 |
| $^{15}N$ atom % excess | 0.039 | 0.039 | 0.001 | 0.80 |
| Total AA, % of OM | 50.3 | 48.1 | 1.7 | 0.19 |
| Total AAN, % of N | 72.9 | 72.6 | 1.4 | 0.89 |

[1]PRG = perennial ryegrass
[2]G = 100% (DM basis) perennial ryegrass; G+RB = 79% perennial ryegrass and 21% rolled barley.
[3]LAB = liquid-associated bacteria; PAB = particle-associated bacteria.

**Table 4.3.** The AA composition of omasal bacteria and protozoa in lactating dairy cows fed fresh PRG[1] non-supplemented or supplemented with rolled barley

| | LAB[2] AA, g/100 g of AA | | | | PAB[2] AA, g/100 g of AA | | | | Protozoa AA, g/100 g of AA | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Treatment[3] | | | | Treatment | | | | Treatment | | | |
| Item | G | G+RB | SEM | *P*-Value | G | G+RB | SEM | *P*-Value | G | G+RB | SEM | *P*-Value |
| EAA | | | | | | | | | | | | |
| Arg | 5.02 | 5.04 | 0.03 | 0.69 | 4.92 | 4.99 | 0.03 | 0.08 | 4.62 | 4.70 | 0.05 | 0.06 |
| His | 1.71 | 1.77 | 0.02 | <0.05 | 1.68 | 1.72 | 0.01 | <0.05 | 1.66 | 1.70 | 0.02 | <0.05 |
| Ile | 5.61 | 5.54 | 0.04 | 0.26 | 5.84 | 5.84 | 0.03 | 0.93 | 6.50 | 6.38 | 0.07 | <0.05 |
| Leu | 7.38 | 7.43 | 0.04 | 0.38 | 7.63 | 7.63 | 0.02 | 0.91 | 7.62 | 7.70 | 0.05 | 0.12 |
| Lys | 7.45 | 7.52 | 0.07 | 0.37 | 7.55 | 7.52 | 0.05 | 0.55 | 9.81 | 9.46 | 0.21 | <0.05 |
| Met | 3.25 | 3.29 | 0.05 | 0.57 | 2.97 | 2.96 | 0.05 | 0.85 | 2.39 | 2.30 | 0.05 | 0.20 |
| Phe | 4.24 | 4.29 | 0.03 | 0.29 | 4.46 | 4.44 | 0.01 | 0.15 | 4.65 | 4.65 | 0.02 | 0.76 |
| Thr | 5.59 | 5.52 | 0.04 | 0.16 | 5.48 | 5.47 | 0.02 | 0.56 | 4.76 | 4.76 | 0.02 | 0.96 |
| Trp | 2.02 | 2.02 | 0.05 | 0.91 | 2.13 | 2.02 | 0.03 | <0.05 | 1.39 | 1.46 | 0.05 | 0.34 |
| Val | 5.77 | 5.74 | 0.03 | 0.44 | 5.71 | 5.67 | 0.03 | 0.20 | 4.68 | 4.74 | 0.04 | 0.08 |
| Total EAA | 48.0 | 48.2 | 0.08 | 0.26 | 48.4 | 48.3 | 0.07 | 0.21 | 48.1 | 47.8 | 0.13 | <0.05 |
| NEAA | | | | | | | | | | | | |
| Ala | 7.33 | 7.21 | 0.04 | <0.05 | 6.86 | 6.81 | 0.04 | 0.22 | 4.49 | 4.58 | 0.08 | 0.08 |
| Asp | 11.63 | 11.55 | 0.08 | 0.50 | 11.62 | 11.60 | 0.05 | 0.68 | 13.03 | 12.72 | 0.15 | <0.01 |
| Cys | 1.15 | 1.12 | 0.02 | 0.19 | 1.22 | 1.24 | 0.02 | 0.44 | 1.72 | 1.64 | 0.05 | 0.23 |
| Glu | 13.94 | 13.93 | 0.05 | 0.82 | 13.75 | 13.96 | 0.04 | <0.01 | 15.97 | 16.16 | 0.07 | 0.07 |
| Gly | 5.31 | 5.27 | 0.02 | 0.29 | 5.32 | 5.29 | 0.02 | 0.28 | 4.34 | 4.43 | 0.05 | <0.05 |
| Pro | 3.50 | 3.57 | 0.08 | 0.43 | 3.72 | 3.68 | 0.04 | 0.46 | 3.57 | 3.83 | 0.08 | <0.01 |
| Ser | 4.45 | 4.47 | 0.02 | 0.33 | 4.53 | 4.54 | 0.02 | 0.57 | 4.29 | 4.34 | 0.03 | 0.06 |
| Tyr | 4.66 | 4.70 | 0.04 | 0.49 | 4.63 | 4.63 | 0.02 | 0.92 | 4.49 | 4.47 | 0.03 | 0.61 |
| Total NEAA | 52.0 | 51.8 | 0.08 | 0.26 | 51.6 | 51.7 | 0.07 | 0.21 | 51.9 | 52.2 | 0.13 | <0.05 |

[1]PRG = perennial ryegrass

[2]LAB = liquid-associated bacteria; PAB = particle-associated bacteria.

[3]G = 100% (DM basis) perennial ryegrass; G+RB = 79% perennial ryegrass and 21% rolled barley.

### *4.4.2. Omasal Flows of OM, NAN, and AA in Bacteria and Protozoa*

Supplementation of PRG with RB increased the flow of PAB OM and PAB NAN at the omasal canal compared with the G diet (Table 4.4). In addition, supplementation of PRG with RB increased the flows of PAB AA, with the exception of Trp and Pro (Table 4.5). The increased flow of PAB in cows supplemented with RB was likely due to a greater amount of fermentable CHO digested in the rumen and greater efficiency of microbial protein synthesis, when compared with the G diet (Chapter 3). This would suggest that nonstructural carbohydrate-degrading bacteria were primarily associated with the particle phase. This is in contrast with Ahvenjarvi et al. (2002) who observed increased flow of LAB when a grass silage based-diet was supplemented with barley. In the current study, PAB N was considered to be all bacterial N flowing in the particle phase, whereas Ahvenjarvi et al. (2002) considered only bacterial N in the large particle phase to be PAB N.

The flows of LAB OM, LAB NAN, and LAB AA numerically increased with the supplementation of RB; however, significant differences were not detected. This might be related to the calculation method utilized in the current study. The LAB flow was calculated by difference using $^{15}N$ APE in the liquid phase after accounting for $^{15}N$ APE in omasal protozoa. Previous investigations have observed large animal-to-animal variation in the measurement of protozoa (Sylvester et al., 2005; Yáñez-Ruiz et al., 2006), which was also observed in the current study. Due to the calculation methods used, this variability likely increased the variation observed in the measurement of LAB flow.

The contribution of LAB and PAB N to the total microbial N omasal flow were unaffected by rolled barely supplementation (Table 4.4). Liquid-associated bacteria accounted for a higher proportion of the microbial N flow than PAB, averaging 43% and 35%, respectively. Although

previous investigations have reported that a large proportion of the bacteria in the rumen are associated with particulate matter (Craig et al., 1987), a higher rate of passage of the liquid phase compared with particulate phase, allows similar contribution from LAB and PAB to total microbial N flow (Hristov and Broderick, 1996). The high liquid passage rate reported for both treatments (Chapter 3) in the current study likely allowed the large contribution from LAB to microbial N omasal flow.

Supplementation of PRG with RB did not affect protozoa contribution to the microbial OM, NAN, and AA omasal flows (Table 4.4 and Table 4.6). On average protozoa accounted for 21.7% of microbial NAN flow and 23.3% of microbial AA flow. The contribution of protozoa to microbial EAA flows ranged, on average, from 17.4% to 27.9% for Thr and Lys, respectively (Table 4.6). Protozoa omasal flow has not previously been quantified in pasture-fed cows. These measured values are higher than previously reported when compared with cows consuming corn-silage based diets (Sylvester et al., 2005; Fessenden et al., 2019a) and grass-silage based diets (Ahvenjärvi et al., 2002). The rapid liquid passage rate (Chapter 3) of cows in the current study ($0.20\ h^{-1}$) is a likely reason for the large contribution of protozoa to total microbial OM, NAN, and AA omasal flow. Sylvester et al. (2009) demonstrated, in vitro, that a higher ruminal passage rate can simultaneously increase the flow of protozoa and decrease their generation time allowing more efficient protein synthesis to be achieved. Chapter 3 reported low rumen protozoal biomass relative to rumen bacterial biomass and extremely high efficiency of protozoal growth, which is in line with the predictions of Firkins et al. (2007). Sok et al. (2017) recently challenged the calculations of Firkins et al. (2007) arguing that, as passage rates are typically first-order fractional rates, a decreased protozoal ruminal pool size would consequently decrease protozoal ruminal outflow rate, justifying their predictions that protozoa contribute less to the total microbial flow

(16.5%). However, in the current study, direct measurement of protozoa rumen pool size and protozoa omasal flow were conducted simultaneously, negating the assumptions required when utilizing fractional outflow rates to describe protozoal passage (Karnati et al., 2007), with results supporting the predictions of Firkins et al. (2007). More in vivo studies are required to further elucidate protozoal metabolism and to evaluate the ability of current mechanistic models to predict such behavior.

**Table 4.4.** The omasal microbial OM and NAN flows in lactating dairy cows fed fresh PRG[1] non-supplemented or supplemented with rolled barley

| | Treatment[2] | | | |
|---|---|---|---|---|
| Item | G | G+RB | SEM | *P*-Value |
| DMI[3], kg/d | 16.1 | 17.1 | 0.4 | <0.01 |
| NAN flow[3], g/d | 373 | 422 | 18 | <0.01 |
| LAB[4] nutrient flow, g/d | | | | |
| OM flow | 1,340 | 1,601 | 169 | 0.16 |
| NAN flow | 137 | 164 | 18 | 0.17 |
| % of microbial NAN flow | 42.5 | 44.0 | 4.0 | 0.70 |
| PAB[4] nutrient flow, g/d | | | | |
| OM flow | 1,264 | 1,523 | 57 | <0.01 |
| NAN flow | 111 | 134 | 4 | <0.01 |
| % of microbial NAN flow | 34.0 | 36.1 | 1.3 | 0.28 |
| Protozoa nutrient flow, g/d | | | | |
| OM flow | 847 | 814 | 103 | 0.75 |
| NAN flow[3] | 79 | 73 | 11 | 0.55 |
| % of microbial NAN flow[3] | 23.5 | 20.0 | 3.2 | 0.24 |

[1]PRG = perennial ryegrass
[2]G = 100% (DM basis) perennial ryegrass; G+RB = 79% perennial ryegrass and 21% rolled barley.
[3]Previously reported in Chapter 3.
[4]LAB = liquid-associated bacteria; PAB = particle-associated bacteria.

**Table 4.5.** The omasal flow of bacterial and protozoal AA in lactating dairy cows fed fresh PRG[1] non-supplemented or supplemented with rolled barley

| | LAB[2] AA flow, g/d | | | | PAB[2] AA flow, g/d | | | | Protozoa AA flow, g/d | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Treatment[3] | | | | Treatment | | | | Treatment | | | |
| Item | G | G+RB | SEM | *P*-Value | G | G+RB | SEM | *P*-Value | G | G+RB | SEM | *P*-Value |
| EAA | | | | | | | | | | | | |
| Arg | 30.3 | 36.1 | 4.0 | 0.18 | 31.6 | 36.5 | 1.4 | <0.05 | 19.5 | 18.7 | 2.8 | 0.78 |
| His | 10.3 | 12.7 | 1.4 | 0.11 | 10.8 | 12.5 | 0.5 | <0.05 | 7.0 | 6.7 | 1.0 | 0.80 |
| Ile | 33.8 | 39.4 | 4.3 | 0.24 | 37.2 | 42.8 | 1.6 | <0.05 | 27.5 | 25.8 | 4.3 | 0.67 |
| Leu | 44.5 | 52.9 | 5.7 | 0.18 | 48.8 | 55.6 | 2.1 | <0.05 | 31.8 | 30.6 | 4.5 | 0.77 |
| Lys | 44.9 | 54.1 | 6.0 | 0.14 | 48.3 | 55.2 | 2.2 | <0.05 | 42.2 | 38.6 | 7.0 | 0.58 |
| Met | 19.5 | 23.8 | 2.7 | 0.13 | 19.1 | 21.9 | 0.8 | <0.05 | 9.9 | 9.3 | 1.4 | 0.67 |
| Phe | 25.5 | 30.6 | 3.3 | 0.17 | 28.5 | 32.5 | 1.2 | <0.05 | 19.6 | 18.7 | 2.9 | 0.74 |
| Thr | 33.7 | 39.5 | 4.4 | 0.21 | 35.6 | 40.5 | 1.5 | <0.05 | 20.0 | 18.9 | 2.9 | 0.71 |
| Trp | 12.1 | 14.4 | 1.6 | 0.21 | 13.6 | 14.7 | 0.5 | 0.11 | 5.7 | 5.7 | 0.8 | 0.98 |
| Val | 34.8 | 40.9 | 4.5 | 0.22 | 36.8 | 41.6 | 1.6 | <0.05 | 19.7 | 18.9 | 2.8 | 0.77 |
| Total EAA | 289.4 | 344.4 | 37.6 | 0.18 | 310.3 | 353.9 | 13.2 | <0.05 | 202.7 | 191.9 | 30.3 | 0.70 |
| NEAA | | | | | | | | | | | | |
| Ala | 44.1 | 51.4 | 5.6 | 0.23 | 44.3 | 50.3 | 1.9 | <0.05 | 18.5 | 18.1 | 2.4 | 0.84 |
| Asp | 70.1 | 82.5 | 9.1 | 0.20 | 75.0 | 86.0 | 3.4 | <0.05 | 54.7 | 51.3 | 8.3 | 0.66 |
| Cys | 6.9 | 8.1 | 0.9 | 0.23 | 8.0 | 9.2 | 0.3 | <0.05 | 7.0 | 6.5 | 0.9 | 0.61 |
| Glu | 84.1 | 99.0 | 10.7 | 0.19 | 88.1 | 102.0 | 3.8 | <0.05 | 66.5 | 64.2 | 9.6 | 0.80 |
| Gly | 31.9 | 37.7 | 4.1 | 0.19 | 34.3 | 38.9 | 1.5 | <0.05 | 18.1 | 17.5 | 2.5 | 0.82 |
| Pro | 20.7 | 25.5 | 2.6 | 0.11 | 24.1 | 27.1 | 1.1 | 0.05 | 14.7 | 15.5 | 2.1 | 0.70 |
| Ser | 26.8 | 31.8 | 3.4 | 0.17 | 28.9 | 33.2 | 1.2 | <0.05 | 17.9 | 17.3 | 2.6 | 0.79 |
| Tyr | 28.0 | 33.6 | 3.7 | 0.16 | 29.4 | 33.9 | 1.3 | <0.01 | 19.0 | 17.9 | 2.8 | 0.68 |
| Total NEAA | 312.5 | 369.5 | 40.0 | 0.19 | 332.0 | 380.6 | 14.4 | <0.05 | 216.7 | 208.0 | 31.3 | 0.76 |
| Total AA | 601.9 | 713.9 | 77.6 | 0.18 | 642.2 | 734.5 | 27.6 | <0.05 | 419.4 | 399.9 | 61.5 | 0.73 |

[1]PRG = perennial ryegrass

[2]LAB = liquid-associated bacteria; PAB = particle-associated bacteria.

[3]G = 100% (DM basis) perennial ryegrass; G+RB = 79% perennial ryegrass and 21% rolled barley.

**Table 4.6.** The protozoal proportion of omasal microbial AA flow in lactating dairy cows fed fresh PRG[1] non-supplemented or supplemented with rolled barley

| Protozoal AA flow, % of microbial AA | Treatment[2] | | SEM | *P*-Value |
|---|---|---|---|---|
| | G | G+RB | | |
| EAA | | | | |
| Arg | 23.4 | 20.7 | 2.9 | 0.36 |
| His | 24.3 | 21.4 | 3.0 | 0.32 |
| Ile | 27.0 | 23.8 | 3.3 | 0.31 |
| Leu | 24.8 | 22.1 | 2.9 | 0.35 |
| Lys | 29.9 | 25.9 | 3.8 | 0.26 |
| Met | 20.2 | 17.5 | 2.8 | 0.30 |
| Phe | 25.8 | 22.9 | 3.1 | 0.33 |
| Trp | 22.0 | 19.6 | 2.9 | 0.38 |
| Thr | 18.1 | 16.7 | 2.2 | 0.54 |
| Val | 21.2 | 19.0 | 2.7 | 0.41 |
| Total EAA | 24.6 | 21.7 | 3.1 | 0.34 |
| NEAA | | | | |
| Ala | 17.3 | 15.6 | 2.3 | 0.45 |
| Asp | 26.7 | 23.5 | 3.3 | 0.31 |
| Cys | 31.3 | 27.6 | 3.1 | 0.22 |
| Glu | 27.2 | 24.3 | 3.2 | 0.35 |
| Gly | 21.2 | 19.0 | 2.6 | 0.41 |
| Pro | 24.2 | 22.7 | 2.7 | 0.60 |
| Ser | 23.8 | 21.2 | 2.9 | 0.35 |
| Tyr | 24.2 | 21.2 | 3.1 | 0.30 |
| Total NEAA | 24.6 | 22.0 | 3.0 | 0.36 |
| Total AA | 24.6 | 21.9 | 3.0 | 0.35 |

[1]PRG = perennial ryegrass

[2]G = 100% (DM basis) perennial ryegrass; G+RB = 79% perennial ryegrass and 21% rolled barley.

### *4.4.3. Omasal Flows of AA in OTD, Microbial, and Non-microbial Fractions*

Rolled barley supplementation to a PRG diet increased the flow of all AA in OTD compared with the G diet resulting in a 228 g/d increase in total AA flow in cows fed the G+RB diet (Table 4.7; 1,964 vs. 2,193 g/d for G vs. G+RB, respectively; $P < 0.01$). Van Vuuren et al. (1993) reported higher duodenal AA flows when a starch-based supplement was provided to pasture-fed cows, although supplementation level was much higher (7 kg DM/d). In contrast, both O'Mara et al. (1997) and Younge et al. (2004) reported no difference in total AA flows when cows fed PRG were supplemented with a fiber-based concentrate; however, AA flows in both studies were numerically increased in supplemented cows. In the current study, the increase in AA flow was most likely due to the greater amount of fermentable CHO digested in the rumen and the greater efficiency of microbial protein synthesis in cows supplemented with RB (Chapter 3). Furthermore, this increased total AA omasal flow when cows consumed the G+RB diet can help explain, at least in part, an increase in milk protein concentration (Chapter 3).

The effects of RB supplementation on total microbial AA flow and non-microbial AA flow are presented in Table 4.8. Microbial AA flow increased for all AA when cows were supplemented with RB, and this was associated with the increase in PAB AA flow. Rolled barley supplementation did not affect the non-microbial AA flow (Table 4.8). The non-microbial AA portion of the total AA flow accounted for 16.5% and 14.7% in cows fed the G and G+RB diets, respectively and for cows consuming the G diet, this indicates 83.5% of the PRG AA were degraded in the rumen. This rumen degradability is likely underestimated as endogenous AA will contribute to a proportion of non-microbial AA; however, the contribution of endogenous AA at the omasal canal is small (Ørskov et al., 1986; Lapierre et al., 2008). To our knowledge, no other dataset exists pertaining to the non-microbial AA contribution to total AA omasal flow in pasture-

fed cows. However, one other study has previously reported the contribution of NANMN to total NAN flow at the omasal canal in pasture-fed cows (Sairanen et al., 2005) with a number of others reporting the NANMN contribution to duodenal NAN flow (Berzaghi et al., 1996; O'Mara et al., 1997; Peyraud et al., 1997; Younge et al., 2004). The rumen degradation of pasture N [1 – (NANMN flow/N intake)] reported in omasal flow studies varied from 87.5% to 88.5%, whereas in the duodenal flow studies it varied from 64.7% to 79.0%. In the duodenal flow studies, the lower estimates of pasture N rumen degradation is likely, at least in part, due to larger endogenous N contributions compared with omasal flow studies. Rumen degradation of pasture N, measured with in situ procedures, support the omasal flow data (Beever et al., 1986; Van Vuuren et al., 1991) and reported rapid rumen degradation rates of PRG N, which exceeded 0.20 $h^{-1}$. Using the data from Chapter 3, the rumen degradation rate of PRG N in vivo could be calculated as both NANMN rumen pool size and NANMN omasal flow were reported and the calculations indicate that the PRG N degraded in vivo at 0.16 $h^{-1}$. Altogether, these observations demonstrate the low contribution from non-microbial AA to total AA flow in cows fed fresh PRG-based diets.

**Table 4.7.** The omasal true digesta flow of AA in lactating dairy cows fed fresh PRG[1] non-supplemented or supplemented with rolled barley

| | Treatment[2] | | | |
|---|---|---|---|---|
| AA flow, g/d | G | G+RB | SEM | *P*-Value |
| EAA | | | | |
| Arg | 86.2 | 97.6 | 3.9 | <0.01 |
| His | 32.9 | 37.6 | 1.5 | <0.01 |
| Ile | 107.4 | 118.9 | 4.8 | <0.01 |
| Leu | 155.2 | 173.5 | 7.0 | <0.01 |
| Lys | 154.4 | 168.5 | 6.9 | <0.01 |
| Met | 51.8 | 58.2 | 2.6 | <0.01 |
| Phe | 92.8 | 103.5 | 4.2 | <0.01 |
| Thr | 105.8 | 116.5 | 4.7 | <0.01 |
| Trp | 36.7 | 40.2 | 1.6 | <0.01 |
| Val | 109.4 | 121.3 | 5.0 | <0.01 |
| Total EAA | 932.4 | 1,035.9 | 41.8 | <0.01 |
| NEAA | | | | |
| Ala | 144.9 | 157.9 | 6.8 | <0.01 |
| Asp | 220.2 | 243.8 | 9.9 | <0.01 |
| Cys | 25.2 | 29.1 | 1.4 | <0.01 |
| Glu | 276.7 | 317.7 | 12.2 | <0.01 |
| Gly | 109.7 | 122.1 | 5.1 | <0.01 |
| Pro | 76.2 | 86.0 | 3.8 | <0.01 |
| Ser | 86.9 | 97.7 | 3.9 | <0.01 |
| Tyr | 92.0 | 102.1 | 4.1 | <0.01 |
| Total NEAA | 1,031.7 | 1,156.6 | 46.6 | <0.01 |
| Total AA | 1,964.1 | 2,192.5 | 88.3 | <0.01 |

[1]PRG = perennial ryegrass

[2]G = 100% (DM basis) perennial ryegrass; G+RB = 79% perennial ryegrass and 21% rolled barley.

**Table 4.8.** The omasal flow of microbial and non-microbial AA in lactating dairy cows fed fresh PRG[1] non-supplemented or supplemented with rolled barley

| | Microbial AA flow, g/d | | | | Non-microbial AA flow, g/d | | | |
|---|---|---|---|---|---|---|---|---|
| | Treatment[2] | | | | Treatment | | | |
| Item | G | G+RB | SEM | *P*-Value | G | G+RB | SEM | *P*-Value |
| EAA | | | | | | | | |
| Arg | 80.0 | 92.6 | 4.1 | <0.01 | 5.7 | 5.4 | 1.0 | 0.82 |
| His | 27.6 | 32.3 | 1.4 | <0.01 | 5.3 | 5.2 | 0.5 | 0.87 |
| Ile | 97.0 | 109.6 | 4.8 | <0.01 | 10.1 | 9.6 | 1.3 | 0.81 |
| Leu | 123.2 | 141.1 | 6.0 | <0.01 | 31.9 | 32.5 | 1.8 | 0.82 |
| Lys | 136.3 | 146.9 | 6.5 | <0.05 | 20.7 | 18.8 | 2.3 | 0.56 |
| Met | 47.9 | 55.6 | 2.3 | <0.01 | 3.9 | 2.6 | 0.9 | 0.27 |
| Phe | 72.4 | 82.9 | 3.5 | <0.01 | 20.3 | 20.6 | 1.3 | 0.83 |
| Thr | 88.1 | 100.1 | 4.1 | <0.01 | 17.7 | 16.5 | 1.3 | 0.52 |
| Trp | 30.9 | 35.3 | 1.5 | <0.01 | 5.8 | 4.9 | 0.4 | 0.13 |
| Val | 90.0 | 102.6 | 4.4 | <0.01 | 19.3 | 18.8 | 1.3 | 0.80 |
| Total EAA | 790.1 | 902.5 | 38.1 | <0.01 | 140.8 | 134.9 | 9.9 | 0.67 |
| NEAA | | | | | | | | |
| Ala | 105.9 | 120.8 | 5.3 | <0.01 | 39.2 | 36.9 | 2.4 | 0.28 |
| Asp | 196.8 | 222.8 | 9.4 | <0.01 | 23.0 | 21.4 | 2.9 | 0.70 |
| Cys | 21.4 | 24.2 | 1.0 | <0.01 | 3.9 | 4.8 | 0.8 | 0.32 |
| Glu | 235.0 | 268.9 | 11.2 | <0.01 | 41.3 | 49.3 | 3.4 | 0.11 |
| Gly | 83.1 | 95.3 | 4.1 | <0.01 | 26.7 | 26.8 | 1.5 | 0.92 |
| Pro | 59.1 | 68.6 | 2.9 | <0.01 | 17.4 | 17.1 | 1.9 | 0.90 |
| Ser | 72.5 | 83.3 | 3.4 | <0.01 | 14.3 | 14.4 | 1.1 | 0.94 |
| Tyr | 75.3 | 86.5 | 3.6 | <0.01 | 16.7 | 15.7 | 1.0 | 0.43 |
| Total NEAA | 849.0 | 970.4 | 40.1 | <0.01 | 182.6 | 186.4 | 12.4 | 0.82 |
| Total AA | 1,639.0 | 1,872.9 | 78.1 | <0.01 | 323.6 | 321.1 | 21.9 | 0.93 |

[1]PRG = perennial ryegrass

[2]G = 100% (DM basis) perennial ryegrass; G+RB = 79% perennial ryegrass and 21% rolled barley.

## 4.5. CONCLUSIONS

In this study, supplementation of PRG with RB appeared to have a minimal effect on the microbial composition of cows consuming fresh PRG-based diets. However, differences in the AA composition among bacteria and protozoa were pronounced emphasizing the importance of including these observations in mechanistic models designed to predict AA flows in lactating dairy cows. Rolled barley supplementation increased microbial AA flow, which is likely due to a greater amount of fermentable CHO digested in the rumen and greater efficiency of microbial protein synthesis, as reported in Chapter 3. Overall, protozoa flow was not affected by diet; however, protozoa contributed 23% of the microbial EAA flow. Rolled barley supplementation had no effect on the non-microbial AA flow and the non-microbial AA flow modestly contributed to total AA flow; accounting for 16.5% and 14.7% in cows fed G and G+RB, respectively. These observations indicate that extensive rumen degradation of PRG AA occurs (83.5%) and subsequently, cows consuming PRG-based diets exhibit a large dependence on microbial AA to support metabolizable AA supply. In conclusion, RB supplementation can increase the omasal flow of microbial AA in cows consuming PRG-based diets. However, more research is required to investigate if this increased AA supply can stimulate higher milk production performance under such dietary conditions.

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## 4.7. REFERENCES

Ahvenjärvi, S., M. Vaga, A. Vanhatalo, and P. Huhtanen. 2018. Ruminal metabolism of grass silage soluble nitrogen fractions. J. Dairy Sci. 101:279–294. https://doi.org/10.3168/jds.2016-12316

Ahvenjärvi, S., A. Vanhatalo, and P. Huhtanen. 2002. Supplementing barley or rapeseed meal to dairy cows fed grass-red clover silage: I. Rumen degradability and microbial flow. J. Anim. Sci. 80:2176– 2187. https://doi.org/10.1093/ansci/80.8.2176

Ahvenjärvi, S., A. Vanhatalo, P. Huhtanen, and T. Varvikko. 2000. Determination of forestomach digestion in lactating dairy cows by omasal or duodenal sampling. Br. J. Nutr. 83:67–77. https://doi.org/10.1017/S0007114500000106

AOAC International. 2005. Official Methods of Analysis. 18th ed. AOAC International, Gaithersburg, MD.

Beever, D. E. and R. C. Siddons. 1986. Digestion and metabolism in the grazing ruminant. Pages 479–497 in Control of Digestion and Metabolism in Ruminants. L. P. Milligan, W. L. Grovum, and A. Dobson, ed. Prentice Hall, Englewood Cliffs, NJ.

Beever, D., M. Dhanoa, H. Losada, R. Evans, S. Cammell, and J. France. 1986. The effect of forage species and stage of harvest on the processes of digestion occurring in the rumen of cattle. Br. J. Nutr. 56:439-454. https://doi.org/10.1079/BJN19860124

Berzaghi, P., J. H. Herbein, and C. E. Polan. 1996. Intake, site, and extent of nutrient digestion of lactating cows grazing pasture. J. Dairy Sci. 79:1581–1589. https://doi.org/10.3168/jds.S0022-0302(96)76520-7

Brito, A. F., G. A. Broderick, and S. M. Reynal. 2007. Effects of different protein supplements on omasal nutrient flow and microbial protein synthesis in lactating dairy cows. J. Dairy Sci. 90:1828-1841. https://doi.org/10.3168/jds.2006-559

Brito, A. F., G. A. Broderick, and S. M. Reynal. 2006. Effect of varying dietary ratios of alfalfa silage to corn silage on omasal flow and microbial protein synthesis in dairy cows. J. Dairy Sci. 89:3939– 3953. https://doi.org/10.3168/jds.S0022-0302(06)72436-5

Craig, W. M., G. A. Broderick, and D. B. Ricker. 1987. Quantitation of microorganisms associated with the particulate phase of ruminal ingesta. J. Nutr. 117:56–62. https://doi.org/10.1093/jn/117.1.56

Denton, B. L., L. E. Diese, J. L. Firkins, and T. J. Hackmann. 2015. Accumulation of reserve carbohydrate by rumen protozoa and bacteria in competition for glucose. Appl. Environ. Microbiol. 81:1832–1838. https://doi.org/10.1128/AEM.03736-14

Elkin, R. G., and J. Griffith. 1985. Hydrolysate preparation for analysis of amino acids in sorghum grains: Effect of oxidative pretreatment. J. Assoc. Off. Anal. Chem. 68:1117–1121.

Fessenden, S. W., T. J. Hackmann, D. A. Ross, E. Block, A. Foskolos, and M. E. Van Amburgh. 2019a. Rumen digestion kinetics, microbial yield, and omasal flows of nonmicrobial, bacterial, and protozoal amino acids in lactating dairy cattle fed fermentation by-products or urea as a soluble nitrogen source. J. Dairy Sci. 102:3036–3052. https://doi.org/10.3168/jds.2018-15448

Fessenden, S. W., A. Foskolos, T. J. Hackmann, D. A. Ross, E. Block, and M. E. Van Amburgh. 2019b. Effects of a commercial fermentation byproduct or urea on milk production, rumen metabolism, and omasal flow of nutrients in lactating dairy cattle. J. Dairy Sci. 102:3023-3035. https://doi.org/10.3168/jds.2018-15447

Firkins, J. L., Z. Yu, and M. Morrison. 2007. Ruminal nitrogen metabolism: perspectives for integration of microbiology and nutrition for dairy. J. Dairy Sci. 90(Suppl. 1), E1–E16. https://doi.org/10.3168/jds.2006-518

France, J., and R. Siddons. 1986. Determination of digesta flow by continuous market infusion. J. Theor. Biol. 121:105–119. https://doi.org/10.1016/S0022-5193(86)80031-5

Gehrke, C. W., L. Wall Sr., J. Absheer, F. Kaiser, and R. Zumwalt. 1985. Sample preparation for chromatography of amino acids: Acid hydrolysis of proteins. J. Assoc. Off. Anal. Chem. 68:811–821.

Hoekstra, N., P. Struik, E. Lantinga, M. E. Van Amburgh, and R. Schulte. 2008. Can herbage nitrogen fractionation in Lolium perenne be improved by herbage management? NJAS-Wagen. J. Life. Sci. 55:167-180. https://doi.org/10.1016/S1573-5214(08)80035-8

Hristov, A. N., and G. A. Broderick. 1996. Synthesis of microbial protein in ruminally cannulated cows fed alfalfa silage, alfalfa hay, or corn silage. J. Dairy Sci. 79:1627–1637. https://doi.org/10.3168/jds.S0022-0302(96)76526-8

Huhtanen, P., P. G. Brotz, and L. D. Satter. 1997. Omasal sampling technique for assessing fermentative digestion in the forestomach of dairy cows. J. Anim. Sci. 75:1380–1392. https://doi.org/10.2527/1997.7551380x

Humphreys, M., U. Feuerstein, M. Vandevalle, and J. Baert. 2010. Ryegrasses. Pages 211–260 in Fodder crops and amenity grasses. B. Boller, U. K. Posselt, and F. Veronesi, ed. New York, NY: Springer Science and Business Media. https://doi.org/10.1007/978-1-4419-0760-8

Hvelplund, T. 1986. The influence of diet on nitrogen and amino acid content of mixed rumen bacteria. Acta Agric. Scand. 36:325–331. https://doi.org/10.1080/00015128609436535

Isaacson, H. R., F. C. Hinds, M. P. Bryant, and F. N. Owens. 1975. Efficiency of energy utilization by mixed rumen bacteria in continuous culture. J. Dairy Sci. 58:1645–1659. https://doi.org/10.3168/jds.S0022-0302(75)84763-1

Karnati, S. K. R., J. T. Sylvester, S. M. Noftsger, Z. Yu, N. R. St-Pierre, and J. L. Firkins. 2007. Assessment of ruminal bacterial populations and protozoal generation time in cows fed different methionine sources. J. Dairy Sci. 90:798–809. https://doi.org/10.3168/jds.S0022-0302(07)71564-3

Keim, J. P. and R. Anrique. 2011. Nutritional strategies to improve nitrogen use efficiency by grazing dairy cows. Chilean J. Agri. Res. 71:623–633. https://doi.org/10.4067/S0718-58392011000400019

Kolver, E. S., and L. D. Muller. 1998. Performance and nutrient intake of high producing Holstein cows consuming pasture or total mixed ration. J. Dairy Sci. 81:1403–1411. https://doi.org/10.3168/jds.S0022-0302(98)75704-2

Korhonen, M., S. Ahvenjärvi, A. Vanhatalo, and P. Huhtanen. 2002. Supplementing barley or rapeseed meal to dairy cows fed grass-red clover silage: II. Amino acid profile of microbial fractions. J. Ani. Sci. 80:2188-2196. https://doi.org/10.1093/ansci/80.8.2188

Landry, J., and S. Delhaye. 1992. Simplified procedure for the determination of tryptophan of foods and feedstuffs from barytic hydrolysis. J. Agric. Food Chem. 40:776–779. https://doi.org/10.1021/jf00017a014

Lapierre, H., S. Binggeli, M. Sok, D. Pellerin, and D. Ouellet. 2019. Estimation of correction factors to determine the true amino acid concentration of protein after a 24-hour hydrolysis. J. Dairy Sci. 102:1205-1212. https://doi.org/10.3168/jds.2018-15392

Lapierre, H., D. R. Ouellet, R. Berthiaume, R. Martineau, G. Holtrop, and G. E. Lobley. 2008.

Distribution of $^{15}N$ in amino acids during $^{15}N$-Leucine infusion: Impact on the estimation of endogenous flows in dairy cows. J. Dairy Sci. 91:2702–2714. https://doi.org/10.3168/jds.2007-0871

Martin, C., L. Bernard, and B. Michalet-Doreau. 1996. Influence of sampling time and diet on amino acid composition of protozoal and bacterial fractions from bovine ruminal contents. J. Anim. Sci. 74:1157–1163. https://doi.org/10.2527/1996.7451157x

Martin, C., A. G. Williams, and B. Michalet-Doreau. 1994. Isolation and characteristics of the protozoal and bacterial fractions from bovine ruminal contents. J. Anim. Sci. 72:2962–2968. https://doi.org/10.2527/1994.72112962x

Mason, V. C., S. Bech-Andersen, and M. Rudemo. 1980. Hydrolysate preparation for amino acid determinations in feed constituents. Z. Tierphysiol. Tierernahr. Futtermittelkd. 43:35–48. https://doi.org/10.1111/j.1439-0396.1980.tb00618.x

National Research Council. 2001. Nutrient Requirements of Dairy Cattle. 7th rev. ed. Natl. Acad. Press, Washington, DC, USA.

O'Mara, F. P., G. K. Stakelum, P. Dillon, J. J. Murphy, and M. Rath. 1997. Rumen fermentation and nutrient flows for cows fed grass and grass supplemented with molassed beet pulp pellets. J. Dairy Sci. 80:2466-2474. https://doi.org/10.3168/jds.S0022-0302(97)76199-X

Ørskov, E. R., N. A. Macleod, and D. J. Kyle. 1986. Flow of nitrogen from the rumen and abomasum in cattle and sheep given protein free nutrients by intragastric infusion. Br. J. Nutr. 56:241–248. https://doi.org/10.1079/BJN19860103

Pacheco, D. and G.C. Waghorn. 2008. Dietary nitrogen − definitions, digestion, excretion and consequences of excess for grazing ruminants. Proc. N. Z.Grassland Assoc. 70:107–116. https://doi.org/10.33584/jnzg.2008.70.2738

Peyraud, J., L. Astigarraga, and P. Faverdin. 1997. Digestion of fresh perennial ryegrass fertilized at two levels of nitrogen by lactating dairy cows. Anim. Feed Sci. Technol. 64:155-171. https://doi.org/10.1016/S0377-8401(96)01056-5

Raffrenato, E., D. A. Ross, and M. E. Van Amburgh. 2018. Development of an in vitro method to determine rumen undigested aNDFom for use in feed evaluation. J. Dairy Sci. 101:9888–9900. https://doi.org/10.3168/jds.2018-15101

Reynal, S. M., and G. A. Broderick. 2005. Effect of dietary level of rumen-degraded protein on production and nitrogen metabolism in lactating dairy cows. J. Dairy Sci. 88:4045–4064. https://doi.org/10.3168/jds.S0022-0302(05)73090-3

Reynal, S. M., G. A. Broderick, and C. Bearzi. 2005. Comparison offour markers for quantifying microbial protein flow from the rumen of lactating dairy cows. J. Dairy Sci. 88:4065–4082. https://doi.org/10.3168/jds.S0022-0302(05)73091-5

Reynal, S. M., G. A. Broderick, S. Ahvenjärvi, and P. Huhtanen. 2003. Effect of feeding protein supplements of differing degradability on omasal flow of microbial and undegraded protein. J. Dairy Sci. 86:1292-1305. https://doi.org/10.3168/jds.S0022-0302(03)73713-8

Rutherfurd, S. M. 2009. Accurate determination of the amino acid content of selected feedstuffs. Int. J. Food Sci. Nutr. 60:53-62. https://doi.org/10.1080/09637480802269957

Sairanen, A., H. Khalili, J. I. Nousiainen, S. Ahvenjärvi, and P. Huhtanen. 2005. The effect of concentrate supplementation on nutrient flow to the omasum in dairy cows receiving freshly cut grass. J. Dairy Sci. 88:1443-1453. https://doi.org/10.3168/jds.S0022-0302(05)72812-5

Sok, M., D. R. Ouellet, J. L. Firkins, D. Pellerin, and H. Lapierre. 2017. Amino acid composition of rumen bacteria and protozoa in cattle. J. Dairy Sci. 100:5241–5249. https://doi.org/10.3168/jds.2016-12447

Sylvester, J. T., S. K. R. Karnati, B. A. Dehority, M. Morrison, G. L. Smith, N. R. St-Pierre, and J. L. Firkins. 2009. Rumen ciliated protozoa decrease generation time and adjust 18S ribosomal DNA copies to adapt to decreased transfer interval, starvation, and monensin. J. Dairy Sci. 92:256-269. https://doi.org/10.3168/jds.2008-1417

Sylvester, J. T., S. K. R. Karnati, Z. Yu, C. J. Newbold, and J. L. Firkins. 2005. Evaluation of a real-time PCR assay quantifying the ruminal pool size and duodenal flow of protozoal nitrogen. J. Dairy Sci. 88:2083–2095. https://doi.org/10.3168/jds.S0022-0302(05)72885-X

Udén, P., P. E. Colucci, and P. J. Van Soest. 1980. Investigation of chromium, cerium and cobalt as markers in digesta. Rate of passage studies. J. Sci. Food Agric. 31:625–632. https://doi.org/10.1002/jsfa.2740310702

Valk, H., I. E. Kappers, and S. Tamminga. 1996. In sacco degradation characteristics of organic matter, neutral detergent fibre and crude protein of fresh grass fertilized with different

amounts of nitrogen. Anim. Feed Sci. Technol. 63:63–87. https://doi.org/10.1016/S0377-8401(96)01024-3

Van den Pol-van Dasselaar, A., D. Hennessy, and J. Isselstein. 2020. Grazing of Dairy Cows in Europe - An In-Depth Analysis Based on the Perception of Grassland Experts. Sustainability 12:1098-1116. https://doi.org/10.3390/su12031098

Van Vuuren, A., C. Van Der Koelen, and J. Vroons-De Bruin. 1993. Ryegrass versus corn starch or beet pulp fiber diet effects on digestion and intestinal amino acids in dairy cows. J. Dairy Sci. 76:2692-2700. https://doi.org/10.3168/jds.S0022-0302(93)77605-5

Van Vuuren, A.M., S. Tamminga, and R. S. Ketelaar. 1991. In-sacco degradation of organic matter and crude protein of fresh grass (Lolium perenne) in the rumen of grazing dairy cows. J. Agric. Sci. 116:429–436. https://doi.org/10.1017/S0021859600078242

Volden, H., L. T. Mydland, and O. M. Harstad. 1999a. Chemical composition of protozoa and bacteria isolated from ruminal contents of dairy cows fed diets differing in dietary nitrogen supplementation. Acta Agric. Scand., Sect. A, Anim. Sci. 49:4, 235-244. https://doi.org/10.1080/090647099423999

Volden, H., O. M. Harstad, and L. T. Mydland. 1999b. Amino acid content and profile of protozoal and bacterial fractions isolated from ruminal contents of lactating dairy cows fed diets differing in nitrogen supplementation. Acta Agric. Scand. A Anim. Sci. 49:245–250. https://doi.org/10.1080/090647099424006

Whitehouse, N. L., V. Olson, C. Schwab, W. Chesbrot, K. Cunningham, and T. Lykos. 1994. Improved techniques for dissociating particle-associated mixed ruminal microorganisms from ruminal digesta solids. J. Anim. Sci. 72:1335–1343. https://doi.org/10.2527/1994.7251335x

Williams, A. G., and G. S. Coleman. 1997. The rumen protozoa. Pages 73–139 in The Rumen Microbial Ecosystem. P. N. Hobson and C. S. Stewart, ed. Elsevier Science Publishers Ltd., London, UK.

Yáñez-Ruiz, D. R., N. D. Scollan, R. J. Merry, and C. J. Newbold. 2006. Contribution of rumen protozoa to duodenal flow of nitrogen, conjugated linoleic acid and vaccenic acid in steers fed silages differing in their water-soluble carbohydrate content. Br. J. Nutr. 96:861–869. https://doi.org/10.1017/BJN20061927

Younge, B., J. Murphy, and M. Rath. 2004. Nutrient metabolism in the rumen and milk production in cows fed on grass-silage and fresh grass based diets. Livest. Prod. Sci. 88:43-54. https://doi.org/10.1016/j.livprodsci.2003.11.003

# CHAPTER 5: REFINEMENT OF THE CORNELL NET CARBOHYDRATE AND PROTEIN SYSTEM V7.0 TO PREDICT THE OMASAL FLOW OF NITROGEN AND AMINO ACIDS, UTILIZING A UNIQUE PASTURE-BASED DATA SET

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## 5.1. ABSTRACT

Within the new dynamic structure of the Cornell Net Carbohydrate and Protein System (**CNCPS v7.0**), a protozoal sub-model has been incorporated to mechanistically describe the influence of protozoa on ruminal behavior and microbial amino acid flow. During model development few in vivo data pertaining to protozoal dynamics from lactating dairy cows were available. More recently, experimental techniques involving rapid protozoa harvesting, omasal sampling, and rumen evacuation have allowed the quantification of in vivo protozoal net generation time, increasing our current understanding of protozoal dynamics. Therefore, the primary objective of this study was to reparameterize the coefficients of the protozoal sub-model in the CNCPS v7.0 to improve predictions of in vivo conditions of the lactating dairy cow. Updates to the microbial AA composition and the passage rate of feed N that is insoluble in borate phosphate buffer but soluble in neutral detergent were also included. A model behavior assessment utilizing a unique pasture-based data set and a model performance evaluation against a literature

data set were performed. Results demonstrated that the reparameterized model (**RP v7.0**) had strong capability to predict the flows of N and AA at the omasal canal in lactating dairy cows consuming a perennial ryegrass-based diet. The flow of non-ammonia, non-microbial N was, however, overestimated for the RP v7.0 compared with the observed data (88 vs. 49 g N $day^{-1}$, respectively). Results from model evaluation against the literature dataset indicated that the prediction of non-ammonia N and microbial N for the RP v7.0 were both accurate and precise with $R^2_{BLUP}$ = 0.97 and 0.97, $R^2_{MP}$ = 0.92 and 0.90, and concordance correlation coefficient = 0.96 and 0.95, respectively. A lower level of systematic bias was observed in the prediction of non-ammonia, non-microbial N flow (1% systematic bias) when compared with the initial model structure (19% systematic bias), as the intercept and the slope of the regression was closer to zero and unity, respectively. Considerable improvement was achieved in the ability of the RP v7.0 to predict AA flow compared with the CNCPS v7.0, likely due to the reparameterized coefficients altering the relative contribution from each N fraction and the updated AA composition of bacteria and protozoa. However, further work is required to identify the sources of discrepancy between predicted and observed Met and Lys flows. Altogether, results suggest a significant improvement in the capacity of the model to predict AA flows at the omasal canal when compared with a literature data set.

**Keywords:** CNCPS, protozoa, evaluation, omasal flow, pasture

## 5.2. INTRODUCTION

In ruminant animals, accurate and precise prediction of their AA supply involves a comprehensive understanding of pre-gastric fermentation and the microbial populations involved. Dietary protein is extensively degraded in the rumen and lost to circulation as ammonia and urea,

or captured and re-synthesized as microbial protein, which subsequently contributes the largest supply of AA to the ruminant. The extent dietary protein degrades depends on a number of factors, such as the composition of the protein, the rate of degradation, level of intake, and rate of passage (Van Soest, 1994). Endogenous secretions can also contribute substantially to AA supply (Lapierre et al., 2008), albeit to a greater degree when samples are obtained from the duodenum compared with the omasum (Ørskov et al., 1986).

Recently, a new dynamic version of the Cornell Net Carbohydrate and Protein System (**CNPCS v7.0**) was developed to further describe the biological mechanisms that affect AA supply (Higgs, 2014; Higgs and Van Amburgh, 2016). One of the major model updates was the inclusion of a protozoal sub-model due to increasing evidence indicating that protozoa contribute substantially to the total microbial AA flow (Firkins et al., 2007). Additionally, protozoal metabolism affects the total microbial AA supply via bacterial predation, as well as nutrient digestion and cycling within the rumen (Newbold et al., 2015).

An initial evaluation of CNPCS v7.0 demonstrated a strong ability to predict non-ammonia nitrogen (**NAN**) flow compared with a literature data set composed of studies utilizing the omasal sampling technique (Higgs, 2014). However, biases were present in the partitioning of this NAN flow as non-ammonia, non-microbial nitrogen (**NANMN**) flow was over predicted, whereas microbial nitrogen (**MicN**) flow was under predicted. In addition, the estimates of EAA flows were over predicted by the model when compared with the literature data set. A more recent evaluation indicated that protozoal N flow was under predicted by 43%, suggesting that the coefficients used to describe protozoal metabolism required refinement (Fessenden et al., 2019).

Due to the difficulty of protozoal isolation, few studies have attempted to directly measure their flow in vivo (Firkins and Yu, 2006). As a result, during initial model parameterization, coefficients

were primarily based on in vitro studies of protozoal species or on data obtained from non-lactating animals, which likely do not represent rumen conditions of modern lactating dairy cows (Firkins et al., 2007). In Chapter 3, rapid protozoal harvesting, rumen evacuation, and the omasal sampling technique were utilized to determine in vivo protozoal generation time in lactating dairy cows. The experimental diets also consisted of immature perennial ryegrass, which based on high aNDFom digestibility and large influxes of innate water, exhibit rapid rumen turnovers. The results from Chapter 3 indicate that protozoa have the capability to grow at faster rates than previously measured (Hristov and Jouany, 2005) and this allows them to maintain their populations in the rumen and not be washed out by the rapid turnover of both the liquid and fiber pools.

Therefore, the primary objective of the current study was to utilize the observations from Chapter 3 in combination with the growing literature of in vivo protozoal dynamics to reparameterize the protozoal sub-model in the CNCPS v7.0. A secondary objective was to describe additional updates, present an evaluation of model performance against a literature data set, and present a preliminary assessment of the ability of the CNCPS to predict AA supply in pasture-fed cows.

## 5.3. MATERIALS AND METHODS

### *5.3.1. Model Reparameterization*

In the CNCPS v7.0, protozoa are comprised of two main groups based on their preferred growth substrates, entodiniomorhids and isotrichids protozoa (i.e. holotrichs; Higgs, 2014). Currently, within the structure of the model, all protozoa are assumed to pass from the rumen in the particle phase (Higgs, 2014); however, in vivo data to support this assumption are limited (Firkins and Yu, 2006). In addition, the data to support this assumption were obtained from animals fed at low levels of intake, which might not accurately represent high-producing animals due to differences in

rumen turnover and rates of passage (Firkins et al., 2007). A number of recent studies, in lactating dairy cows have measured large quantities of protozoa leaving the rumen in the liquid phase (Ahvenjarvi et al., 2002; Karnati et al., 2007; Fessenden et al., 2019). Furthermore, as reported in Chapter 3, protozoal generation time was shorter than that of the liquid retention time, indicating the ability of the protozoa population to pass from the rumen at a similar rate as the liquid phase. While isotrichids have demonstrated the ability to 'attach' to particles in the rumen (Diaz et al., 2014), the predominant entodiniomorphids lack this ability (Dehority, 1984; Firkins et al., 2020). Given these observations and that isotrichids typically represent 20% of the total rumen protozoal population (Clarke et al., 1964), the model was reparameterized so that 20% of the rumen protozoa pass with the particle phase, whereas the remaining 80% pass with the liquid phase. The appropriateness of this partition between liquid and particle phase on model predictions was assessed using the data and observations from Chapter 3.

In Chapter 3, protozoal generation time was less than 5 h, which coincided with the faster rumen turnover of liquid and fiber pools from the rumen. Due to the higher passage rate in high-producing cows or cows fed highly digestible pasture, protozoal lysis rate has been suggested to reduce, when compared with cows fed at low levels of intake (Wells and Russell, 1996; Firkins et al., 2007). Firkins et al. (2007) also suggested that a faster protozoa rumen passage would improve the efficiency of protozoal cell growth, as previously demonstrated for the efficiency of bacterial cell growth (Isaacson et al., 1975; Harrison et al., 1976). Studies investigating the effect of increased dilution rate on protozoal generation time support these suggestions (Dehority, 2004; Sylvester et al., 2009). Given the relationship between dilution rate and lysis rate described by Wells and Russell (1996), a lower protozoal lysis rate was assigned to the protozoal pool in the reparameterized model (10% of the passage rate) compared with the current version of the model

(50% of the passage rate). This was done through an iterative process where the predictions of protozoal rumen pool size and flow to the omasum were assessed against the data of Chapter 3.

To calculate total protozoal predation of bacterial N, the CNCPS v7.0 assumes 50% of the engulfed bacterial N is incorporated into the protozoal cell, while the remaining 50% is excreted back into the ruminal environment. This determines the net requirement for N by protozoa and was based on in vitro data from Wallace and McPherson, (1987). However, Coleman and Sandford, (1979) demonstrated that protozoa grown in vivo digest and release 75-80% less bacteria when compared with protozoa grown in vitro, which is likely due to the commonly used in vitro experimental technique of pulse dosing bacteria to starved protozoa (Diaz et al., 2014). This technique also removes the protozoal engulfment of starch particulate matter, which limits protozoal internal capacity and the amount of bacteria ingested (Williams and Coleman, 1992; Hristov and Jouany, 2005). Furthermore, protozoal engulfment of bacteria can be a slow continuous process and faster ruminal passage likely lessens predation of bacteria by protozoa (Firkins et al., 2020). Koenig et al. (2000) demonstrated that defaunation of sheep did not affect the absolute amount of bacterial N recycled. Therefore, based on lower ingestion of bacterial N in vivo and a limited time to recycle the N in cows with higher ruminal turnover, the excretion of bacterial N by protozoa was reduced from 50% to 10% in the reparameterized model. This 80% reduction in predation corresponds to the discrepancy between in vitro and in vivo measurements described by Coleman and Sandford, (1979) and aligns with the observations in Chapter 3.

The bacterial and protozoal AA composition were also updated using the data set described in Chapter 4. This data set represented a complete description of the AA composition of liquid-associated bacteria (n = 30), particle-associated bacteria (n = 30), and protozoa (n = 30). The AA determined included the sulphur AA, Met and Cys, which underwent a pre-oxidation step before

acid hydrolysis to ensure adequate recovery. The concentration of Trp was also determined, which has regularly been omitted from microbial AA analysis (Sok et al 2017; Lapierre et al., 2019).

Finally, ruminal digestion in the CNCPS is dependent on the competition between rates of degradation and passage (kd/kd + kp), originally described by Waldo et al. (1972). In previous evaluations of the CNCPS, predictions of NANMN outflow from the rumen were overestimated (Van Amburgh et al., 2015; Higgs, 2014). In Chapter 3, the in vivo digestion rate and passage rate of NANMN could be quantified as rumen pool size and omasal flow were measured simultaneously. The calculated NANMN passage rate approximated that of the aNDFom passage rate; however, in the current CNCPS v7.0 the NANMN rate of passage approximates that of the particle passage rate. Therefore, to reflect the slower NANMN passage rate from the rumen, the insoluble PB1 pool in the reparameterized model was assigned to flow with the aNDFom passage rates instead of the particle passage rate. The aNDFom passage rate equations currently used in CNCPS v7.0 were developed for the NorFor system (NorFor, 2011) and use DMI, the aNDFom concentration of the diet, and BW as factors in the equations. Considering the PB1 pool is primarily comprised of the N associated with the total dietary fiber, passing from the rumen at the same rate as aNDFom likely more appropriately reflects the behavior of this fraction. Adjustment of the PA2 pool passage rate was not performed as extensive data now supports that soluble AA and peptides escape from the rumen in the liquid phase (Choi et al., 2002; Reynal et al., 2007).

### *5.3.2. Model Behavior Assessment and Evaluation*

While a number of studies have measured N and AA flows in pasture-fed cows, duodenal sampling was performed, which has a number of drawbacks reducing the applicability of these studies for the current model behavior assessment. The combination of the acidic abomasal

environment and large endogenous N abomasal secretions (Ørskov et al., 1986; Lapierre et al., 2008) reduces the ability of these studies to accurately describe feed N degradation (Broderick et al., 2010). In addition, incomplete marker recovery at the duodenum can result in unrealistically low digestion data, which fall outside biological limits (Huhtanen et al., 2010). For example, in a number of studies investigating the digestion of pasture-based diets, that incorporated duodenal sampling, negative portions of hind-gut digestion of total NDF were reported (Van Vuuren et al., 1992; Peyraud et al., 1997), suggesting unrepresentative digesta sampling or marker dysfunction (Huhtanen et al., 2010). Therefore, to assess the ability of the CNCPS to describe N fraction flow and AA flow in cows consuming a pasture diet, the omasal sampling experiment described in Chapters 3 and 4 was utilized. All relevant data required to conduct a model simulation were obtained from the perennial ryegrass-only treatment and entered into the CNCPS v7.0 (Higgs, 2014). The same data were entered into an in-development version of the CNCPS, which included the model reparameterizations discussed in the current study (**RP v7.0**). The model-predicted omasal flows of all N fractions and AA were compared with those measured during the experiment. Dietary details, level of intake, and animal parameters used as inputs for the model behavior assessment exercise can be found in Chapter 3.

A comprehensive evaluation of RP v7.0 predictions of NAN, MicN, NANMN, and AA flow at the omasal canal, using a previously compiled data set was conducted (Higgs, 2014). In the evaluation of N flows, 16 published studies with 61 treatment means were evaluated, whereas to evaluate individual AA flows 11 studies with 43 treatment means were utilized [Table 5.1; Adapted from Higgs (2014)]. Criteria used for study selection and a description regarding data set construction can be found in Higgs (2014).

**Table 5.1.** Omasal sampling studies used to evaluate model N and AA flows[1]

| Study | Amino acid flows reported |
|---|---|
| Ahvenjärvi et al. (1999) | |
| Ahvenjärvi et al. (2002) | x |
| Ahvenjärvi et al. (2006) | |
| Brito et al. (2006) | x |
| Brito et al. (2007a) | x |
| Brito et al. (2007b) | x |
| Brito et al. (2009) | x |
| Broderick and Reynal (2009) | x |
| Choi et al. (2002) | |
| Korhonen et al. (2002) | x |
| Owens et al. (2008a) | |
| Owens et al. (2008b) | |
| Reynal et al. (2007) | x |
| Reynal and Broderick (2003) | x |
| Reynal and Broderick (2005) | x |
| Vanhatalo et al. (2009) | x |

[1]Adapted from Higgs (2014)

### *5.3.3. Statistical Analysis*

Statistical analysis was conducted using SAS version 9.4 (SAS Institute Inc. Cary, NC). A similar method as described by Higgs (2014) was applied. The simulation data were analyzed by a mixed model using the restricted maximum likelihood procedure:

$$Y_{ij} = \beta_0 + s_i + \beta_1 X_{ij} + \varepsilon_{ij},$$

where $Y_{ij}$ = the expected outcome for the dependent variable $Y$ observed at level $j$ of the continuous variable $X$ in study $i$, $\beta_0$ = the overall intercept across all studies, $s_i$ = the random effect of study $i$, $\beta_1$ = the overall slope of $Y$ on $X$ across all studies, $X_{ij}$ = the model-predicted data associated with level $j$ of the continuous variable $X$ in study $i$, and $\varepsilon_{ij}$ = random variation.

The squared sample correlation coefficients reported were based on either the BLUP ($R^2_{BLUP}$) or model predictions using a mean study effect ($R^2_{MP}$). Potential bias and confounding factors were assessed through examination of the conditional residuals. To indicate accuracy, root mean square prediction errors (RMSPE) were reported (Tedeschi, 2006). Furthermore, MSPE was decomposed to assess error due to mean bias, systematic bias, and random variation (Bibby and Toutenburg, 1977). Finally, to assess agreement between the actual and predicted values, the concordance correlation coefficient (CCC; Lin, 1989) was used. In this calculation, the Pearson correlation coefficient reflects the precision of the predictions, whereas the bias correction factor reflects accuracy.

## 5.4. RESULTS AND DISCUSSION

### *5.4.1. Reparameterized Model Behavior in Pasture-Fed Animals*

To assess the capability of the CNCPS v7.0 and the RP v7.0 to predict N and AA flows at the omasal canal in pasture-fed dairy cows, model-predicted estimates were compared with those described in Chapters 3 and 4 (Table 5.2). The predicted MicN flow was increased for the RP v7.0 (311 g N/d) and was in closer agreement with the observed data (324 g N/d) compared with the CNCPS v7.0. (241 g N/d). This increase in MicN flow was due to increased bacterial and protozoal N flow for the RP v7.0 compared with the CNCPS v. 7.0. The greater passage rate and reduced lysis rate of protozoa for the RP v7.0 allowed a greater protozoal N flow out of the rumen. In addition, a lower predation of bacterial N for the RP v7.0 allowed a greater bacterial N outflow from the rumen, more closely replicating the observed data. In both versions of the model, NANMN flow was overestimated compared with the observed data; however, reassigning the passage rate of the PB1 pool in the RP v7.0 reduced the overestimation of NANMN flow. Total NAN flow was slightly overestimated for the RP v7.0 compared with the observed data (400 vs. 373 g N $day^{-1}$, respectively; Table 5.2) due to the increased accuracy of prediction of MicN flow and an overestimation of NANMN flow. A considerable flow of peptide N and free AA N were predicted to escape ruminal digestion for both versions of the model (data not shown). Although the outflow of peptides and free AA from the rumen has been comprehensively described for cows fed total mixed rations (Choi et al., 2002; Reynal et al., 2007), data currently does not exist for pasture-fed cows. More research is warranted to further elucidate the supply of N in pasture-fed cows and to develop novel data sets to assess the accuracy and precision of prediction from mechanistic models, such as the CNCPS.

To the authors' knowledge, capability to predict the AA supply in pasture-fed cows has not been described previously. Overall, the prediction of AA flow for the RP v7.0 was in closer agreement with the observed data compared with the CNCPS v7.0. (Table 5.2). However, the supply of Arg, Met, and Trp was slightly over predicted for the RP v7.0 compared with the observed data and the CNCPS v7.0. This suggests that the coefficient reparameterization of the CNCPS v7.0 increased the ability of the model to predict the N and AA flows in pasture-fed cows. Indeed, for a number of AA, the RP v7.0 predicted the observed omasal flow with high accuracy (Table 5.2). Future work, incorporating omasal sampling, is required to develop comprehensive evaluation datasets for pasture-fed cows to further investigate the capability of mechanistic models to predict N and AA flows.

**Table 5.2.** The N and AA flows observed at the omasal canal in perennial ryegrass-fed lactating dairy cows (Chapters 3 and 4) and the predicted flows by either the CNCPS v7.0 or the RP v7.0

| Omasal flow[1], g/d | Observed[2] | CNCPS v7.0 | RP v7.0 |
|---|---|---|---|
| NAN | 373 | 345 | 400 |
| Microbial N | 324 | 241 | 311 |
| Bacterial N | 248 | 178 | 227 |
| Protozoal N | 79 | 62 | 85 |
| NANMN | 49 | 105 | 88 |
| Arg | 83 | 87 | 97 |
| His | 31 | 34 | 34 |
| Ile | 97 | 92 | 102 |
| Leu | 152 | 138 | 151 |
| Lys | 148 | 138 | 148 |
| Met | 50 | 47 | 57 |
| Phe | 91 | 96 | 96 |
| Thr | 101 | 91 | 97 |
| Trp | 35 | 35 | 40 |
| Val | 106 | 99 | 105 |

[1]NAN = non-ammonia nitrogen; NANMN = non-ammonia, non-microbial nitrogen
[2]Observed AA flow (Chapter 4) uncorrected for the effect of hydrolysis time

### *5.4.2. Model Evaluation*

Previous evaluations of the CNCPS v7.0 indicated a strong ability to predict NAN flow at the omasal canal (Higgs, 2014). However, within this NAN flow, biases were present where NANMN flow was over predicted and MicN flow was slightly under predicted. In the current evaluation, the flow of NAN was predicted accurately and precisely by the RP v7.0 when compared with the observed literature data (Figure 5.1; Table 5.3). The flow of MicN, was also predicted accurately and precisely ($R^2_{BLUP}$ = 0.97; root mean square error = 22.9; CCC = 0.95). The current evaluation indicated that there was no systematic or mean bias in the prediction of MicN flow and that all error was due to random variation (Figure 5.2; Table 5.3). Fleming et al. (2019) noted a similar partitioning of the prediction error when evaluating MicN flow prediction, using the model proposed by Roman-Garcia et al. (2016). However, a lower level of accuracy and precision was reported (CCC = 0.68; Fleming et al., 2019) when compared with the current evaluation. The evaluation dataset constructed by Fleming et al. (2019) contained studies that utilized both duodenal or omasal sampling techniques. This likely contributed, at least in part, to the lower accuracy and precision for reasons discussed previously (Broderick et al., 2010). Unfortunately, both the previous (Higgs, 2014) and current evaluations could not systematically evaluate the protozoal sub-model flow predictions as the number of studies quantifying protozoal N outflow from the rumen are still lacking in the literature (Firkins et al., 2020). However, a wide range of predicted protozoa N flow was observed in the current study (7 to 28% of MicN flow) indicating that the reparameterized protozoal coefficient allowed high model sensitivity in the partitioning of MicN between bacterial N and protozoal N flow.

In the current evaluation, a lower level of systematic bias was observed in the prediction of NANMN flow (1% systematic bias) when compared with the evaluation of Higgs (2014; 19%

systematic bias), as the intercept and the slope were closer to zero and unity, respectively (Figure 5.3). Therefore, based on the current evaluation, the RP v7.0 can more accurately predict NANMN flow. However, the precision of prediction was not improved as assessed based on both the $R^2_{BLUP}$ and $R^2_{MP}$ (Table 5.3), which could be, at least in part, related to the difficulty in determining the flow of NANMN in vivo. The NANMN flow is generally calculated as the difference between total NAN flow and MicN flow; therefore, the variance in both of these measures will contribute to observed variation in NANMN flow. Overall, the increased prediction accuracy of NANMN flow, in the current evaluation, encourages the adoption of the PB1 passage rate reassignment.

In the initial evaluation of the CNCPS v7.0 (Higgs, 2014), the AA flow for all EAA were over predicted by the model when compared with the literature data set. This was surprising as the prediction of total NAN flow was evaluated to be accurate and precise. A number of factors could be responsible for this discrepancy. Within the structure of the CNCPS v7.0, NAN flow is calculated as the sum of bacterial, protozoal, feed, and endogenous N flow from the rumen. Theoretically, if two or more fractions of N were estimated incorrectly, the inaccuracies could offset one another and result in an accurate estimate of the NAN flow. However, each N fraction is assigned a specific AA composition and when recombined, might result in an under- or over-prediction of the observed AA flow. Other possible factors for the over prediction of AA flow could be error in the AA composition or AA content assigned to each N fraction

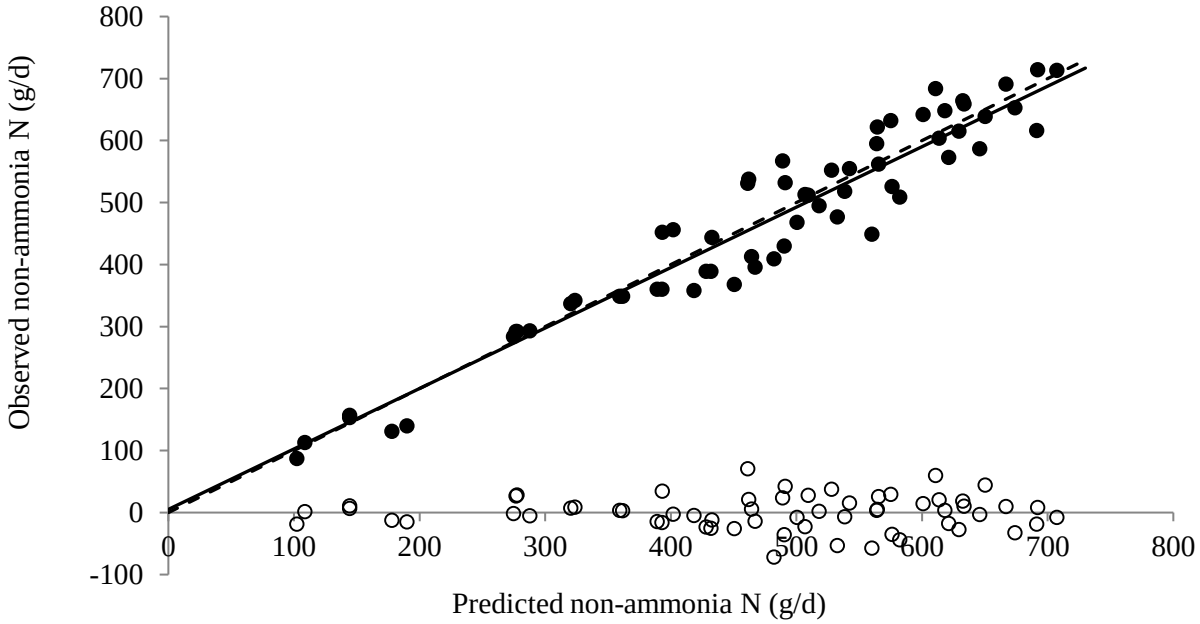



**Figure 5.1.** Observed (literature dataset) versus Cornell Net Carbohydrate and Protein System (CNCPS; reparameterized v7.0) predictions (●) for non-ammonia N flow at the omasal canal. The solid line (—) represents the linear regression and the dashed line (- - -) is the unity line. Conditional residuals from the mixed model analysis are also presented (○).

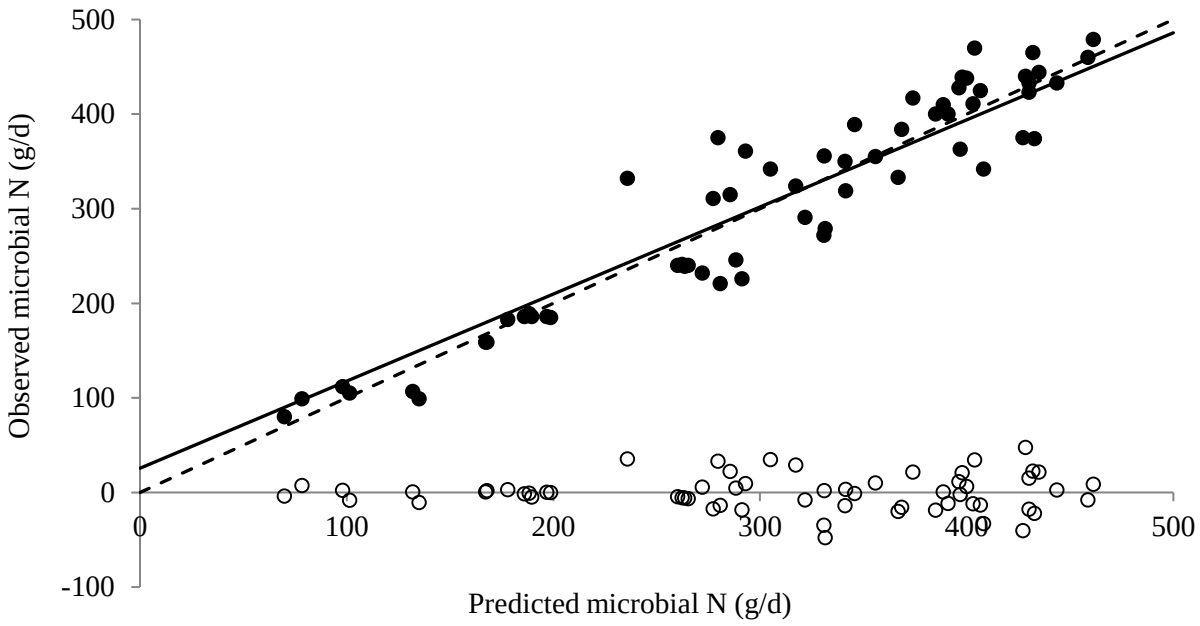



**Figure 5.2.** Observed (literature dataset) versus Cornell Net Carbohydrate and Protein System (CNCPS; reparameterized v7.0) predictions (●) for microbial N flow at the omasal canal. The solid line (—) represents the linear regression and the dashed line (- - -) is the unity line. Conditional residuals from the mixed model analysis are also presented (○).

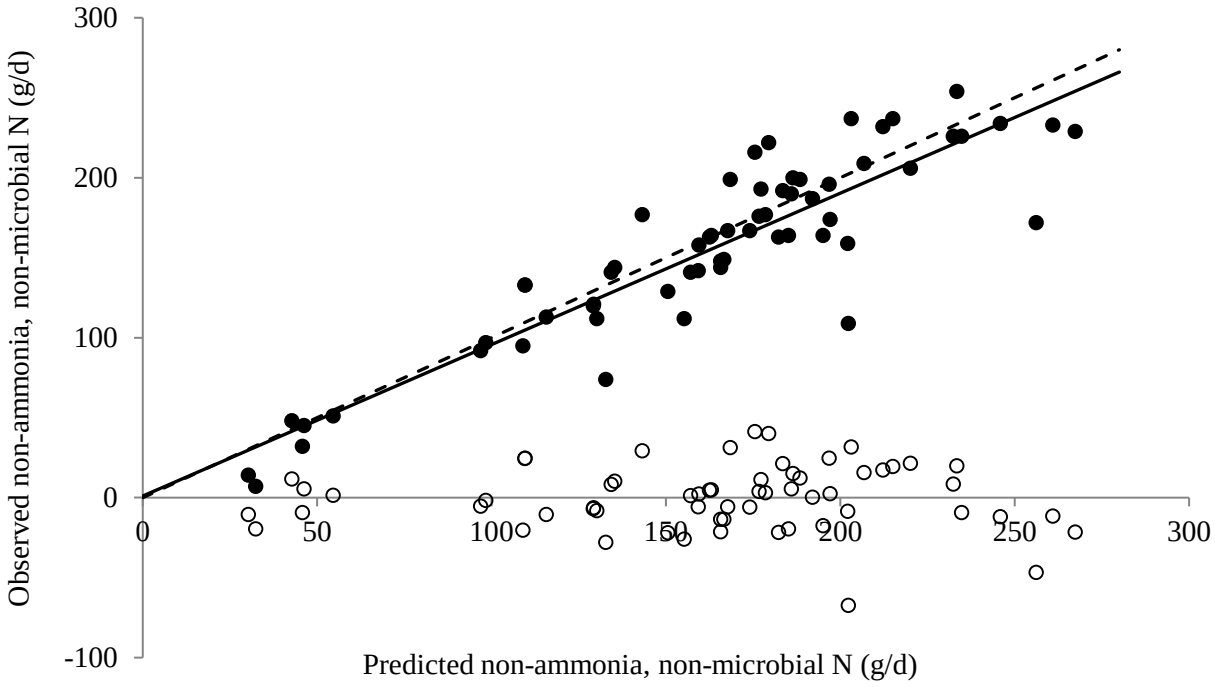



**Figure 5.3.** Observed (literature dataset) versus Cornell Net Carbohydrate and Protein System (CNCPS; reparameterized v7.0) predictions (●) for non-ammonia, non-microbial N flow at the omasal canal. The solid line (—) represents the linear regression and the dashed line (- - -) is the unity line. Conditional residuals from the mixed model analysis are also presented (○).

**Table 5.3.** Prediction adequacy of the reparameterized Cornell Net Carbohydrate and Protein System v7.0 model for N and essential AA predicted flows at the omasal canal (g/d) compared with a literature dataset of omasal sampling studies

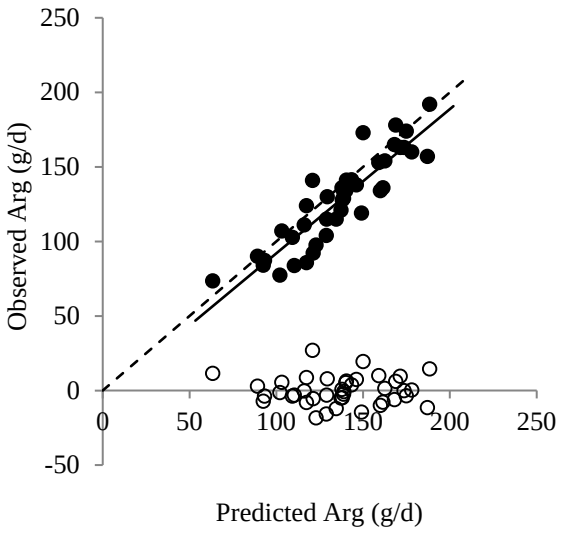
| | | | | | | Variance component (%)[5] | | | | MSPE Partitioned (%)[8] | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Item[1] | $R^2_{BLUP}$[2] | $R^2_{MP}$[3] | RMSE[4] | slope | intercept | study | residual | CCC[6] | RMSPE[7] | $U^M$ | $U^S$ | $U^R$ |
| NAN | 0.97 | 0.92 | 30.5 | 0.98 | 5.1 | 56.4 | 43.6 | 0.96 | 44.5 | 2% | 0% | 98% |
| Microbial N | 0.97 | 0.90 | 22.9 | 0.92 | 25.6 | 67.6 | 32.4 | 0.95 | 36.1 | 0% | 0% | 100% |
| NANMN | 0.88 | 0.81 | 21.9 | 0.95 | 1.0 | 28.6 | 71.4 | 0.90 | 26.2 | 6% | 1% | 92% |
| Arg | 0.91 | 0.80 | 10.4 | 0.97 | -4.8 | 45.4 | 54.6 | 0.86 | 16.2 | 31% | 1% | 69% |
| His | 0.89 | 0.63 | 4.7 | 0.97 | -1.0 | 66.4 | 33.6 | 0.78 | 8.2 | 5% | 11% | 83% |
| Ile | 0.83 | 0.77 | 10.6 | 0.76 | 22.5 | 21.0 | 79.0 | 0.80 | 17.3 | 42% | 14% | 44% |
| Leu | 0.90 | 0.86 | 19.2 | 1.01 | 3.3 | 21.5 | 78.5 | 0.92 | 21.4 | 5% | 1% | 95% |
| Lys | 0.90 | 0.62 | 11.2 | 0.73 | 9.5 | 71.0 | 29.0 | 0.47 | 46.3 | 74% | 8% | 18% |
| Met | 0.93 | 0.41 | 4.0 | 0.70 | 11.0 | 85.8 | 14.2 | 0.50 | 15.2 | 43% | 13% | 44% |
| Phe | 0.90 | 0.51 | 11.4 | 0.92 | 23.2 | 74.7 | 25.3 | 0.63 | 25.3 | 25% | 2% | 73% |
| Thr | 0.90 | 0.80 | 10.6 | 0.94 | 8.9 | 39.2 | 60.8 | 0.89 | 13.1 | 1% | 0% | 99% |
| Val | 0.85 | 0.75 | 11.5 | 0.80 | 16.8 | 33.7 | 66.3 | 0.77 | 20.5 | 48% | 9% | 44% |

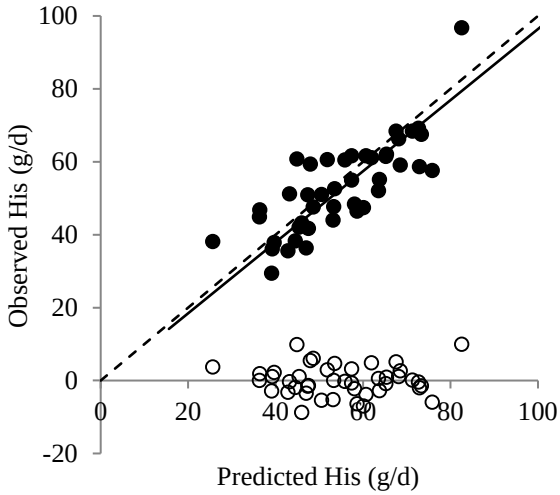
[1]NAN = non-ammonia N; NANMN = non-ammonia, non-microbial N.
[2]$R^2_{BLUP}$ = squared sample correlation coefficient based on BLUP.
[3]$R^2_{MP}$ = squared sample correlation coefficient based on model-predicted estimates.
[4]RMSE = Root mean square error.
[5]Percentage of variance related to the effect of study and random variation.
[6]Concordance correlation coefficient.
[7]RMSPE = Root mean square prediction error.
[8]MSPE = Mean square prediction error partitioned to: $U^M$= mean bias; $U^S$ = systematic bias; $U^R$ = random variation. $U^M + U^S + U^R = 100$.

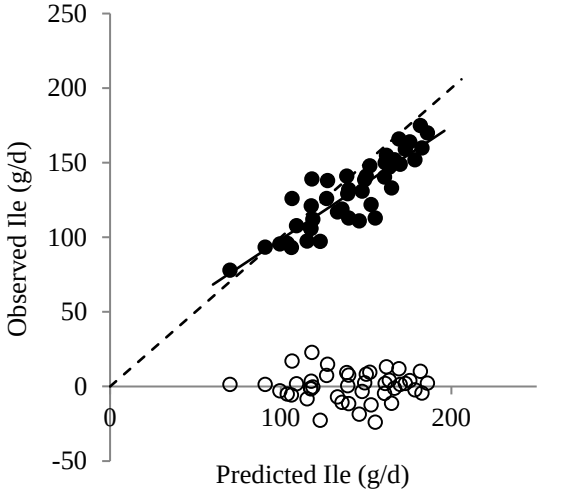
In the current evaluation, considerable improvement in the ability of the CNCPS to predict AA flow was achieved (Table 5.3; Figure 5.4) compared with the evaluation of Higgs (2014). The improvement in AA flow prediction for the RP v7.0 compared with the CNCPS v7.0 is likely due to the reparameterized coefficients altering the relative contribution from each N fraction and the updated AA composition of bacteria and protozoa obtained from Chapter 4. Compared with the evaluation of Higgs (2014), lower levels of systematic bias were observed for all AA flows, as the intercepts and slopes were closer to zero and unity, respectively, except for Met (Figure 5.4). Determination of the sulphur containing AA can be challenging and requires pre-oxidation with performic acid to ensure complete recovery of the AA (Elkin and Griffith, 1984). However, this pre-oxidation step is not commonly reported in the literature, reducing our ability to assess if adequate Met recoveries were achieved across all studies (Fessenden, 2016). This might have led to some of the over-prediction of Met flow observed in the current evaluation, as the updated AA composition incorporated into the RP v7.0 utilized the pre-oxidation step.

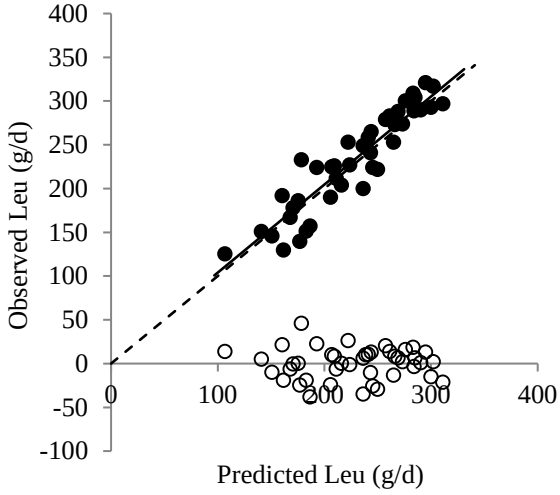
In the evaluation of Higgs (2014), Lys flow was substantially over-predicted, and although not as severe, a similar over-prediction was observed in the current evaluation. It is difficult to ascertain the reason for this. Higgs (2014) suggested that the regular use of formalin as an agent to stop bacterial cells from lysing, might have contributed to some of the discrepancy between predicted and observed Lys flows. Samples treated with formalin have previously been reported to reduce Lys content by more than 30% (Volden and Harstad, 1998); however, not all studies in the literature data set utilized formalin. Fessenden (2016) suggested that the microbial AA composition data from Clark et al. (1992), which has been implemented in the CNCPS, might overestimate Lys content as the majority of data reviewed used a single hydrolysis time-point to determine microbial AA concentrations. There is mounting evidence that a single hydrolysis time-

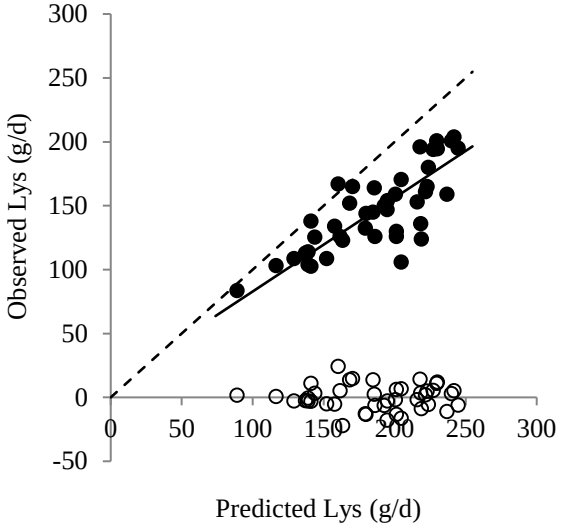
point might underestimate the true AA concentrations of many proteins, especially the branched-chain AA (Rutherfurd et al., 2008; Fessenden et al., 2017; Chapter 2). Lapierre et al. (2019) recently proposed AA specific correction factors that can be applied to all protein matrixes to account for the incomplete AA recovery when using a single hydrolysis time-point. These correction factors could have been applied in the current evaluation; however, the observed AA flow data from the literature data set would also have had to be corrected as all studies utilized a single hydrolysis time-point. If performed in the current evaluation, all proteins would have been corrected by the same factor and therefore, the evaluation would have resulted in a similar over prediction of the Lys flow. Further research is warranted as to whether protein specific correction factors are required and, if so, should be incorporated into future evaluations of the CNCPS.

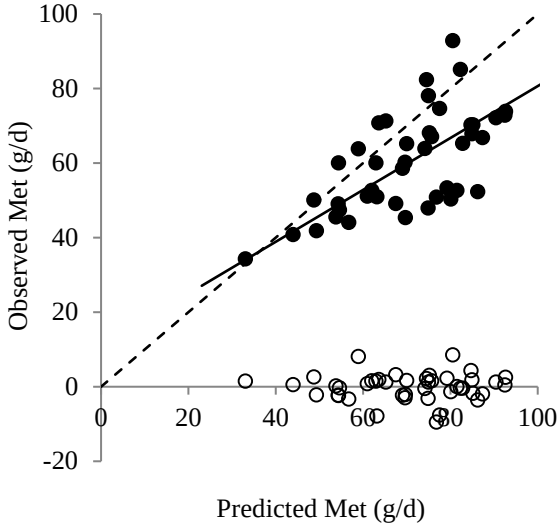

Observed Arg (g/d)
Predicted Arg (g/d)
250
200
150
100
50
0
-50
0
50
100
150
200
250


Observed His (g/d)
Predicted His (g/d)
100
80
60
40
20
0
-20
0
20
40
60
80
100


Observed Ile (g/d)
Predicted Ile (g/d)
250
200
150
100
50
0
-50
0
100
200


Observed Leu (g/d)
Predicted Leu (g/d)
400
350
300
250
200
150
100
50
0
-50
-100
0
100
200
300
400


Observed Lys (g/d)
Predicted Lys (g/d)
300
250
200
150
100
50
0
-50
0
50
100
150
200
250
300


Observed Met (g/d)
Predicted Met (g/d)
100
80
60
40
20
0
-20
0
20
40
60
80
100

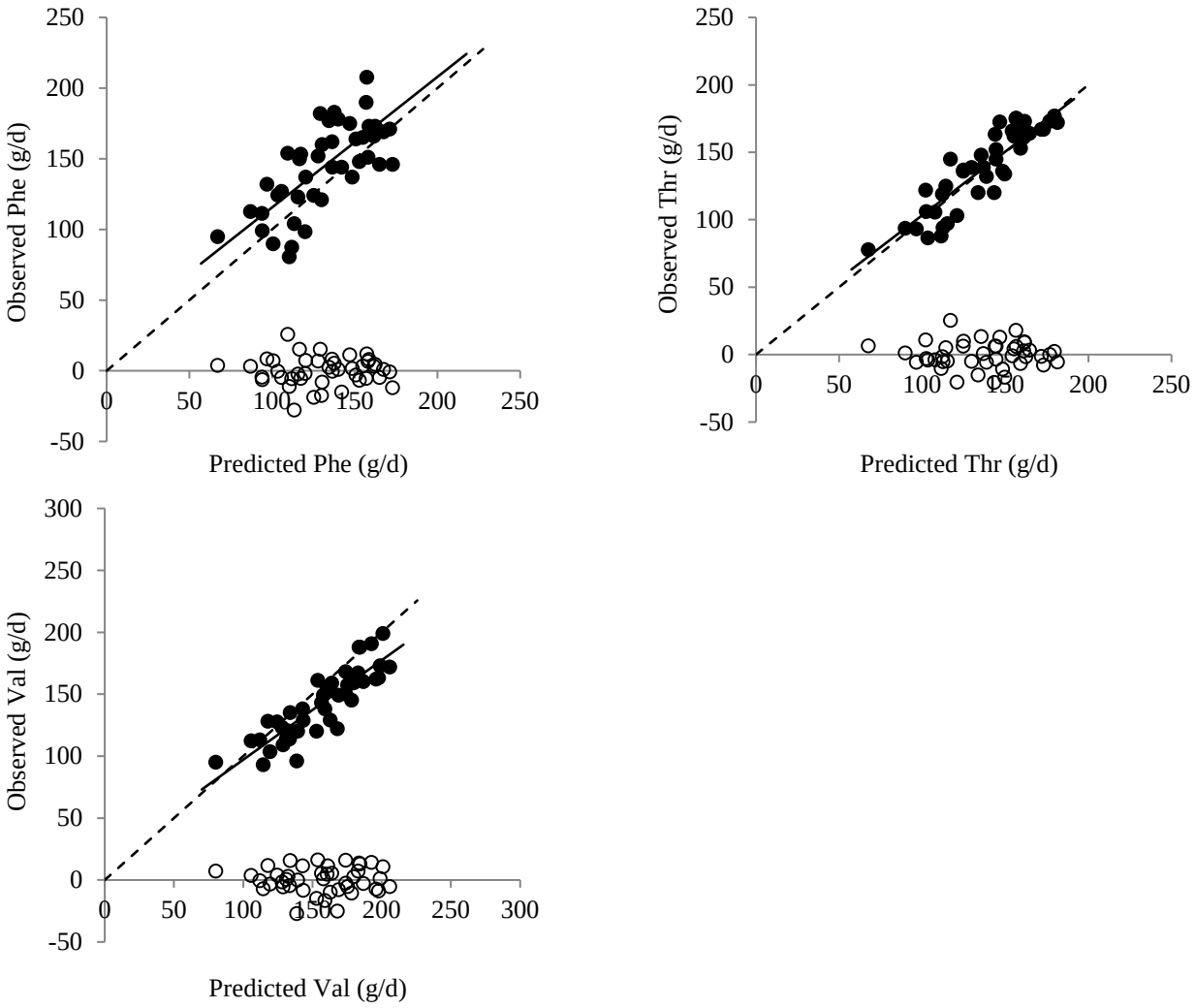



**Figure 5.4.** Observed (literature dataset) versus Cornell Net Carbohydrate and Protein System (CNCPS; reparameterized v7.0) predictions (●) for essential AA flow at the omasal canal. The solid line (—) represents the linear regression and the dashed line (- - -) is the unity line. Conditional residuals from the mixed model analysis are also presented (○).

Finally, in the evaluation of Fleming et al. (2019) the authors suggested that inadequate techniques when conducting AA analysis have resulted in an underestimation of true AA concentrations in the majority of laboratories. Those authors suggested that during acid hydrolysis of the AA too much time or too high a temperature might be used, reducing total AA recovery. This would imply that the RP v7.0 is actually under predicting the omasal flows of AA; however, the literature data set of AA flows used in the current evaluation would also likely be underestimated. Future work should focus on the effect of acid hydrolysis procedures and AA recovery on the omasal flow of AA. Overall, the RP v7.0, in the current evaluation, increased the ability of the model to predict AA flow at the omasal canal when compared with the literature data set. However, further work is required to refine the models ability to predict Met and Lys flows.

## 5.5. CONCLUSIONS

A number of recent in vivo studies have increased our understanding of protozoal dynamics in lactating dairy cows. Reparamaterizing the coefficients of the CNCPS v7.0 to more accurately represent these biological mechanisms, in combination with a number of other model updates, improved the capacity of the model to predict N and AA flows at the omasal canal, when compared with a literature data set. However, further work is required to refine the models ability to predict Met and Lys flows. Lastly, prediction of AA flows for the reparameterized CNCPS v7.0 were in general agreement with observed AA flows obtained from pasture-fed lactating dairy cows; however, the flows of Arg, Met and Trp were slightly over predicted. While this indicates strong prediction capability of the reparameterized CNCPS v7.0 for pasture-based systems, more data is required to systematically evaluate the model under such dietary conditions.

## 5.6. ACKNOWLEDGMENTS

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## 5.7. REFERENCES

Ahvenjärvi, S., E. Joki-Tokola, A. Vanhatalo, S. Jaakkola, and P. Huhtanen. 2006. Effects of replacing grass silage with barley silage in dairy cow diets. J. Dairy Sci. 89:1678-1687. https://doi.org/10.3168/jds.S0022-0302(06)72235-4

Ahvenjärvi, S., A. Vanhatalo, and P. Huhtanen. 2002. Supplementing barley or rapeseed meal to dairy cows fed grass-red clover silage: I. Rumen degradability and microbial flow. J. Anim. Sci. 80:2176– 2187. https://doi.org/10.1093/ansci/80.8.2176

Ahvenjärvi, S., A. Vanhatalo, P. Huhtanen, and T. Varvikko. 1999. Effects of supplementation of a grass silage and barley diet with urea, rapeseed meal and heat-moisture-treated rapeseed cake on omasal digesta flow and milk production in lactating dairy cows. Acta Agriculturae Scandinavica, Section A - Animal Science 49:179 - 189. https://doi.org/10.1080/090647099424097

Bibby, J. and H. Toutenburg. 1977. Prediction and improved estimation in linear models. Wiley, London, UK.

Brito, A. F., G. F. Tremblay, H. Lapierre, A. Bertrand, Y. Castonguay, G. Bélanger, R. Michaud, C. Benchaar, D. R. Ouellet, and R. Berthiaume. 2009. Alfalfa cut at sundown and harvested as baleage increases bacterial protein synthesis in late-lactation dairy cows. J. Dairy Sci. 92:1092-1107. https://doi.org/10.3168/jds.2008-1469

Brito, A. F., G. A. Broderick, J. J. O. Colmenero, and S. M. Reynal. 2007a. Effects of feeding formate-treated alfalfa silage or red clover silage on omasal nutrient flow and microbial protein synthesis in lactating dairy cows. J. Dairy Sci. 90:1392-1404. https://doi.org/10.3168/jds.S0022-0302(07)71625-9

Brito, A. F., G. A. Broderick, and S. M. Reynal. 2007b. Effects of different protein supplements on omasal nutrient flow and microbial protein synthesis in lactating dairy cows. J. Dairy Sci. 90:1828-1841. https://doi.org/10.3168/jds.2006-559

Brito, A. F., G. A. Broderick, and S. M. Reynal. 2006. Effect of varying dietary ratios of alfalfa silage to corn silage on omasal flow and microbial protein synthesis in dairy cows. J. Dairy Sci. 89:3939-3953. https://doi.org/10.3168/jds.S0022-0302(06)72436-5

Broderick, G. A., P. Huhtanen, S. Ahvenjärvi, S. M. Reynal, and K. J. Shingfield. 2010. Quantifying ruminal nitrogen metabolism using the omasal sampling technique in cattle - A meta-analysis. J. Dairy Sci. 93:3216-3230. https://doi.org/10.3168/jds.2009-2989

Broderick, G. A. and S. M. Reynal. 2009. Effect of source of rumen-degraded protein on production and ruminal metabolism in lactating dairy cows. J. Dairy Sci. 92:2822-2834. https://doi.org/10.3168/jds.2008-1865

Choi, C. W., S. Ahvenjarvi, A. Vanhatalo, V. Toivonen, and P. Huhtanen. 2002. Quantitation of the flow of soluble non-ammonia nitrogen entering the omasal canal of dairy cows fed grass silage based diets. Anim. Feed Sci. Technol. 96:203-220. https://doi.org/10.1016/S0377-8401(01)00348-0

Clark, J. H., T. H. Klusmeyer, and M. R. Cameron. 1992. Microbial protein synthesis and flows of nitrogen fractions to the duodenum of dairy cows. J. Dairy Sci. 75:2304-2323. https://doi.org/10.3168/jds.S0022-0302(92)77992-2

Clarke, R. 1964. Ciliates of the rumen of domestic cattle (Bos taurus L.). N.Z. J. Agric. Res. 7:248-257. https://doi.org/10.1080/00288233.1964.10416409

Coleman, G. and D. C. Sandford. 1979. The engulfment and digestion of mixed rumen bacteria and individual bacterial species by single and mixed species of rumen ciliate protozoa grown in vivo. J. Agri. Sci. 92:729-742. https://doi.org/10.1017/S0021859600053971

Dehority, B. A. 2004. In vitro determination of generation times for Entodinium exiguum, Ophryoscolex purkynjei, and Eudiplodinium maggii. J. Eukaryot. Microbiol. 51:333–338. https://doi.org/10.1111/j.1550-7408.2004.tb00575.x

Dehority, B. A. 1984. Evaluation of subsampling and fixation procedures used for counting rumen protozoa. Appl. Environ. Microbiol. 48:182–185. https://doi.org/10.1128/aem.48.1.182-185.1984

Diaz, H. L., J. R. Knapp, S. K. R. Karnati, B. A. Dehority, and J. L. Firkins. 2014. Effects of wortmannin, sodium nitroprusside, insulin, genistein, and guanosine triphosphate on chemotaxis and cell growth ofEntodiniumcaudatum, Epidinium caudatum, and mixed ruminal protozoa. J. Dairy Sci. 97:2244–2253. https://doi.org/10.3168/jds.2013-7429

Elkin, R. and J. Griffith. 1984. Hydrolysate preparation for analysis of amino acids in sorghum grains: effect of oxidative pretreatment. J. AOAC 68:1117-1121. https://doi.org/10.1093/jaoac/68.6.1117

Fessenden, S. W., T. J. Hackmann, D. A. Ross, E. Block, A. Foskolos, and M. E. Van Amburgh. 2019. Rumen digestion kinetics, microbial yield, and omasal flows of nonmicrobial, bacterial, and protozoal amino acids in lactating dairy cattle fed fermentation by-products or urea as a soluble nitrogen source. J. Dairy Sci. 102:3036–3052. https://doi.org/10.3168/jds.2018-15448

Fessenden, S. W., T. J. Hackmann, D. A. Ross, A. Foskolos, and M. E. Van Amburgh. 2017. Ruminal bacteria and protozoa composition, digestibility, and amino acid profile determined by multiple hydrolysis times. J. Dairy Sci. 100:7211-7226. https://doi.org/10.3168/jds.2016-12531

Fessenden, S. W. 2016. Amino acid supply in lactating dairy cattle. Ph.D. Dissertation, Cornell Univ., Ithaca, NY.

Firkins, J. L., Z. Yu, T. Park, and J. E. Plank. 2020. Extending Burk Dehority's perspectives on the role of ciliate protozoa in the rumen. Front Microbiol 11:123. https://doi.org/10.3389/fmicb.2020.00123

Firkins, J. L., Z. Yu, and M. Morrison. 2007. Ruminal nitrogen metabolism: perspectives for integration of microbiology and nutrition for dairy. J. Dairy Sci. 90:1–16. https://doi.org/10.3168/jds.2006-518

Firkins, J. L., and Z. Yu. 2006. Characterisation and quantification of the microbial populations in the rumen. Pages 19–54 in Ruminant Physiology, Digestion, Metabolism and Impact of Nutrition on Gene Expression, Immunology and Stress. K. Sejrsen, T. Hvelplund, and M. O. Nielsen, ed. Wageningen: Wageningen Academic Publishers,.

Fleming, A. J., H. Lapierre, R. R. White, H. Tran, P. J. Kononoff, R. Martineau, W. P. Weiss, and M. D. Hanigan. 2019. Predictions of ruminal outflow of amino acids in dairy cattle. J. Dairy Sci. 102:10947–10963. https://doi.org/10.3168/jds.2019-16301

Harrison, D. G., D. E. Beever, D. J. Thomson, and D.F. Osbourn. 1976. Manipulation of fermentation in the rumen. J. Sci. Food Agri. 27:617-620. https://doi.org/10.1002/jsfa.2740270706

Higgs, R. and M. E. Van Amburgh. 2016. Evolution of the CNCPS-Development of V7. Pages 125-146 in Proc. Cornell Nutrition Conference, Syracuse, NY. Cornell University, Ithaca, NY.

Higgs, R. 2014. Development of a dynamic rumen and gastro-intestinal model in the Cornell Net Carbohydrate and Protein System to predict the nutrient supply and requirements of dairy cattle. Ph.D. Dissertation, Cornell Univ., Ithaca, NY.

Hristov, A. N. and J. P. Jouany. 2005. Factors affecting the efficiency of nitrogen utilization in the rumen. Pages 117–166 in Nitrogen and Phosphorus Nutrition of Cattle and Environment. A. N. Hristov, and E. Pfeffer, ed. Wallingford: CAB International,

Huhtanen, P., S. Ahvenjärvi, G. A. Broderick, S. M. Reynal, and K. J. Shingfield. 2010. Quantifying ruminal digestion of organic matter and neutral detergent fiber using the omasal sampling technique in cattle—A meta-analysis. J. Dairy Sci. 93:3203-3215. https://doi.org/10.3168/jds.2009-2988

Isaacson, H. R., F. C. Hinds, M. P. Bryant, and F. N. Owens. 1975. Efficiency of energy utilization by mixed rumen bacteria in continuous culture. J. Dairy Sci. 58:1645–1659. https://doi.org/10.3168/jds.S0022-0302(75)84763-1

Karnati, S. K. R., J. T. Sylvester, S. M. Noftsger, Z. Yu, N. R. St-Pierre, and J. L. Firkins. 2007. Assessment of ruminal bacterial populations and protozoal generation time in cows fed different methionine sources. J. Dairy Sci. 90:798–809. https://doi.org/10.3168/jds.S0022-0302(07)71564-3

Koenig, K. M., C. J. Newbold, F. M. McIntosh, and L. M. Rode. 2000. Effects of protozoa on bacterial nitrogen recycling in the rumen. J. Anim. Sci. 78:2431–2445. https://doi.org/10.2527/2000.7892431x

Korhonen, M., A. Vanhatalo, and P. Huhtanen. 2002. Effect of protein source on amino acid supply, milk production, and metabolism of plasma nutrients in dairy cows fed grass silage. J. Dairy Sci. 85:3336-3351. https://doi.org/10.3168/jds.S0022-0302(02)74422-6

Lapierre, H., S. Binggeli, M. Sok, D. Pellerin, and D. Ouellet. 2019. Estimation of correction factors to determine the true amino acid concentration of protein after a 24-hour hydrolysis. J. Dairy Sci. 102:1205-1212. https://doi.org/10.3168/jds.2018-15392

Lapierre, H., D. R. Ouellet, R. Berthiaume, R. Martineau, G. Holtrop, and G. E. Lobley. 2008. Distribution of $^{15}N$ in amino acids during $^{15}N$-Leucine infusion: Impact on the estimation of endogenous flows in dairy cows. J. Dairy Sci. 91:2702–2714. https://doi.org/10.3168/jds.2007-0871

Lin, L. I. K. 1989. A concordance correlation coefficient to evaluate reproducibility. Biometrics. 45:255–268. https://doi.org/10.2307/2532051

Newbold, C. J., G. de la Fuente, A. Belanche, E. Ramos-Morales, and N. R. McEwan. 2015. The role of ciliate protozoa in the rumen. Front. Microbiol. 6:1313. https://doi.org/10.3389/fmicb.2015.01313

NorFor. 2011. The Nordic feed evaluation system. Wageningen Academic Publishers, The Netherlands.

Ørskov, E. R., N. A. Macleod, and D. J. Kyle. 1986. Flow of nitrogen from the rumen and abomasum in cattle and sheep given protein free nutrients by intragastric infusion. Br. J. Nutr. 56:241-248. https://doi.org/10.1079/BJN19860103

Owens, D., M. McGee, and T. Boland. 2008a. Effect of grass regrowth interval on intake, rumen digestion and nutrient flow to the omasum in beef cattle. Anim. Feed Sci. Technol. 146:21-41. https://doi.org/10.1016/j.anifeedsci.2007.11.012

Owens, D., M. McGee, T. Boland, and P. O'Kiely. 2008b. Intake, rumen fermentation and nutrient flow to the omasum in beef cattle fed grass silage fortified with sucrose and/or supplemented with concentrate. Anim. Feed Sci. Technol. 144:23-43. https://doi.org/10.1016/j.anifeedsci.2007.09.032

Peyraud, J., L. Astigarraga, and P. Faverdin. 1997. Digestion of fresh perennial ryegrass fertilized at two levels of nitrogen by lactating dairy cows. Anim. Feed Sci. Technol. 64:155-171. https://doi.org/10.1016/S0377-8401(96)01056-5

Reynal, S. M. and G. A. Broderick. 2005. Effect of dietary level of rumen-degraded protein on production and nitrogen metabolism in lactating dairy cows. J. Dairy Sci. 88:4045-4064. https://doi.org/10.3168/jds.S0022-0302(05)73090-3

Reynal, S. M. and G. A. Broderick. 2003. Effects of feeding dairy cows protein supplements of varying ruminal degradability. J. Dairy Sci. 86:835-843. https://doi.org/10.3168/jds.S0022-0302(03)73666-2

Reynal, S., I. Ipharraguerre, M. Lineiro, A. Brito, G. Broderick, and J. Clark. 2007. Omasal flow of soluble proteins, peptides, and free amino acids in dairy cows fed diets supplemented with proteins of varying ruminal degradabilities. J. Dairy Sci. 90:1887-1903. https://doi.org/10.3168/jds.2006-158

Roman-Garcia Y, R. R. White, and J. L. Firkins. 2016. Meta-analysis of postruminal microbial nitrogen flows in dairy cattle. I. Derivation of equations. J. Dairy Sci. 99:7918–7931. https://doi.org/10.3168/jds.2015-10661

Rutherfurd, S., P. Moughan, D. Lowry, and C. Prosser. 2008. Amino acid composition determined using multiple hydrolysis times for three goat milk formulations. Int. J. Food Sci. Nutr. 59:679-690. https://doi.org/10.1080/09637480701705424

Sok, M., D. R. Ouellet, J. L. Firkins, D. Pellerin, and H. Lapierre. 2017. Amino acid composition of rumen bacteria and protozoa in cattle. J. Dairy Sci. 100:5241–5249. https://doi.org/10.3168/jds.2016-12447

Sylvester, J. T., S. K. R. Karnati, B. A. Dehority, M. Morrison, G. L. Smith, N. R. St-Pierre, and J. L. Firkins. 2009. Rumen ciliated protozoa decrease generation time and adjust 18S ribosomal DNA copies to adapt to decreased transfer interval, starvation, and monensin. J. Dairy Sci. 92:256-269. https://doi.org/10.3168/jds.2008-1417

Sylvester, J. T., S. K. R. Karnati, Z. Yu, C. J. Newbold, and J. L. Firkins. 2005. Evaluation of a real-time PCR assay quantifying the ruminal pool size and duodenal flow of protozoal nitrogen. J. Dairy Sci. 88:2083–2095. https://doi.org/10.3168/jds.S0022-0302(05)72885-X

Tedeschi, L. O. 2006. Assessment of the adequacy of mathematical models. Agric. Systems 89:225-247. https://doi.org/10.1016/j.agsy.2005.11.004

Van Amburgh, M. E., E. Collao-Saenz, R. Higgs, D. Ross, E. Recktenwald, E. Raffrenato, L. Chase, T. Overton, J. Mills, and A. Foskolos. 2015. The Cornell Net Carbohydrate and Protein System: Updates to the model and evaluation of version 6.5. J. Dairy Sci. 98:6361-6380. https://doi.org/10.3168/jds.2015-9378

Van Soest, P. J. 1994. Nutritional ecology of the ruminant. 2nd Ed. Cornell Univ. Press, Ithaca, NY.

Van Vuuren, A. M., F. Krol-Kramer, R.A. Van Der Lee, and H. Corbijn. 1992. Protein digestion and intestinal amino acid in dairy cows fed fresh Lolium perenne with different nitrogen contents. J. Dairy Sci. 75:2215-2225. https://doi.org/10.3168/jds.S0022-0302(92)77982-X

Vanhatalo, A., K. Kuoppala, S. Ahvenjarvi, and M. Rinne. 2009. Effects of feeding grass or red clover silage cut at two maturity stages in dairy cows. 1. Nitrogen metabolism and supply of amino acids. J. Dairy Sci. 92:5620-5633. https://doi.org/10.3168/jds.2009-2249

Volden, H. and O. M. Harstad. 1998. Amino acid composition of bacteria harvested from the rumen of dairy cows fed three diets differing in protein content and rumen protein degradability at two levels of intake. Acta Agric. Scand. A Anim. Sci. 48:210–215. https://doi.org/10.1080/09064709809362422

Waldo D.R., L. W. Smith, and E. L. Cox. 1972. Model of cellulose disappearance from the rumen. J. Dairy Sci. 55:125–129. https://doi.org/10.3168/jds.S0022-0302(72)85442-0

Wallace, R. J. and C. A. McPherson. 1987. Factors affecting the rate of breakdown of bacterial protein in rumen fluid. Br. J. Nutr. 58:313–323. https://doi.org/10.1079/BJN19870098

Wells, J. E. and J. B. Russell. 1996. Why do many ruminal bacteria die and lyse so quickly? J. Dairy Sci. 79:1487-1495. https://doi.org/10.3168/jds.S0022-0302(96)76508-6

# CHAPTER 6: THE EFFECT OF CONCENTRATE SUPPLEMENT TYPE ON MILK PRODUCTION, NUTRIENT INTAKE, AND TOTAL-TRACT NUTRIENT DIGESTIBILITY IN MID-LACTATION, SPRING-CALVING DAIRY COWS GRAZING PERENNIAL RYEGRASS (*LOLIUM PERENNE* L.) PASTURE

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## 6.1. ABSTRACT

The objective of this study was to evaluate the effect of concentrate supplement type on milk production, nutrient intake, and total-tract nutrient digestion in lactating dairy cows grazing mid-season perennial ryegrass (*Lolium perenne* L.; **PRG**) pasture. Eighty lactating dairy cows averaging (mean ± SD) 98 ± 25 DIM and 518 ± 64 kg of body weight were stratified based on pre-study milk production and parity and randomly assigned to 1 of 4 dietary treatments. The 4 dietary treatments were a non-supplemented PRG control (**GO**); PRG supplemented with 4.4 kg DM of citrus pulp and 0.067 kg DM of urea (**CITRUS**); PRG supplemented with 0.8 kg DM of rumen-protected soybean meal (**RP-SBM**); PRG supplemented with 3.1 kg DM of a combination of rumen-protected soybean meal and citrus pulp (**MIX**). The study consisted of a 2-wk adaptation period and a 10-wk period of data collection. Weekly measurements of milk production, body weight, body condition score, and feeding and rumination time were made. Nutrient intake and total-tract digestibility were measured during wk 6 of the study. All data were analyzed using the

Proc Mixed procedure in SAS (SAS Institute Inc., Cary, NC). High soil moisture deficit recorded during the study reduced pasture growth rate and substantially altered the chemical composition of the mid-season PRG offered to the cows. Total dry matter intake was increased in cows fed CITRUS compared with cows fed GO and RP-SBM, but was similar to cows fed MIX (18.0, 15.9, 16.4, and 17.2 ± 0.41 kg dry matter/d, respectively). The apparent total-tract neutral detergent fiber digestibility of cows fed the CITRUS diet was lower compared with the GO and RP-SBM diets, but was similar to the MIX diet (0.67, 0.70, 0.70, and 0.69 ± 0.01 g/g, respectively). The energy-corrected milk (**ECM**) yield of cows fed MIX was highest (23.7 kg/d), CITRUS intermediate (22.2 kg/d), and GO lowest (20.8 kg/d; P < 0.01). Cows fed RP-SBM produced more ECM (22.9 kg/d; *P* < 0.01) compared with cows fed GO and produced similar ECM compared with cows fed CITRUS and MIX diets. The RP-SBM diet increased milk protein yield compared with the GO diet (*P* < 0.01), tended to increase milk protein yield compared with the CITRUS diet (*P* = 0.06), and was similar to the MIX diet. Milk fat content and the composition of milk fat were not influenced by treatment. The results demonstrate that for cows consuming pasture-based diets, increasing metabolizable protein supply allowed higher milk yield as metabolizable protein was more limiting than metabolizable energy. However, due to the conditions experienced during this experiment, caution is recommended when extrapolating these results to cows consuming typical mid-season PRG pastures.

**Key words:** dairy cow, milk production, perennial ryegrass, supplementation

## 6.2. INTRODUCTION

In temperate regions of the world, pasture-based systems are a robust method of milk production via the consumption of home-grown, human-inedible forage (Laisse et al., 2018). High

pasture inclusion levels in dairy cow diets can support a resilient business model for the producer and an animal welfare friendly image, while concurrently minimizing the system's environmental impact (Dillon et al., 2008; van Vuuren and Chilibroste, 2013). Since the elimination of the European Union milk quotas in 2015, Ireland's milk production has increased by over 50%, primarily through increased cow numbers and increased milk yield per cow (Kelly et al., 2020). It is likely that further expansion will be hindered due to land availability, labor resources, and increasing societal pressure on issues such as climate change (Hoekstra et al., 2020). There is, however, opportunity to further increase individual cow performance by strategically manipulating dietary nutrient supply in pasture-based systems.

The CP concentration of perennial ryegrass (*Lolium perenne* L.; **PRG**) pasture has generally been assumed adequate to maintain animal performance (Wales et al, 1999; Bargo et al., 2003). However, PRG protein has been demonstrated to undergo extensive rumen proteolysis and substantial pre-duodenal losses (Beever et al., 1986; Chapter 3) creating potential scenarios where increased MP supply could increase milk yield (Berzaghi et al., 1996; O'Mara et al., 2000). However, there is contrasting evidence in the literature as to the effectiveness of supplementation of rumen-protected protein (**RP-P**) supplements to grazing dairy cows (Delaby et al., 1996; Astigarraga et al., 2002). Metabolizable energy intake is thought to be first limiting milk yield of grazing animals due to lower DMI and slower rates of ruminal degradation (Kolver, 2003). Concentrate supplements containing high levels of fermentable carbohydrate (**F-CHO**) have typically been incorporated to overcome these physical limitations and increase the energy density of pasture-based diets (Bargo et al., 2003; Keim et al., 2011). Chapter 3 reported that supplementation of PRG-based diets with F-CHO can increase MP supply, via increased microbial protein synthesis and thus, a potential secondary mechanism might be involved in achieving higher

animal performance (Broderick, 2017). Consequently, when F-CHO supplements are offered, it is difficult to differentiate whether increased milk production is achieved via increased ME supply, AA supply, or a synergistic effect of both. Ultimately, if a greater understanding is attained of the nutrients first limiting milk yield of PRG-fed cows, a higher and more consistent milk production response to supplementation could be achieved.

In this study, we utilized the Cornell Net Carbohydrate and Protein System (**CNCPS** v7.0; Higgs and Van Amburgh, 2016), which has previously been incorporated in the design of experimental diets, to help separate nutritional interactions and mechanisms responsible for increased productive performance (Fessenden et al., 2020). It was hypothesized that supplementing with F-CHO or low levels of a RP-P ingredient would increase milk production, to a similar extent, over that of cows fed a PRG-only control. Furthermore, it was hypothesized that supplementation with F-CHO and RP-P combined would result in a synergistic effect, outperforming supplementation of each ingredient alone. Therefore, the objective of this experiment was to investigate the effect of concentrate supplement type on milk production, nutrient intake, and total-tract nutrient digestion in mid-lactation dairy cows fed PRG-based diets.

## 6.3. MATERIALS AND METHODS

This study was approved by the Teagasc Animal Ethics Committee and conducted under experimental license (AE19132-P081) from the Health Products Regulatory Authority under European directive 2010/63/EU and S.I. no. 543 of 2012. The study was undertaken at Teagasc Clonakilty Agricultural College, Co. Cork, Ireland (51°63’N, 08°85’E; 25-70 m above sea level) from June – August 2018. Meteorological data was obtained from a weather station located at Timoleague, Co. Cork, Ireland (Agricultural Catchments Program, Teagasc). Data recorded

comprised of daily rainfall (mm), air temperatures (°C), wind speed (m/s) and soil temperature (°C), and from this data, soil moisture deficit (**SMD**; mm) was subsequently calculated (Schulte et al., 2005).

### *6.3.1. Cows, Experimental Design, and Treatment Administration*

Eighty lactating dairy cows averaging (mean ± SD) 98 ± 25 DIM and 518 ± 64 kg of BW were enrolled in a 2-wk pre-study acclimation and covariate period. During this period, all cows were offered, daily, the same diet of approximately 14 kg DM of PRG and 5 kg DM of a dairy concentrate. At the end of the 2-wk acclimation period, cows were stratified based on pre-study milk production and parity, and randomly assigned to 1 of 4 treatments for 10 wk of experimental data collection. The four dietary treatments were a non-supplemented PRG control (**GO**); PRG supplemented with 4.4 kg DM of citrus pulp and 0.067 kg DM of urea (**CITRUS**); PRG supplemented with 0.8 kg DM of rumen-protected soybean meal (**RP-SBM**); PRG supplemented with 3.1 kg DM of a combination of rumen-protected soybean meal and citrus pulp (**MIX**; Table 6.1). The experimental diets were formulated using CNCPS v7.0 (Higgs and Van Amburgh, 2016) because it uses a three pool system to define fiber digestion (Raffrenato et al., 2019) to describe rumen fiber fill and turnover and incorporates protozoa and endogenous protein into the MP and AA predictions (Higgs and Van Amburgh, 2016). One of the objectives was to formulate DMI that would remain isocaloric on an ME basis, but differ in the source of nutrients. The average mid-season PRG chemical composition from the previous 3 yr at the research station was assumed to be representative of the forage the cows would consume. Citrus pulp was included in the CITRUS diet at a level to achieve increased total DMI and hence, increased predicted ME supply over that of the GO control. In addition, a small quantity of urea (67 g) was added to prevent a potential

deficiency in rumen ammonia. Given microbial cell growth is directly related to F-CHO availability in the CNCPS, the CITRUS diet was predicted to increase MP supply over that of the GO control (2,036 g and 1,781 g MP/d for CITRUS and GO, respectively). A heat-treated- (**HT**) soybean meal, which included synthetic methionine (ByPro, Devenish Nutrition Ltd., Belfast, Northern Ireland), was included in the RP-SBM diet to match the predicted MP supply of the CITRUS diet (2,069 g MP/d). The low inclusion levels of the HT-soybean meal was formulated so that the predicted ME supply for the RP-SBM diet would be similar to the GO control diet and less than the CITRUS diet (49, 48, and 52 Mcals ME/d for RP-SBM, GO, and CITRUS, respectively). The ruminal proportional protein disappearance for the HT-soybean meal has previously been described by Sheehy et al. (2020). Finally, the MIX diet was formulated to contain a slightly lower level of the HT-soybean meal, compared with the RP-SBM diet, plus the addition of citrus pulp (2,174 g MP/d and 51 Mcals ME/d). Ingredient composition and feeding level of the supplements are presented in Table 6.1. The supplements were offered to the respective cows in self-locking stalls (O'Donovan Engineering, Coachford, Co. Cork, Ireland) immediately after both am and pm milking (0730 and 1530 h), in two equal meals. Cows were observed while consuming the supplements and refusals were recorded if present.

**Table 6.1.** Ingredient composition and feeding level of the experimental supplements

| | Experimental supplements[1] | | |
|---|---|---|---|
| Ingredient, % of DM | CITRUS | RP-SBM | MIX |
| Citrus pulp | 92.2 | - | 72.3 |
| HT-Soybean meal[2] | - | 95.1 | 21.8 |
| Molasses | 2.9 | 3.0 | 3.0 |
| Cal-Mag[3] | 2.2 | 3.4 | 2.2 |
| Mineral mix[4] | 2.2 | 1.7 | 2.2 |
| Urea | 1.4 | - | - |
| Total feeding level, kg DM/d | 4.8 | 0.8 | 3.1 |

[1]CITRUS = primarily citrus pulp-based supplement; RP-SBM = primarily rumen-protected soybean meal-based supplement; MIX = combination of citrus pulp and RP-SBM based supplement.
[2]BYPRO (Devenish Nutrition Ltd., Belfast, Northern Ireland) contained heat-treated (HT) soybean meal and synthetic methionine.
[3]CalMag (McDonnells Bros, Coolagown, Fermoy, Co. Cork, Ireland).
[4]Dairy Mineral Mix (McDonnells Bros, Coolagown, Fermoy, Co. Cork, Ireland) contained the following: 0.47 mg/kg of selenium, 1.4 mg/kg of cobalt, 7.0 mg/kg of iodine, 47 mg/kg of copper 47.0 mg/kg of manganese, 76.0 mg/kg zinc, 19.0 g/kg of magnesium, 9,500 IU/kg of vitamin A, 1,900 IU/kg of vitamin D, 9.0 IU/kg of vitamin E.

### *6.3.2. Grazing Management and Diet Chemical Analysis*

The grazing area comprised 20.6 ha, permanently subdivided into 19 paddocks, ranging in size from 0.7 to 2.5 ha. All cows grazed together as a single group and had ad libitum access to fresh water. For the majority of the experiment, cows were allocated a 36-h residence time within each paddock or until a targeted post-grazing residual sward height of 4 to 4.5 cm was achieved. Pasture supply was managed by weekly monitoring of farm pasture cover (O'Donovan et al., 2002). Pasture cover observations were recorded on PastureBase Ireland (Hanrahan et al., 2017) with the generated output from the software aiding the grazing management decisions for the coming week. During the 10-wk experimental period, below average rainfall resulted in a high SMD, causing a reduction in pasture growth rate. In order to maintain PRG in the cow's diet an additional area of 5.2 ha was allocated to the grazing area.

Pasture DM yield (kg DM/ha) was determined prior to grazing in each paddock by harvesting two strips (1.2 m x 10 m) with an Etesia mower (Etesia UK. Ltd., Warwick, UK.). The cut material was collected and weighed, and a sub-sample removed to determine DM concentration. One hundred grams of the sampled material was dried for 15 h at 90 °C for DM determination. Using a rising pasture plate meter with a steel plate (diameter 355 mm and 3.2 kg/m2; Jenquip, Feilding, New Zealand) ten pasture height measurements were recorded before and after harvesting for both strips. As a result of the recorded measurements above, sward density was calculated as follows:

$$\text{Sward density (kg DM/cm per ha)} = \text{pasture yield (kg DM/ha)} \,/\, [\text{precutting height (cm)} - \text{postcutting height (cm)}]$$

The average paddock pre-grazing yield above a cutting height of 4 cm was then calculated according to the following formula:

$$\text{Pregrazing yield (kg DM/ha)} = [\text{pregrazing sward height (cm)} - 4\text{ cm}] \times \text{sward density (kg DM/cm per ha)}$$

Pre-grazing and post-grazing sward height above ground level was determined on each paddock prior to and immediately after grazing by taking between 30 and 50 measurements across the diagonal of the paddock using the rising plate meter described above. Pasture allowance was calculated as proposed by Delaby et al. (1998).

Prior to grazing of each paddock, a selection of pasture was sampled with a Gardena hand shears (Accu 60, Gardena International GmbH, Ulm, Germany) at 4 cm above ground level, which was representative of that consumed by the cows. Immediately after harvesting, samples were frozen at - 20ºC. Subsequently, the samples were first bowl-chopped and then freeze dried (LS40+chamber, MechaTech Systems Ltd., Bristol, U.K.) at - 55°C for 120 h. Concentrate supplements were sampled weekly and dried at 90°C for 15 h and analyzed for DM. Additional weekly samples were oven dried at 60°C for 48 h. Dried samples of pasture and concentrate supplements were ground through a 1-mm screen using a Cyclotech 1093 Sample Mill (Foss, DK-3400 Hillerød, Denmark) and stored for subsequent chemical analysis.

The dried pasture samples were pooled weekly and analyzed for chemical composition using wet chemical methods (CPM Plus Package) by Cumberland Valley Analytical Services (Waynesboro, PA). Experimental supplements were pooled, over approximately 3-wk periods, and analyzed as described for pasture. In addition to this analysis, all feed samples were analyzed to determine the in vitro amylase- and sodium sulfite-treated NDF corrected for ash residue (**aNDFom**) digestibility at Cornell University, Ithaca, NY, following the procedures described by Raffrenato et al. (2018a). The time points selected for PRG were 12, 30, 120 and 240 h, with the

12 h time point included to more precisely quantify the rate of degradation (Chapter 2). For the citrus pulp and rumen-protected soybean meal ingredients, the non-forage fiber time points of 12, 72 and 120 h were selected as described by Zontini (2016). Water-soluble carbohydrates (**WSC**) were determined according to the procedures of Hall (2014).

### *6.3.3. Animal Measurements and Analysis*

Cows were milked at 0730 h and 1530 h daily throughout the study. Weekly milk production was determined from individual milk yields (kg) recorded at each milking (Dairymaster, Causeway, Co. Kerry, Ireland). Milk fat, crude protein, and anhydrous lactose concentrations were determined weekly from one successive PM and AM milking sample from each cow. These samples were analysed using a Fourier transform mid-infrared spectrophotometer (Lactoscope FTA; Delta Instruments, Drachten, the Netherlands) at Centenary Cooperative Creamery (Thurlus, Co. Tipperary, Ireland). Milk fatty acid (**FA**) analysis was also conducted at Centenary Cooperative Creamery as described by Woolpert et al. (2016), using partial least squares chemometric prediction models based on the mid-infrared spectra described by Wojciechowski and Barbano (2016). Calibration for measurement of fat, protein, anhydrous lactose, and FA parameters was performed using modified milk calibration samples (Kaylegian et al., 2006) prior to beginning the experiment. Weekly milk solids (**MSo;** kg fat + protein) and energy corrected milk (**ECM**; Tyrrell and Reid, 1965) were then calculated. Body weight was recorded weekly after AM milking on exiting from the milking parlor using an electronic scale and Winweigh software package (Tru-test Limited, Auckland, New Zealand). Body condition score was recorded weekly as the average of two trained scorers using a 1–5 scale (where 1 = emaciated and 5 = extremely fat) with 0.25 increments (Edmonson et al., 1989).

Individual cow pasture DMI was estimated using the n-alkane technique (Mayes et al., 1986) as modified by Dillon and Stakelum (1989). Briefly, each cow was dosed orally twice daily after milking with a paper bung saturated with 760 mg of n-dotriacontane (C32) for a 12-d period during wk 5 and 6 of the experiment (Dove and Mayes, 2006). During the last 6 d of C32 administration, fecal grab samples from each cow were obtained twice daily prior to milking and immediately frozen at − 20°C. Fecal samples were later pooled per cow, a subsample was placed in a forced air oven at 60°C until completely dried, and then ground to pass a 1-mm screen on a Cyclotech mill. During the same 6-d period, pasture samples were obtained using Gardena hand shears at 4 cm above ground level to represent pasture consumed by the cows. The n-alkane concentrations were analyzed by gas chromatography (GC; Varian 3400 series, Varian, Darmstadt, Germany; Dove and Mayes, 2006), which used direct saponification (Dillon, 1993). The column was a 30 m×0.53 mm in side diameter capillary column type SPB1 with 0.5 μmol L-1 film thickness. The ratio of pasture tritriacontane (C33) to dosed C32 was then used to calculate estimated daily pasture DMI as follows:

$$\text{Daily pasture intake (kg of DM/cow)} = \frac{F_i/F_j \text{ x } (D_j + I_s \text{ x } S_j) - I_s \text{ x } S_i}{P_i - (F_i/F_j \text{ x } P_j)}$$

Where $F_i$, $S_i$, and $P_i$ are the concentrations (mg/kg of DM) of the natural odd chain n-alkanes in feces, supplement, and pasture, respectively, $F_j$, $S_j$, and $P_j$ are the concentrations (mg/kg of DM) of the even chain n-alkane in feces, supplement, and pasture, respectively, $D_j$ is the dose rate (mg/d) of the even chain n-alkane, and $I_s$ is the daily supplement intake (kg DM/d). Concentrate supplements, offered and refused, were measured twice daily at each feeding event. Fecal samples were additionally analyzed for DM, OM (AOAC, 2005), aNDFom (Mertens, 2002), and

undigested aNDFom after 240 h of in vitro fermentation (uNDFom; Raffrenato et al., 2018a) and then utilized to calculate nutrient fecal output and apparent total-tract digestibility (**TTD**) using the fecal concentration of uNDFom as an internal marker as described by Huhtanen et al. (1994).

All cows were equipped with a MooMonitor + collar device (Dairymaster, Tralee, Ireland) to record feeding, ruminating and resting time. The MooMonitor + has recently been validated to accurately monitor feeding and rumination time in pasture-based dairy cows (Werner et al., 2019). The collar device, which contains a box with a 3-axis accelerometer, was placed on the right side of the cow's neck. From the recorded measurements, data analysis with a generic algorithm, which was developed by Dairymaster, was performed on-board to identify specific patterns for the different categories of feeding, rumination, and resting. The output data were then collated into 24-h daily summaries.

Blood samples were collected after the morning milking on 3 occasions during the experiment (wk 4, 6, and 8) via coccygeal vein puncture. Blood samples were collected into tubes containing sodium heparin, centrifuged (3,000 × g for 20 min at 4°C), and plasma was harvested and stored at - 20°C. Plasma samples were later analyzed for plasma urea nitrogen (**PUN**) using enzymatic tests (Kit No. UR3825, Randox Laboratories Ltd., Antrim, Northern Ireland; University College Dublin, Co. Dublin, Ireland).

### *6.3.4. Statistical Analysis*

Data were analyzed in a repeated measurements model using the MIXED procedure of SAS version 9.4 (SAS Institute Inc. Cary, NC) by the following model:

$$Y_{\text{ijkl}} = \mu + T_i + W_j + TW_{ij} + C_{k:i} + BX_{ki} + Pl + \varepsilon_{ijkl}$$

where $Y_{ijkl}$ = dependent variable, $\mu$ = overall mean, $T_i$ = fixed effect of treatment i, $W_j$ = fixed effect of week j, $TW_{ij}$ = fixed interaction of treatment i and week j, $C_{k:i}$ = random effect of cow k within treatment i, $BX_{ki}$ = the covariate adjustment for each cow, $P_l$ = fixed effect of parity l, and $\varepsilon_{ijkl}$ = residual error. Covariate adjustments were applied where appropriate. The repeated measures analysis was based on week. Using the Akaike's information criterion, an autoregressive of order 1 covariance structure provided the best fit to the data. Data describing intake, rumination per unit of intake, and TTD were analyzed using SAS MIXED procedures. The model included the fixed effects of treatment and parity. Cow was included as the random effect. No repeated statement was used in the model. In all cases, the degrees of freedom were determined using the Kenward-Rogers option. Means were determined using the least squares means statement, and multiple comparisons between treatment means were made using the Tukey–Kramer method. Statistical significance was considered at $P \leq 0.05$ and trends were considered at $0.05 < P \leq 0.10$.

## 6.4. RESULTS

### *6.4.1. Meteorological data*

Mean monthly air and soil temperature, total rainfall, and mean SMD data, during the experimental period and the previous 4-yr averages, are presented in Table 6.2. Mean monthly air and soil temperature during the experiment were, overall, similar to the previous 4-yr averages. Rainfall for the months of June and July was 36% of the previous 4-year average; 60 mm compared with a 4-year average of 160 mm. This extended period of low rainfall led to drought conditions (i.e. a period of 15 or more consecutive days to none of which is credited 0.2 mm or more of precipitation; Met Eireann, 2018) and extremely high SMD during wk 2 to 7 of the experiment (Table 6.2).

**Table 6.2.** Meteorological data during the 10-wk experimental period (June 14 to August 22, 2018) compared with the previous 4-yr average (2014-2017)

| | Month | | | |
|---|---|---|---|---|
| | May | Jun | Jul | Aug |
| Mean air temperature (°C) | | | | |
| 2018 | 11.5 | 15.2 | 16.9 | 14.8 |
| 4-yr average | 11.2 | 13.7 | 14.9 | 14.2 |
| Mean soil temperature (°C) | | | | |
| 2018 | 12.1 | 15.7 | 17.4 | 16.4 |
| 4-yr average | 12.2 | 15.3 | 16.6 | 16.0 |
| Total rainfall (mm) | | | | |
| 2018 | 62 | 18 | 42 | 70 |
| 4-yr average | 70 | 87 | 73 | 91 |
| Mean soil moisture deficit (mm) | | | | |
| 2018 | 10 | 45 | 77 | 48 |
| 4-yr average | 25 | 30 | 29 | 17 |

### *6.4.2. Grazing Characteristics and Diet Chemical Composition*

The reduced rainfall and high SMD experience caused a reduction in pasture growth rate, compared with the previous 4-yr average (52 ± 19 vs. 72 ± 8 kg DM/ha per day, respectively). Pre-grazing yield was 2,051 ± 290 kg DM/ha; however, this varied across the experimental period reaching a peak (2,574 kg DM/ha) in wk 6. Pasture DM concentration was 26 ± 6%, was also variable, and reached a peak (37%) in wk 7, which coincided with peak SMD (88.5 mm). Pasture pre- and post-grazing compressed sward heights were 9.6 ± 1.8 and 4.4 ± 0.3 cm above ground level, respectively. Pasture allowance (> 4 cm) was 18.7 ± 1.6 kg DM/cow per day on average across the 10-wk of the experiment.

Pasture chemical composition, presented in Figure 6.1, was also influenced by the reduced rainfall and high SMD experienced during the experiment. Pasture CP concentration was 14.3 ± 3.6% on average but was extremely variable, reaching a nadir of 11% CP in wk 7, and a peak of 20% CP during wk 8 to 10. Water-soluble carbohydrate concentration averaged 19.7 ± 4% but was elevated from wk 1 to 7 averaging 22% WSC before reducing to 14% WSC during wk 8 to 10. Both aNDFom and uNDFom reached their highest concentrations (46.8% of DM and 24.1% of aNDFom, respectively) during wk 5 to 8 coinciding with peak SMD. As a result of this variability, chemical composition of the pasture offered was divided into 3 distinct periods: Grazing Period 1 ‘pre-peak SMD’ (corresponding to wk 1 to 4), Grazing Period 2 ‘peak SMD’ (corresponding to wk 5 to 7), and Grazing Period 3 ‘post-peak SMD’ (corresponding to wk 8 to 10) which is presented in Table 6.3. The chemical composition of the experimental supplements are in Table 6.3.

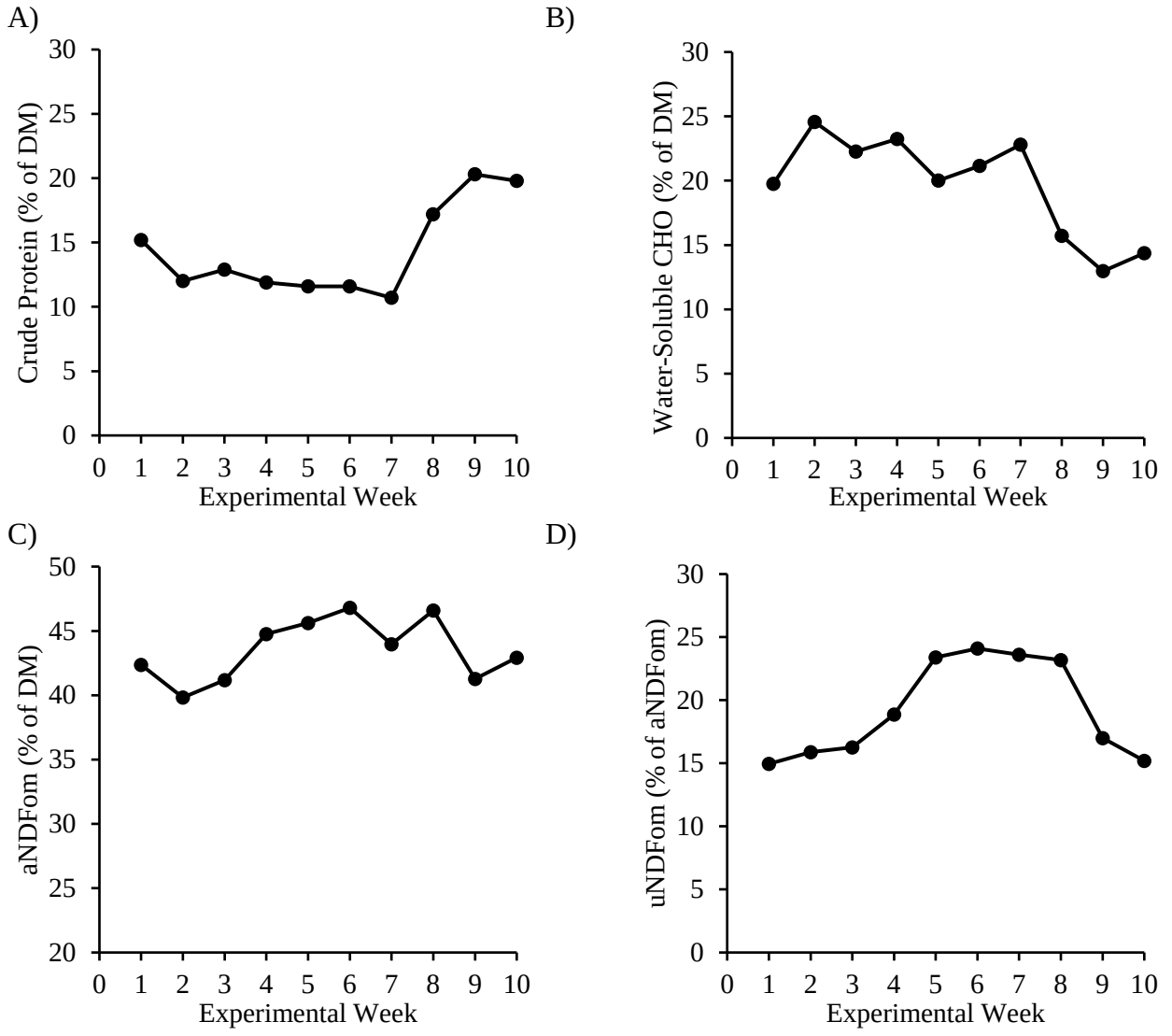


**Figure 6.1.** Changes in pasture A) Crude Protein (% of DM) B) Water-Soluble Carbohydrate (% of DM) C) aNDFom (% of DM) and D) uNDFom (% of aNDFom) offered to dairy cows over the 10-wk experimental period (June 14 to August 22, 2018).

**Table 6.3.** Chemical composition of pasture and concentrate supplements used in the experiment

| | Experimental feedstuffs[1] | | | | | |
|---|---|---|---|---|---|---|
| Chemical composition[2] | GP1 | GP2 | GP3 | CITRUS | RP-SBM | MIX |
| DM, % | 23.2 | 33.8 | 21.0 | 87.3 | 88.0 | 87.5 |
| CP, % of DM | 13.0 | 11.3 | 19.1 | 8.9 | 45.0 | 20.6 |
| Soluble N, % of N | 43.0 | 42.3 | 43.4 | 48.5 | 7.8 | 18.5 |
| NDIN, % N | 11.0 | 12.6 | 8.8 | 22.4 | 2.9 | 9.1 |
| ADIN, % N | 4.7 | 5.6 | 3.5 | 16.4 | 1.2 | 4.1 |
| aNDFom, % of DM | 42.0 | 45.5 | 43.6 | 25.1 | 8.7 | 18.3 |
| 12-h uNDFom, % of aNDFom | 61.8 | 70.1 | 65.5 | 59.8 | 40.8 | - |
| 30-h uNDFom, % of aNDFom | 47.6 | 39.9 | 45.4 | - | - | - |
| 72-h uNDFom, % of aNDFom | - | - | - | 21.4 | 16.0 | - |
| 120-h uNDFom, % of aNDFom | 19.9 | 26.2 | 20.3 | 17.6 | 10.1 | - |
| 240-h uNDFom, % of aNDFom | 16.5 | 23.7 | 18.5 | - | - | - |
| ADF, % of DM | 24.6 | 26.4 | 25.4 | 17.8 | 5.6 | 14.7 |
| ADL, % of DM | 2.5 | 3.5 | 3.1 | 2.8 | 1.5 | 2.6 |
| WSC, % of DM | 22.5 | 21.3 | 14.3 | 27.0 | 11.5 | - |
| Starch, % of DM | 1.6 | 0.9 | 1.2 | 2.2 | 2.6 | 2.1 |
| Ether extract, % of DM | 2.9 | 2.7 | 3.7 | 2.5 | 2.0 | 2.4 |
| Ash, % of DM | 7.3 | 6.9 | 8.1 | 9.5 | 12.3 | 15.9 |

[1]GP1 = Perennial ryegrass experimental wk 1 to 4; GP2 = Perennial ryegrass experimental wk 5 to 7; GP3 Perennial ryegrass experimental wk 8 to 10; CITRUS = primarily citrus pulp-based supplement; RP-SBM = primarily rumen-protected soybean meal-based supplement; MIX = combination of citrus pulp and RP-SBM based supplement.

[2]aNDFom = amylase- and sodium sulfite-treated NDF corrected for ash residue; uNDFom = undigested amylase- and sodium sulfite treated NDF corrected for ash residue; WSC = Water-Soluble Carbohydrate.

### *6.4.3. Milk Production and Milk Composition*

Cows fed diets containing concentrate supplement (CITRUS, RP-SBM, and MIX) had higher milk yield than cows fed the GO control diet ($P < 0.01$; Table 6.4). Energy corrected milk yield was affected by diet ($P < 0.01$), where MIX was highest, CITRUS intermediate, and the GO diet lowest. The cows fed the RP-SBM diet had higher ECM compared with the GO diet ($P < 0.01$) but similar to cows fed CITRUS and MIX diets and had higher milk protein content than the CITRUS diet ($P < 0.01$) but similar to cows fed the GO and MIX diets. Milk fat content and fatty acid composition was not significantly influenced by diet. Compared with cows fed the GO diet, milk lactose content was significantly higher ($P < 0.01$) for the cows fed the CITRUS diet, while RP-SBM and MIX diets were similar to the cows fed the GO diet.

Compared with the GO diet, the cows fed both the RP-SBM and MIX diets produced higher daily milk fat, protein, lactose and MSo yield ($P < 0.01$; Table 6.4; Figure 6.2). Compared with the GO diet, the cows fed the CITRUS diet produced higher daily milk protein and lactose yield ($P < 0.01$), while there was no difference in milk fat and MSo yield. Compared with the CITRUS diet, the cows fed the MIX diet produced higher milk protein yield ($P < 0.01$), while the cows fed the RP-SBM tended to produced higher milk protein yield ($P = 0.06$). There was a significant diet by week of experiment interaction for all milk yield variables, with the exception of fat yield (Table 6.4) and this is likely due to the deficiency in rainfall and subsequent soil moisture deficit.

**Table 6.4.** Effect of concentrate supplement type on milk production and composition in mid-lactation dairy cows grazing perennial ryegrass

| | Diet[1] | | | | | *P*-value | | |
|---|---|---|---|---|---|---|---|---|
| Item | GO | CITRUS | RP-SBM | MIX | SEM | Diet (D) | Week (W) | D*W |
| Milk yield, kg/d | 18.4[a] | 20.4[b] | 20.3[b] | 21.4[b] | 0.31 | <0.01 | <0.01 | <0.01 |
| ECM[2], kg/d | 20.8[a] | 22.2[b] | 22.9[bc] | 23.7[c] | 0.37 | <0.01 | <0.01 | <0.01 |
| Fat, % | 4.28 | 4.10 | 4.24 | 4.16 | 0.093 | 0.52 | <0.01 | 0.27 |
| De novo[3], g/100 g milk | 1.05 | 1.01 | 1.06 | 1.05 | 0.027 | 0.65 | <0.01 | 0.50 |
| Mixed[3], g/100 g milk | 1.39 | 1.33 | 1.36 | 1.35 | 0.033 | 0.67 | <0.01 | 0.38 |
| Preformed[3], g/100 g milk | 1.57 | 1.53 | 1.57 | 1.53 | 0.033 | 0.63 | <0.01 | 0.13 |
| Mean unsaturation[3] | 0.21 | 0.20 | 0.20 | 0.20 | 0.006 | 0.17 | <0.01 | 0.12 |
| Crude protein, % | 3.41[ab] | 3.30[a] | 3.49[b] | 3.38[ab] | 0.032 | <0.01 | <0.01 | 0.20 |
| Lactose, % | 4.35[a] | 4.44[b] | 4.36[a] | 4.38[ab] | 0.020 | <0.01 | <0.01 | 0.47 |
| Fat, kg/d | 0.79[a] | 0.84[ab] | 0.87[b] | 0.89[b] | 0.018 | <0.01 | <0.01 | 0.18 |
| Crude protein, kg/d | 0.62[a] | 0.67[b] | 0.71[bc] | 0.72[c] | 0.010 | <0.01 | <0.01 | <0.01 |
| Lactose, kg/d | 0.8[a] | 0.91[b] | 0.89[b] | 0.94[b] | 0.015 | <0.01 | <0.01 | <0.01 |
| Milk solids[4], kg/d | 1.41[a] | 1.50[ab] | 1.57[bc] | 1.61[c] | 0.026 | <0.01 | <0.01 | <0.05 |

[1]GO = Perennial ryegrass (PRG) only control; CITRUS = PRG plus citrus pulp-based supplement; RP-SBM = PRG plus rumen-protected soybean meal-based supplement; MIX = PRG plus combination of citrus pulp and RP-SBM based supplement.
[2]Estimated according to Tyrrell and Reid, (1965).
[3]De novo = fatty acids C4 to C14; Mixed = fatty acids C16, C16:1, and C17; = fatty acids greater than or equal to C18; Mean unsaturation = double bonds per fatty acids
[4]Milk solids = kg fat + protein.
[a-c]Means within row with different superscripts are significantly different ($P < 0.05$)

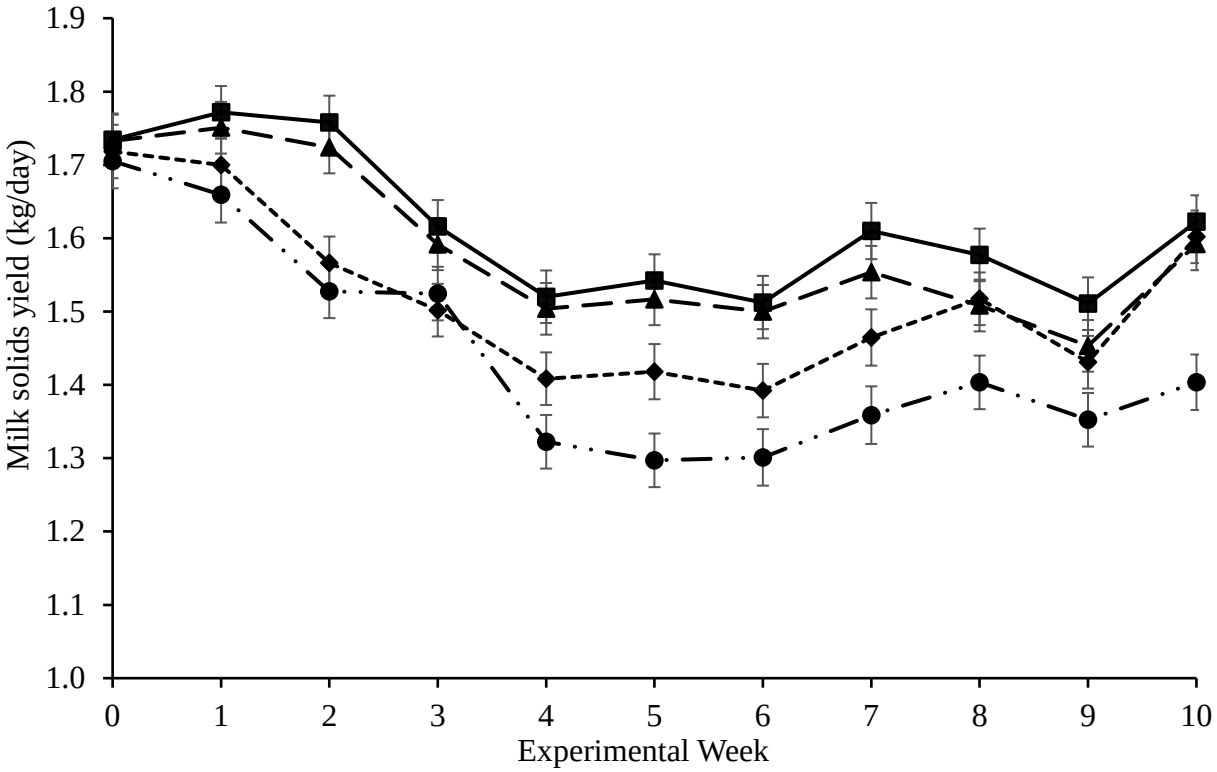


**Figure 6.2.** Effect of concentrate supplement type on milk solids yield (lsmean ± SEM) across the 10-wk experimental period (June 14 to August 22, 2018) for cows fed perennial ryegrass-only diet (●); perennial ryegrass plus citrus pulp-based supplement (♦); perennial ryegrass plus rumen protected soybean meal-based supplement (▲); and perennial ryegrass plus combination of citrus pulp and rumen-protected soybean meal-based supplement (■).

### *6.4.4. Dry Matter Intake, Total-Tract Nutrient Digestibility, and Plasma Urea Nitrogen*

Table 6.5 shows the effect of concentrate supplement type on intake of DM, OM, aNDFom and its fractions during experimental wk 6. Cows fed CITRUS and MIX diets had lower pasture DMI than the GO diet ($P < 0.01$); however, the total DM and OM intake of cows fed the CITRUS diet was higher than the GO and RP-SBM diets ($P < 0.01$). Accordingly, cows fed CITRUS and MIX diets consumed less aNDFom from pasture compared with cows fed the GO and RP-SBM diets ($P < 0.01$). All diets achieved similar total aNDFom intake (Table 6.5; $P = 0.82$). Feed efficiency (ECM/DMI) was higher for the cows fed RP-SBM and MIX diets compared with cows fed CITRUS ($P < 0.01$) and similar to cows fed GO (1.37, 1.34, 1.18, and 1.24 ± 0.05 kg/kg for RP-SBM, MIX, CITRUS, and GO diets, respectively).

The effect of concentrate supplement type on apparent TTD of DM, OM, aNDFom, and potentially digestible aNDFom (**pdNDFom**) is in Table 6.5. Overall, diet had no effect on TTD of OM ($P = 0.15$); however, cows fed the CITRUS diet had lower TTD of DM than the RP-SBM diet ($P < 0.05$), and similar to the GO and MIX diets. Cows fed CITRUS had lower TTD of aNDFom and pdNDFom than GO and RP-SBM ($P < 0.01$), and similar to the MIX diet. The cows fed the CITRUS diet had reduced PUN levels compared with cows fed the GO, RP-SBM and MIX diets ($P < 0.01$; Figure 6.3) and cows fed the RP-SBM diet had higher PUN than MIX ($P < 0.01$) but similar to the GO diet. There was a significant diet by week interaction for PUN ($P < 0.01$; Figure 6.3).

**Table 6.5.** Effect of concentrate supplement type on intake[1] of DM, OM, aNDFom, pdNDFom, and uNDFom, apparent total-tract digestibility, and metabolizable energy supply in mid-lactation dairy cows grazing perennial ryegrass

| | Diet[2] | | | | | |
|---|---|---|---|---|---|---|
| Item[3] | GO | CITRUS | RP-SBM | MIX | SEM | *P*-value |
| Intake, kg/d | | | | | | |
| Total DM | 15.9[a] | 18.0[b] | 16.4[a] | 17.2[ab] | 0.41 | <0.01 |
| Pasture DM | 15.9[a] | 13.1[b] | 15.6[a] | 14.1[b] | 0.42 | <0.01 |
| Supplement DM | 0.0 | 4.8 | 0.8 | 3.1 | - | - |
| Total OM | 14.8[a] | 16.8[b] | 15.3[a] | 16.1[ab] | 0.39 | <0.01 |
| Total aNDFom | 7.3 | 7.2 | 7.3 | 7.1 | 0.19 | 0.82 |
| Pasture aNDFom | 7.3[a] | 6.1[b] | 7.2[a] | 6.5[b] | 0.19 | <0.01 |
| Total pdNDFom | 5.6 | 5.5 | 5.6 | 5.5 | 0.15 | 0.93 |
| Pasture pdNDFom | 5.6[a] | 4.6[b] | 5.5[a] | 5.0[b] | 0.15 | <0.01 |
| Total uNDFom | 1.7 | 1.6 | 1.7 | 1.6 | 0.05 | 0.25 |
| Pasture uNDFom | 1.7[a] | 1.4[b] | 1.7[a] | 1.5[b] | 0.05 | <0.01 |
| Apparent total-tract digestion, g/g | | | | | | |
| DM | 0.72[ab] | 0.70[a] | 0.72[b] | 0.72[ab] | 0.006 | <0.05 |
| OM | 0.75 | 0.74 | 0.75 | 0.75 | 0.005 | 0.15 |
| aNDFom | 0.70[a] | 0.67[b] | 0.70[a] | 0.69[ab] | 0.006 | <0.01 |
| pdNDFom | 0.92[a] | 0.87[b] | 0.92[a] | 0.90[ab] | 0.008 | <0.01 |
| ME supply[4], Mcals/d | 39 | 44 | 40 | 42 | - | - |

[1]Estimated using the n-alkane technique during wk 6 of the experiment

[2]GO = Perennial ryegrass (PRG) only control; CITRUS = PRG plus citrus pulp-based supplement; RP-SBM = PRG plus rumen-protected soybean meal-based supplement; MIX = PRG plus combination of citrus pulp and RP-SBM based supplement.

[3]aNDFom = amylase- and sodium sulfite-treated NDF corrected for ash residue; pdNDFom = potentially digestible aNDFom; uNDFom = undigested amylase- and sodium sulfite treated NDF corrected for ash residue.

[4]ME supply = metabolizable energy supply calculated using the Cornell Net Carbohydrate and Protein System, v.7.0.

[a-c]Means within row with different superscripts are significantly different ($P < 0.05$).

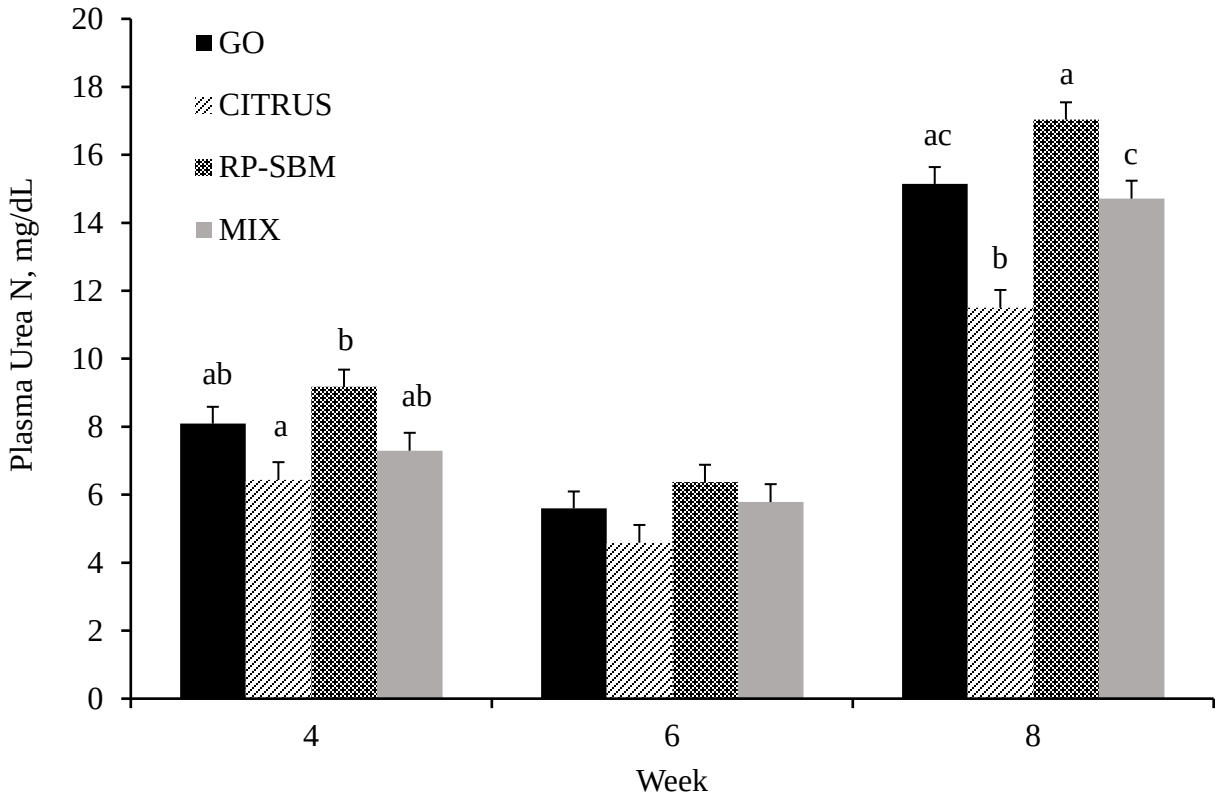


**Figure 6.3.** Effect of concentrate supplement type on plasma urea nitrogen (lsmean ± SEM) in mid-lactation dairy cows grazing perennial ryegrass. GO = Perennial ryegrass (PRG) only; CITRUS = PRG plus citrus pulp-based supplement; RP-SBM = PRG plus rumen-protected soybean meal-based supplement; MIX = PRG plus combination of citrus pulp and rumen-protected soybean meal-based supplement.

### *6.4.5. Feeding and Ruminating Behavior, Body Weight, and Body Condition Score*

Concentrate supplement type had no effect on feeding time (Table 6.6). Cows fed CITRUS and MIX diets ruminated less compared with cows fed the GO and RP-SBM diets ($P < 0.01$). When rumination time was expressed per kg of aNDFom or uNDFom intake, there was no effect of diet (Table 6.6). However, we observed a tendency for cows fed CITRUS to ruminate less per kg of pdNDFom compared with cows fed the GO diet ($P = 0.09$). Conversely, cows fed CITRUS and MIX diets had greater resting time compared with cows fed the GO diet ($P < 0.01$). Feeding time, resting time and ruminating time were affected by week of experiment (Table 6.6). During wk 4 to 6 of the experiment, feeding and resting time were the lowest whereas time ruminating peaked.

The effect of concentrate supplement type on BW and BCS is in Table 6.7. The cows fed the MIX diet had higher BW ($P < 0.05$) compared with cows fed the GO diet and numerically the highest BW change but overall, BW change, BCS, and BCS change were not affected by diet (Table 6.7). Due to the soil moisture deficiency, week had an effect on BW and BCS ($P < 0.01$) as both reduced to the lowest levels during wk 4 to 7.

**Table 6.6.** Effect of concentrate supplement type on feeding, resting, and ruminating time in mid-lactation dairy cows grazing perennial ryegrass

| | Diet[1] | | | | | *P*-value | | |
|---|---|---|---|---|---|---|---|---|
| Item[2] | GO | CITRUS | RP-SBM | MIX | SEM | Diet (D) | Week (W) | D*W |
| Feeding time (min/d) | 610 | 602 | 626 | 607 | 8.2 | 0.15 | <0.01 | 0.24 |
| Resting time (min/d) | 246[a] | 276[b] | 255[ab] | 272[b] | 6.4 | <0.01 | <0.01 | <0.05 |
| Ruminating time (min/d) | 515[a] | 489[b] | 511[a] | 487[b] | 6.1 | <0.01 | <0.01 | <0.01 |
| Ruminating time (min/kg aNDFom per d) | 82 | 73 | 79 | 79 | 2.9 | 0.15 | - | - |
| Ruminating time (min/kg pdNDFom per d) | 108 | 94 | 104 | 103 | 3.7 | 0.09 | - | - |
| Ruminating time (min/kg uNDFom per d) | 346 | 319 | 335 | 342 | 12.4 | 0.46 | - | - |

[1]GO = Perennial ryegrass (PRG) only control; CITRUS = PRG plus citrus pulp-based supplement; RP-SBM = PRG plus rumen-protected soybean meal-based supplement; MIX = PRG plus combination of citrus pulp and RP-SBM based supplement.
[2]aNDFom = amylase- and sodium sulfite-treated NDF corrected for ash residue; pdNDFom = potentially digestible aNDFom; uNDFom = undigested amylase- and sodium sulfite treated NDF corrected for ash residue.
[a-b]Means within row with different superscripts are significantly different ($P < 0.05$)

**Table 6.7.** Effect of concentrate supplement type on body weight and body condition score in mid-lactation dairy cows grazing perennial ryegrass

| | Diet[1] | | | | | *P*-value | | |
|---|---|---|---|---|---|---|---|---|
| Item | GO | CITRUS | RP-SBM | MIX | SEM | Diet (D) | Week (W) | D*W |
| BW, kg | 517[a] | 521[ab] | 526[ab] | 529[b] | 2.78 | <0.05 | <0.01 | <0.01 |
| BW change, kg/week | -0.69 | 0.44 | 0.53 | 1.19 | 0.98 | 0.57 | <0.01 | <0.01 |
| BCS | 2.94 | 2.94 | 2.92 | 2.94 | 0.02 | 0.94 | <0.01 | 0.32 |
| BCS change, score/week | -0.01 | -0.01 | -0.01 | -0.01 | 0.01 | 0.97 | <0.01 | <0.05 |

[1]GO = Perennial ryegrass (PRG) only control; CITRUS = PRG plus citrus pulp-based supplement; RP-SBM = PRG plus rumen-protected soybean meal-based supplement; MIX = PRG plus combination of citrus pulp and RP-SBM based supplement.
[a-b]Means within row with different superscripts are significantly different ($P < 0.05$)

## 6.5. DISCUSSION

In this experiment, we utilized the CNCPS to create diets that could investigate the effect of increased MP supply, when F-CHO are supplemented, without the confounding increase in ME supply. This was achieved by including low levels of a RP-P ingredient in a PRG-based diet (RP-SBM) and comparing it to a diet supplemented with F-CHO only (CITRUS). This allowed inference for factors first limiting milk production, when cows consuming PRG are supplemented with F-CHO (Brun-Lafleur et al., 2010). However, for the majority of this study (wk 1 to 7), reduced rainfall and high SMD substantially altered the chemical composition of the PRG offered in comparison with typical mid-season swards (Figure 6.1; O'Neill et al., 2013). Due to the altered chemical composition of the PRG observed in this study, care must be taken when extrapolating the results to cows grazing typical PRG pasture.

### *6.5.1. Milk Production and Milk Composition*

Supplementation of grazing dairy cows with a F-CHO ingredient or low levels of a RP-P ingredient, in the current study, increased ECM yield to a similar extent over that of cows fed a PRG-only diet (Table 6.4). Similar predicted ME supply, using the CNCPS, was achieved for cows fed the RP-SBM and GO diets (40 and 39 Mcals ME/d, respectively; Table 6.5). Thus, the increased milk production for cows offered F-CHO or RP-P was likely mediated through increased MP supply rather than increased ME supply. A number of studies have demonstrated increased milk production when grazing dairy cows have been supplemented with RP-P ingredients to increase MP supply such as protected casein (Stobbs et al., 1977; Rogers et al., 1980; Minson, 1981) formaldehyde-treated soybean meal (Delaby et al., 1995, Delagarde et al., 1997; Astigarraga et al., 2002) or fishmeal (O'Mara et al., 2000). In contrast, Rusdi and Van Houtert, (1997) and

Delaby et al. (1996) showed no response to protected soybean or fishmeal and the contrasting evidence as to the effect of RP-P ingredients on milk production reported in the literature could be due to variation in the chemical composition and digestibility of PRG (Delagarde et al., 2000; Lovett et al, 2005). Consequently, this might cause different nutrients to first-limit milk production in grazing dairy cows at different times of the year, stages of growth or under different climatic conditions.

A high milk response to supplement (calculated as the difference in milk produced between unsupplemented and supplemented treatment divided by supplement DMI) was achieved in the current study for the RP-SBM diet (2.36 kg of milk/kg of supplement). This response was much higher than the response achieved for the CITRUS diet (0.42 kg of milk/kg of supplement) and to previous responses observed in supplemented grazing cows (Bargo et al., 2003). The low CP and high uNDFom concentrations of the PRG in the current study might have elevated the milk production response in cows fed the RP-SBM diet, as demonstrated by Delaby et al. (1996) for cows consuming high versus low CP pasture. However, both Delagarde et al. (1997) and O'Mara et al. (2000) previously observed high milk response to RP-P ingredients in cows grazing PRG swards.

Cows fed the MIX diet had higher milk production performance than the CITRUS and GO diets, but similar performance to the RP-SBM diet. Furthermore, when the RP-P ingredient was included in the diet, milk protein yield increased compared with cows fed the CITRUS or GO diets. This suggests that the RP-P ingredient included in the MIX diet had a greater influence in overcoming the limiting nutrients for milk production than the F-CHO as there was no synergistic effect of supplementing both ingredients. Under the conditions experienced in this study,

increasing MP supply allowed higher milk production performance to be achieved as MP was more limiting than ME supply.

Seasonal and climatic factors can alter the chemical composition of PRG swards leading to highly variable factors that limit milk production performance (Delagarde et al., 2000). While increasing MP supply enhanced milk yield compared with the GO diet in the current study, high levels of N intake can increase environmental N loss as a result of increased urinary N excretion (Mulligan et al., 2004). Offering excessive amounts of RP-P supplements to cows grazing adequate CP pastures can potentially enhance these negative environmental outcomes and reduce the milk response to supplement observed (Stakelum, 1993; O'Mara et al 2000). Therefore, it is essential to achieve a further understanding of the MP supply and nutrients first limiting milk production performance of cows grazing PRG swards.

### *6.5.2. Dry Matter Intake, Total-Tract Nutrient Digestibility, and Plasma Urea Nitrogen*

Both Mulligan et al. (2004) and Reid et al. (2015) observed higher DMI in cows grazing PRG when offered citrus pulp-based concentrates, which is in agreement with the findings of the current study. Although cows offered citrus pulp-based supplements in all three studies achieved considerably higher energy intake, greater milk production performance was not observed when compared with cows consuming protein-based supplements. Solomon et al. (2000) and Hall et al. (2010) observed reduced DMI when citrus pulp was included in the diet; however, in both of those studies, citrus pulp was used as a corn grain substitute in total mixed rations. In the current study, citrus pulp replaced PRG containing elevated levels of aNDFom and uNDFom, which has slower degradation rates than rapidly degrading corn grain.

Dry matter intake can be inhibited by physical fill limitations if the rate of digesta emptying is slow (Forbes, 2007). In Chapter 3, physical fill did not seem to limit the intake capacity of animals grazing PRG; however, a lower aNDFom intake was observed in that study in comparison with the current study (1.1% vs. 1.4% of BW, respectively). The similar aNDFom intakes achieved across all diets in the current study suggests that the experimental cows consuming PRG elevated in both aNDFom and uNDFom, might have reached an aNDFom intake capacity of approximately 1.4% of BW. The CITRUS diet, as formulated, had a lower concentration of aNDFom compared with GO, and this probably allowed the higher total DMI be achieved prior to reaching an aNDFom intake capacity. The aNDFom intake capacity seemed indiscriminate of the source of aNDFom (i.e. pasture vs. by-product) and this aNDFom intake capacity likely governed the substitution rates (i.e. the decrease in pasture DMI per kg of supplemental DMI; Kellaway and Porta, 1993) observed in the current study (0.58, 0.38, and 0.58 kg of pasture/kg of supplement for CITRUS, RP-SBM, and MIX diets, respectively). The rate of degradation and indigestible proportion of aNDFom can also affect rumen physical fill (Mertens and Ely, 1979; Mertens, 1997). The intake of uNDFom was similar among all diets in the current study but higher than that of Chapter 3 (0.32% vs. 0.13% of BW).

Apparent TTD of all nutrients were lower in the current study in comparison with previous investigations of cows consuming PRG (Beecher et al., 2014; Garry, 2016). Throughout the experiment, both aNDFom and uNDFom concentrations of the PRG offered were elevated in comparison with Irish mid-season PRG (Chapter 3). The mean concentrations observed in the current study were similar to that of Australian and South African PRG samples (Raffrenato et al., 2018b) for both aNDFom (44.2% vs. 43.5% of DM, respectively) and uNDFom (19.2% vs. 19.3% of aNDFom, respectively). Thus, some of the reduced TTD, observed in the current study, is likely

attributable to the elevated aNDFom and uNDFom concentrations, which seem indicative of the adverse growing conditions.

As a proportion of the RP-SBM ingredient degrades in the rumen (Sheehy et al., 2020), it is plausible that this ingredient might have supported higher rumen N balance. However, cows fed the MIX, RP-SBM and GO diets had similar aNDFom TTD, suggesting that this mechanism was not involved in allowing the MIX and RP-SBM diets to achieve higher milk production performance than the GO diet. Further, Schor and Gagliostro (2001) reported higher milk production performance in cows grazing similar CP swards (15% of DM) as the current study, when offered a rumen undegradable protein source (blood meal) compared with a rumen degradable protein source (soybean meal). Increased DMI has also been reported to reduce TTD, due to a faster rate of passage through the rumen (Krizsan et al., 2012), which might also have contributed to the reduced TTD observed in cows fed the CITRUS diet in the current study.

In a meta-analysis quantifying ruminal N metabolism, Broderick et al. (2010) reported that zero ruminal N-balance (where omasal N outflow equaled N intake) occurred when the diet averaged 14.7% CP, where ruminal ammonia-N and MUN were 7.1 and 8.3 mg/dL. In the current study, the PRG offered averaged 14% CP across the 10-wk experimental period and 11.6% CP during wk 6, resulting in low PUN levels (~6 mg/dL; Figure 6.3) across all experimental diets when TTD was determined. Negative rumen N balance can impact nutrient digestibility (Hoover, 1986; Lee et al., 2012; Higgs, 2014) and this likely contributed additionally to the reduced TTD observed in the current study. Furthermore, cows fed the CITRUS diet had lower aNDFom TTD, compared with the GO and RP-SBM diets, which might have affected their ability to support microbial protein synthesis in the rumen. Supplementary urea (67 g) was included in the design of the CITRUS diet

to prevent low rumen N-balance; however, it seems not enough urea was included to counteract the low CP content of PRG in this study.

### *6.5.3. Feeding and Ruminating Behavior*

A reduction in feeding time due to concentrate supplementation is commonly reported in cows consuming pasture-based diets (McGilloway and Mayne, 1996). In the current study, feeding time was not affected by diet and averaged 611 min/d among all diets. This might be due, at least in part, to the type and amount of concentrate supplement offered as both Bargo et al., (2002) and Pulido and Leaver (2001) offered higher amounts of a starch-based supplement. Other variables such as method of measuring feeding time might have also influence these results.

Although similar levels of total aNDFom intake were achieved by cows on all diets, cows fed the CITRUS and MIX diets ruminated less compared with the GO and RP-SBM diets. The inherent rate of citrus pulp pdNDFom degradation was faster than the PRG offered in the current study (5.7 %/h vs 5.0%/h, respectively). This potentially higher rate of pdNDFom degradation of cows fed CITRUS and MIX diets might have led to the observed reduction in rumination time. However, rumination time for all diets were in an acceptable range and comparable to other PRG-based diets (Gregorini et al., 2013; O'Sullivan et al., 2019) suggesting that the aNDFom from the citrus pulp ingredient was adequately stimulatory for rumination and rumen health.

## 6.6. CONCLUSION

High SMD drastically reduced pasture growth rate and significantly altered the chemical composition of mid-season PRG offered during this experiment. Cows fed the CITRUS diet increased total DMI but reduced aNDFom TTD compared with cows fed the GO diet. Similar

aNDFom intakes were observed for all diets suggesting that the experimental cows, consuming PRG elevated in both aNDFom and uNDFom, reached an aNDFom intake capacity of approximately 1.4% of BW. Supplementation of grazing dairy cows with a F-CHO or a RP-P ingredient, under the conditions experienced during this experiment, increased milk production performance to a similar extent over that of cows fed a PRG-only diet. As the RP-SBM and GO diets achieved similar ME intake, it seems likely that the increased milk production performance was mediated through increased MP supply. Furthermore, when the RP-P ingredient was included in the diet, milk protein yield increased compared with cows fed the CITRUS and GO diets. Therefore, under the conditions experienced during this experiment, increasing MP supply allowed higher milk production performance to be achieved as MP was more limiting than ME supply. While care must be taken extrapolating these results to typical mid-season PRG, this study indicates that there is a requirement to further understand the MP supply and potential nutrients first limiting milk production performance of cows grazing PRG swards.

## 6.7. ACKNOWLEDGMENTS

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## 6.8. REFERENCES


AOAC International. 2005. Official Methods of Analysis. 18th ed. AOAC International, Gaithersburg, MD

Astigarraga, L., J. L. Peyraud, and L. Delaby. 2002. Effect of nitrogen fertiliser rate and protein supplementation on the herbage intake and the nitrogen balance of grazing dairy cows. Anim. Res. 51:279-293. https://doi.org/10.1051/animres:2002022.

Bargo, F., L. D. Muller, E. S. Kolver, and J. E. Delahoy. 2003. Invited review: Production and digestion of supplemented dairy cows on pasture. J. Dairy Sci. 86:1–42. https://doi.org/10.3168/jds.S0022-0302(03)73581-4.

Bargo, F., L. D. Muller, J. E. Delahoy, and T. W. Cassidy. 2002. Milk response to concentrate supplementation ofhigh producing dairy cows grazing at two pasture allowances. J. Dairy Sci. 85:1777– 1792. https://doi.org/10.3168/jds.S0022-0302(02)74252-5.

Beecher, M., F. Buckley, S. M. Waters, T. Boland, D. Enriquez-Hidalgo, M. Deighton, M. O'Donovan, and E. Lewis. 2014. Gastrointestinal tract size, total-tract digestibility, and rumen microflora in different dairy cow genotypes. J. Dairy Sci. 97:3906-3917. https://doi.org/10.3168/jds.2013-7708.

Beever, D. E., H.R. Losada, S.B. Cammell, R. T. Evans, and M. J. Haines. 1986. Effect of forage species and season on nutrient digestion and supply in grazing cattle. Br. J. Nutr 56:209-225. https://doi.org/10.1079/BJN19860101

Berzaghi, P., J. Herbein, and C. Polan. 1996. Intake, site, and extent of nutrient digestion of lactating cows grazing pasture. J. Dairy Sci. 79:1581-1589. https://doi.org/10.3168/jds.S0022-0302(96)76520-7.

Broderick, G. A. 2017. Review: Optimizing ruminant conversion of feed protein to human food protein. Animal 12:1722–1734. https://doi.org/10.1017/S1751731117002592.

Broderick, G., P. Huhtanen, S. Ahvenjärvi, S. Reynal, and K. Shingfield. 2010. Quantifying ruminal nitrogen metabolism using the omasal sampling technique in cattle—A meta-analysis. J. Dairy Sci. 93:3216-3230. https://doi.org/10.3168/jds.2009-2989.

Brun-Lafleur, L., L. Delaby, F. Husson, and P. Faverdin. 2010. Predicting energy× protein interaction on milk yield and milk composition in dairy cows. J. Dairy Sci. 93:4128-4143. https://doi.org/10.3168/jds.2009-2669.

Delaby, L., J. L. Peyraud, A. Bouttier, and J. -R. Reccatte. 1998. Effet d'une réduction simultanée de la fertilisation azotée et du chargement sur les performances des vaches laitières et la valorisation du pâturage. Ann. Zootech 47:17–39. https://hal.archives-ouvertes.fr/hal-00889711.

Delaby, L., J. Peyraud, R. Vérité, and B. Marquis. 1996. Effect of protein content in the concentrate and level of nitrogen fertilization on the performance of dairy cows in pasture. Ann. Zootech. 327-341.

Delaby, L., Peyraud, J., Vérité, R. and Marquis, B. 1995. Effet de la complémentation protéique sur les performances des vaches laitières au pâturage conduit à 2 niveaux de fertilisation. Ann. Zootech. 44:173-188. https://hal.archives-ouvertes.fr/hal-00889176.

Delagarde, R., J. Peyraud, L. Delaby, and P. Faverdin. 2000. Vertical distribution of biomass, chemical composition and pepsin—cellulase digestibility in a perennial ryegrass sward: interaction with month of year, regrowth age and time of day. Anim. Feed Sci. Technol. 84:49-68. https://doi.org/10.1016/S0377-8401(00)00114-0.

Delagarde, R., J. Peyraud, and L. Delaby. 1997. The effect of nitrogen fertilization level and protein supplementation on herbage intake, feeding behaviour and digestion in grazing dairy cows. Anim. Feed Sci. Technol. 66:165-180.

Dillon, P., T. Hennessy, L. Shalloo, F. Thorne, and B. Horan. 2008. Future outlook for the Irish dairy industry: a study of international competitiveness, influence of international trade reform and requirement for change. Int. J. Dairy Technol. 61:16-29. https://doi.org/10.1111/j.1471-0307.2008.00374.x.

Dillon, P., 1993. The use of n-alkanes as markers to determine intake, botanical composition of available or consumed herbage in studies of digesta kinetics with dairy cows. Ph.D Dissertation. National University of Ireland, Dublin, Ireland.

Dillon, P. and G. Stakelum. 1989. Herbage and dosed alkanes as a grass measurement technique for dairy cows. Irish J. Agr. Food Res. 28:104.

Edmonson, A., I. Lean, L. Weaver, T. Farver, and G. Webster. 1989. A body condition scoring chart for Holstein dairy cows. J. Dairy Sci. 72:68-78. https://doi.org/10.3168/jds.S0022-0302(89)79081-0.

Fessenden, S., D. Ross, E. Block, and M. E. Van Amburgh. 2020. Comparison of milk production, intake, and total-tract nutrient digestion in lactating dairy cattle fed diets containing either

wheat midds and urea, commercial fermentation by-product, or rumen-protected soybean meal. J. Dairy Sci. 103:5090-5101. https://doi.org/10.3168/jds.2019-17744.

Forbes, J. M. 2007. Voluntary food intake and diet selection in farm animals. CAB International, Wallingford, UK.

Garry, B. 2016. The effect of animal and sward factors on the in vivo organic matter digestibility of perennial ryegrass. PhD Dissertation. School of Agriculture and Food Science, Univ. College Dublin, Belfield, Dublin 4, Ireland.

Gregorini, P., E. Minnee, W. Griffiths, and J. Lee. 2013. Dairy cows increase ingestive mastication and reduce ruminative chewing when grazing chicory and plantain. J. Dairy Sci. 96:7798-7805. https://doi.org/10.3168/jds.2013-6953.

Hall, M. B. 2014. Selection of an empirical detection method for determination of water-soluble carbohydrates in feedstuffs for application in ruminant nutrition. Anim. Feed Sci. Technol. 198:28-37. https://doi.org/10.1016/j.anifeedsci.2014.08.009.

Hall, M. B., C. C. Larson, and C. J. Wilcox. 2010. Carbohydrate source and protein degradability alter lactation, ruminal, and blood measures. J. Dairy Sci. 93:311-322. https://doi.org/10.3168/jds.2009-2552.

Hanrahan, L., A. Geoghegan, M. O'Donovan, V. Griffith, E. Ruelle, M. Wallace, and L. Shalloo. 2017. PastureBase Ireland: A grassland decision support system and national database. Comput. Electron. Agric. 136:193-201. https://doi.org/10.1016/j.compag.2017.01.029.

Higgs, R. and M. E. Van Amburgh. 2016. Evolution of the CNCPS-Development of V7. Pages 125-146 in Proc. Cornell Nutrition Conference, Syracuse, NY. Cornell University, Ithaca, NY.

Higgs, R., L. Chase, D. Ross, and M. E. Van Amburgh. 2015. Updating the Cornell Net Carbohydrate and Protein System feed library and analyzing model sensitivity to feed inputs. J. Dairy Sci. 98:6340-6360. https://doi.org/10.3168/jds.2015-9379.

Higgs, R. 2014. Development of a dynamic rumen and gastro-intestinal model in the Cornell Net Carbohydrate and Protein System to predict the nutrient supply and requirements of dairy cattle. PhD Dissertation. Department of Animal Science, Cornell Univ., Ithaca, NY.

Hoekstra N. J., R. P. O. Schulte, P. J. Forrestal, D. Hennessy, D. J. Krol, G. J. Lanigan, C. Müller, L. Shalloo, D. P. Wall, and K. G. Richards. 2020. Scenarios to limit environmental nitrogen

losses from dairy expansion. Sci. Total Environ. 707: 134606. https://doi.org/10.1016/j.scitotenv.2019.134606.

Hoover, W. 1986. Chemical factors involved in ruminal fiber digestion. J. Dairy Sci. 69:2755-2766. https://doi.org/10.3168/jds.S0022-0302(86)80724-X.

Huhtanen, P., K. Kaustell, and S. Jaakkola. 1994. The use of internal markers to predict total digestibility and duodenal flow of nutrients in cattle given six different diets. Anim. Feed Sci. Technol. 48:211-227. https://doi.org/10.1016/0377-8401(94)90173-2.

Kaylegian, K. E., G. Houghton, J. Lynch, J. Fleming, and D. Barbano. 2006. Calibration of infrared milk analyzers: Modified milk versus producer milk. J. Dairy Sci. 89:2817-2832. https://doi.org/10.3168/jds.S0022-0302(06)72555-3.

Keim, J. P., and R. Anrique. 2011. Nutritional strategies to improve nitrogen use efficiency by grazing dairy cows. Chil. J. Agric. Res. 71:623–633. http://doi.org/10.4067/S0718-58392011000400019

Kolver, E. S. 2003. Nutritional limitations to increased production on pasture-based systems. Proceedings of the Nutrition Society 62:291-300. https://doi.org/10.1079/PNS2002200.

Krizsan, S., L. Nyholm, J. Nousiainen, K.-H. Südekum, and P. Huhtanen. 2012. Comparison of in vitro and in situ methods in evaluation of forage digestibility in ruminants. J. Anim. Sci 90(9):3162-3173. https://doi.org/10.2527/jas.2011-4347.

Laisse, S., R. Baumont, P. Veysset, M. Benoit, P. Madrange, B. Rouillé, and J. L. Peyraud. 2018. The net contribution of ruminant production to the protein supply for humans. Pages 718–720 in General meeting of the European Grassland Federation. Cork, Ireland. https://hal.archives-ouvertes.fr/hal-01827929.

Läpple, D., and G. Sirr. 2019. Dairy intensification and quota abolition: A comparative study of production in Ireland and the Netherlands. EuroChoices (Uckfield) 18:26–32. https://doi.org/10.1111/1746-692X.12213.

Lee, C., A. N. Hristov, K. S. Heyler, T. W. Cassidy, H. Lapierre, G. A. Varga, and C. Parys. 2012. Effects of metabolizable protein supply and amino acid supplementation on nitrogen utilization, milk production, and ammonia emissions from manure in dairy cows. J. Dairy Sci. 95:5253-5268. https://doi.org/10.3168/jds.2012-5366.

Lovett, D., L. Stack, S. Lovell, J. Callan, B. Flynn, M. Hawkins, and F. O'Mara. 2005. Manipulating enteric methane emissions and animal performance of late-lactation dairy

cows through concentrate supplementation at pasture. J. Dairy Sci. 88:2836-2842. https://doi.org/10.3168/jds.S0022-0302(05)72964-7.

Mayes, R., C. Lamb, and P. M. Colgrove. 1986a. The use of dosed and herbage n-alkanes as markers for the determination of herbage intake. J. Agric. Sci. 107:161-170. https://doi.org/10.1017/S0021859600066910.

McGilloway, D. A., and C. S. Mayne. 1996. The importance of grass availability for the high genetic merit dairy cow. Page 135 in Recent Advances in Animal Nutrition. P. C. Garnsworthy, J. Wiseman, and W. Haresign, ed. Nottingham Univ. Press. UK.

Mertens, D. R. 2002. Gravimetric determination of amylase-treated neutral detergent fiber in feeds with refluxing in beakers or crucibles: collaborative study. J. AOAC Int. 85:1217-1240.

Mertens, D. R. 1997. Creating a system for meeting the fiber requirements of dairy cows. J. Dairy Sci. 80:1463-1481. https://doi.org/10.3168/jds.S0022-0302(97)76075-2.

Mertens, D. R. and L. O. Ely. 1979. A dynamic model of fiber digestion and passage in the ruminant for evaluating forage quality. J. Anim. Sci. 49:1085-1095. https://doi.org/10.2527/jas1979.4941085x.

Met Éireann, 2018. Warm Dry Weather of June and July 2018. Dublin, Ireland: Met Éireann. Available at https://www.met.ie/cms/assets/uploads/2018/08/DryWarmWx06072018SS.pdf (Accessed 14 March 2020).

Minson, D. 1981. The effects of feeding protected and unprotected casein on the milk production of cows grazing ryegrass. J. Agric. Sci. 96:239-241. https://doi.org/10.1017/S0021859600032056.

Mulligan, F., P. Dillon, J. Callan, M. Rath, and F. O'mara. 2004. Supplementary concentrate type affects nitrogen excretion of grazing dairy cows. J. Dairy Sci. 87:3451-3460. https://doi.org/10.3168/jds.S0022-0302(04)73480-3.

O'Donovan, M., P. Dillon, M. Rath, and G. Stakelum. 2002. A comparison of four methods of herbage mass estimation. Ir. J. Agric. Food Res. 17-27. https://www.jstor.org/stable/25562447.

O'Mara, F. P., J. J. Murphy, and M. Rath. 2000. The effect of concentrate supplements differing in ruminal protein degradability on milk production and blood metabolite concentrations of dairy cows grazing perennial ryegrass pasture. Livest. Prod. Sci. 64:183-191. https://doi.org/10.1016/S0301-6226(99)00150-5.

O'Neill, B., E. Lewis, M. O'Donovan, L. Shalloo, F. Mulligan, T. Boland, and R. Delagarde. 2013. Evaluation of the Graze In model of grass dry-matter intake and milk production prediction for dairy cows in temperate grass-based production systems. 1–Sward characteristics and grazing management factors. Grass Forage Sci. 68:504-523. https://doi.org/10.1111/gfs.12023.

O'Sullivan, M., P. Dillon, K. O'Sullivan, K. Pierce, N. Galvin, M. Egan, and F. Buckley. 2019. Intake, efficiency, and feeding behavior characteristics of Holstein-Friesian cows of divergent Economic Breeding Index evaluated under contrasting pasture-based feeding treatments. J. Dairy Sci. 102:8234-8246. https://doi.org/10.3168/jds.2019-16371.

Pulido, R. G., and J. D. Leaver. 2001. Quantifying the influence of sward height, concentrate level, and initial milk yield on the milk production and grazing behaviour of continuously stocked dairy cows. Grass For. Sci. 56:57–67. https://doi.org/10.1046/j.1365-2494.2001.00246.x

Raffrenato, E., C. Nicholson, and M. E. Van Amburgh. 2019. Development of a mathematical model to predict pool sizes and rates of digestion of 2 pools of digestible neutral detergent fiber and an undigested neutral detergent fiber fraction within various forages. J. Dairy Sci. 102:351-364. https://doi.org/10.3168/jds.2018-15102

Raffrenato, E., D. Ross, and M. E. Van Amburgh. 2018a. Development of an in vitro method to determine rumen undigested aNDFom for use in feed evaluation. J. Dairy Sci. 101:9888-9900. https://doi.org/10.3168/jds.2018-15101.

Raffrenato, E., R. Lombard, L. Erasmus, D. McNeill, D. Barber, M. Callow, and D. Poppi. 2018b. Prediction of indigestible NDF in South African and Australian forages from cell wall characteristics. Anim. Feed Sci. Technol. 246:104-113. https://doi.org/10.1016/j.anifeedsci.2018.08.009.

Reid, M., M. O'Donovan, J. P. Murphy, C. Fleming, E. Kennedy, and E. Lewis. 2015. The effect of high and low levels of supplementation on milk production, nitrogen utilization efficiency, and milk protein fractions in late-lactation dairy cows. J. Dairy Sci. 98:5529-5544. https://doi.org/10.3168/jds.2014-9016.

Rogers, G., R. Porter, T. Clarke, and J. Stewart. 1980. Effect of protected casein supplements on pasture intake, milk yield and composition of cows in early lactation. Aus. J. Agric. Res. 31:1147-1152. https://doi.org/10.1071/AR9801147.

Rusdi, and M. F. J. Van Houtert. 1997. Responses to protected amino acids or protected protein in dairy cows grazing ryegrass pastures in early or late lactation. Proc. N.Z. Soc. Anim. Prod. 120-125.

Schor, A. and G. A. Gagliostro. 2001. Undegradable protein supplementation to early-lactation dairy cows in grazing conditions. J. Dairy Sci. 84:1597-1606. https://doi.org/10.3168/jds.S0022-0302(01)74593-6.

Schulte, R. P. O., J. Diamond, K. Finkele, N. M. Holden, and A. J. Brereton. 2005. Predicting the soil moisture conditions of Irish grasslands. Irish J. Agr. Food Res. 44:95–110. https://www.jstor.org/stable/25562535.

Sheehy, M., F. Mulligan, S. Taylor, and A. Fahey. 2020. Effects of a novel heat-treated protein and carbohydrate supplement on feed consumption, milk production, and cheese yield in early-lactation dairy cows. J. Dairy Sci. 103:4315-4326. https://doi.org/10.3168/jds.2019-17468.

Solomon, R., L. E. Chase, D. Ben-Ghedalia, and D. E. Bauman. 2000. The effect of nonstructural carbohydrate and addition of full fat extruded soybeans on the concentration of conjugated linoleic acid in the milk fat of dairy cows. J. Dairy Sci. 83:1322-1329. https://doi.org/10.3168/jds.S0022-0302(00)74998-8.

Stakelum, G. K. N., 1993. Supplementary Feeding and Herbage Intake of the Dairy Cow. Ph.D. National Univ. of Ireland, Dublin, Ireland.

Stobbs, T., D. Minson, and M. Mcleod, 1977. The response of dairy cows grazing a nitrogen fertilized grass pasture to a supplement of protected casein. J. Agric. Sci. 89:137-141. https://doi.org/10.1017/S0021859600027295.

Tyrrell, H. F., and J. T. Reid. 1965. Prediction of the energy value of cow's milk. J. Dairy Sci. 48:1215–1223. https://doi.org/10.3168/jds.S0022-0302(65)88430-2.

Van Amburgh, M. E., E. Collao-Saenz, R. Higgs, D. Ross, E. Recktenwald, E. Raffrenato, L. Chase, T. Overton, J. Mills, and A. Foskolos. 2015. The Cornell Net Carbohydrate and Protein System: Updates to the model and evaluation of version 6.5. J. Dairy Sci. 98:6361-6380. https://doi.org/10.3168/jds.2015-9378.

Van Vuuren, A. and P. Chilibroste. 2013. Challenges in the nutrition and management of herbivores in the temperate zone. Animal 7:19-28. https://doi.org/10.1017/S1751731111001741.

Wales, W., P. Doyle, and D. Dellow. 1999. Degradabilities of dry matter and crude protein from perennial herbage and supplements used in dairy production systems in Victoria. Aust. J. Exp. Agric. 39:645-656. https://doi.org/10.1071/EA98156.

Werner, J., C. Umstatter, L. Leso, E. Kennedy, A. Geoghegan, L. Shalloo, M. Schick, and B. O'Brien. 2019. Evaluation and application potential of an accelerometer-based collar device for measuring grazing behavior of dairy cows. Animal. 13:2070-2079. https://doi.org/ 10.1017/S1751731118003658.

Wojciechowski, K. L. and D. M. Barbano. 2016. Prediction of fatty acid chain length and unsaturation of milk fat by mid-infrared milk analysis. J. Dairy Sci. 99:8561-8570. https://doi.org/10.3168/jds.2016-11248.

Woolpert, M., H. Dann, K. Cotanch, C. Melilli, L. Chase, R. Grant, and D. Barbano. 2016. Management, nutrition, and lactation performance are related to bulk tank milk de novo fatty acid concentration on northeastern US dairy farms. J. Dairy Sci. 99:8486-8497. https://doi.org/10.3168/jds.2016-10998.

Zontini, A., M,. 2016. Characterization of fiber fractions in plant by-products and modeling fiber digestion with application in CNCPS 7.0. PhD. Department of Animal Science, Cornell Univ., Ithaca, NY.

# CHAPTER 7: SUMMARY AND FUTURE WORK

## 7.1. SUMMARY

Due to the growing demand for animal-based protein and the unique ability of ruminants to convert human inedible feed into highly nutritious food, dairy production systems will continue to play a vital role in global food and nutrition security. In pasture-based production systems, it is likely that perennial ryegrass (**PRG**) will remain the predominant or base pasture species due to many favorable traits, such as early spring growth, high digestibility, and large dry matter yield. However, increasing environmental challenges will require pasture-based systems to adapt and innovate. Nutritional strategies currently exist to increase the nutrient use efficiency, productivity, and sustainability of the system; however, to ascertain the optimal strategy, a robust understanding of the nutrient supply from PRG is required.

In Chapter 2, new and updated feed chemistry methods were performed to characterize the nutritive value of PRG. Results demonstrated that the aNDFom fraction of immature PRG comprises of a large potentially digestible pool, which degrades at a rapid rate and requires a 12-h in vitro fermentation time point to more accurately describe the degradation behavior. In addition, a large proportion of the PRG N was estimated to be soluble and highly degradable in the rumen based on the N-fractionation scheme employed by the Cornell Net Carbohydrate and Protein System (**CNCPS**). Chapter 2 also highlighted that inadequate recovery of PRG amino acids (**AA**) occurs when single hydrolysis time points are performed.

The objectives of Chapters 3 and 4 were to evaluate the effect of rolled barley supplementation on rumen metabolism, omasal flow of nutrients, and microbial dynamics in lactating dairy cows fed PRG-based diets. The omasal sampling technique, rumen evacuation, microbial isolation and AA analysis were utilized in the study. Cows supplemented with rolled barley did not increase

overall performance and reduced ruminal and total-tract aNDFom digestibility; however, this was not mediated through a reduction in reticulorumen pH. Flow of non-ammonia N and bacterial N at the omasal canal was increased in supplemented cows compared with non-supplemented. Rolled barley supplementation increased microbial AA flow, which was likely due to the greater amount of fermentable carbohydrate digested in the rumen and a greater efficiency of microbial protein synthesis. Extensive rumen degradation of the PRG AA indicated that cows consuming PRG-based diets exhibit a large dependence on microbial AA to support metabolizable AA supply. Protozoa N flow was not affected by diet; however, protozoa did supply a considerable amount of the microbial N for both diets. Furthermore, liquid retention time and protozoa generation time averaged 5 h and 4.1 h, respectively, which indicated that protozoa have the capability to grow and leave the rumen at much higher rates than previously assumed.

From this enhanced understanding of protozoal dynamics, the primary objective of Chapter 5 was to reparameterize the coefficients of the protozoal sub-model in the CNCPS v7.0 to better represent in vivo conditions of the lactating dairy cow. Considerable improvement was achieved in the ability of the model to predict AA flow, when compared with a literature data set. However, discrepancies between predicted and observed Met and Lys flows indicated that further refinement is required while all other AA are predicted with fairly high accuracy. In addition, prediction of AA flows were in general agreement with the measurements from Chapter 4 for PRG-fed cows; however, the flows of Arg, Met and Trp were slightly over predicted.

Finally, in Chapter 6, nutritional strategies to optimize productivity from PRG-based diets were investigated. The results demonstrated that increased metabolizable protein (**MP**) supply allowed higher milk yield to be achieved, as MP was more limiting than metabolizable energy (**ME**). However, a high soil moisture deficit experienced during the study altered the chemical

composition of the PRG, reducing the ability to extrapolate the results to cows consuming typical PRG pastures.

Overall, this dissertation provides an enhanced understanding of the nutrient supply from PRG-based diets and highlights opportunities to increase the productivity and efficiency of pasture-based systems.

## 7.2. FUTURE WORK

In Chapter 2, for the first time, the in vitro aNDFom digestibility method, developed by Raffrenato et al. (2018, 2019), was applied to PRG pasture. Results demonstrated that the method had the ability to distinguish samples based on season and drought conditions. Future work should incorporate a larger data set to evaluate the effect of other factors on the fractionation of aNDFom and the associated rates of degradation, such as plant maturity and cultivar. Furthermore, the method should be utilized for evaluations of other pasture species and in plant breeding programs to directly quantify cell wall degradation. In vivo studies, as conducted for total mixed ration systems (Cotanch et al., 2014; Zontini et al., 2015), should be undertaken to assess the impact of aNDFom fractions on variables such as DMI, rumination, rumen turnover, and milk production for pasture-fed cows.

When evaluating the capability of CNCPS v7.0 to predict rumen degraded aNDFom, model predicted estimates were in close agreement with the observed measurements for the PRG-only diet from Chapter 3 (4,309 vs. 4,217 g of rumen degraded aNDFom, respectively; Figure 7.1). This indicates that the current structure of the model, in combination with the in vitro aNDFom digestibility method, can predict rumen aNDFom degradation with high accuracy. However, when rolled barley was supplemented, the model overestimated rumen degraded aNDFom, when

compared with the observed measurements (3,965 vs. 3,540 g of rumen degraded aNDFom, respectively; Figure 7.1). While the structure of the CNCPS can account for the effect of low rumen pH and low rumen ammonia on aNDFom degradation, other factors such as the requirement of cellulolytic bacteria for branched-chain VFA (Van Soest, 1994) or the 'carbohydrate effect' described by Mould et al. (1983), might not be fully captured. The Nordic feed evaluation system utilizes a non-linear 'rumen load index' (NorFor, 2011) to account for the negative associative effect on aNDFom degradation, when increased levels of starch and sucrose are included in the diet (Khalili and Huhtanean, 1991; Oba and Allen, 2003). Interestingly, during NorFor model evaluation the rumen load index was not negatively correlated with observed rumen pH, but was negatively correlated with observed reductions in rumen NDF degradation. Future work should investigate the causative factors of reduced aNDFom degradation and incorporate this information into the mechanistic structure of the CNCPS v.7.0.

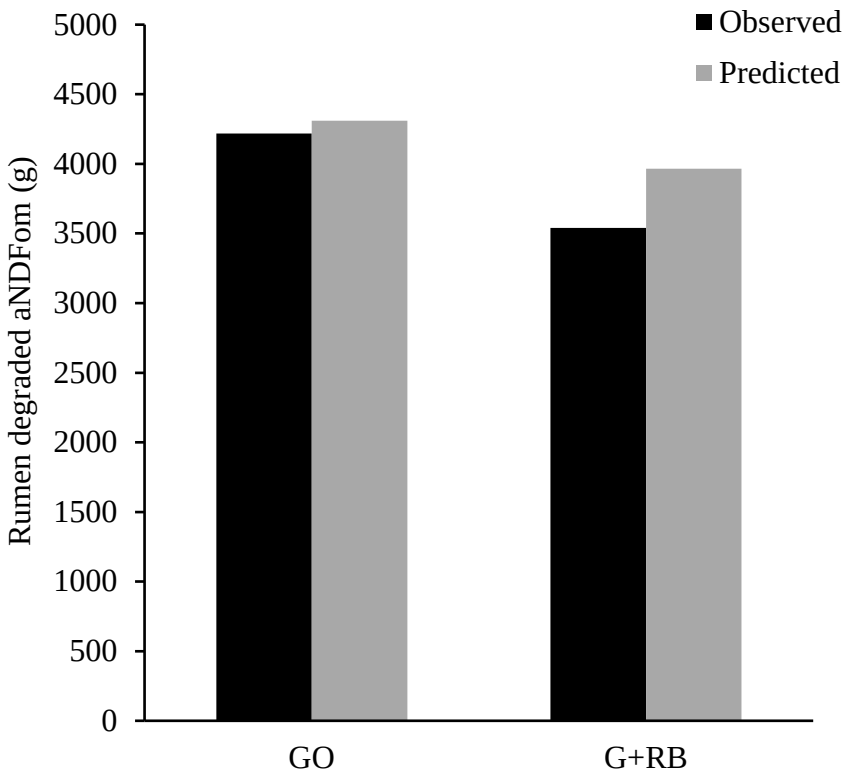


**Figure 7.1.** Observed (Chapter 3) versus predicted Cornell Net Carbohydrate and Protein System (Reparameterized CNCPS v7.0; Chapter 5) prediction for rumen degraded aNDFom.

In Chapters 3 and 4, extensive rumen degradation of PRG AA and a low contribution to the total AA flow, are in agreement with the review by Beever and Siddons (1986). Future research, incorporating the omasal sampling technique should be directed towards further understanding the AA supply of pasture-fed lactating dairy cows. Studies should also be performed to investigate if extensive rumen degradation of PRG AA is consistent across seasons.

The experiment conducted in Chapter 6 should be repeated under more typical climatic conditions to investigate if the supply of AA limit the milk yield of pasture-fed lactating dairy cows. More specific nutritional strategies could be investigated based on the enhanced understanding developed during the current research. For example Table 7.1 shows the predicted EAA supply (Reparameterized CNCPS v7.0; Chapter 5) for the PRG-only diet from Chapters 3 and 4, compared with the optimal supply of each EAA relative to ME, as described by Higgs (2014; g digested AA/Mcal ME). Due to the high energy density of PRG (2.75-2.9 Mcals ME kg/DM; Keim et al., 2011) and the extensive rumen degradation of the ingested AA, it seems likely that the metabolizable supply of EAA per unit of ME is below optimal for PRG-fed cows. Interestingly, His seems to have the largest discrepancy between the predicted and optimal supply of EAA per unit of ME. Lee et al. (2012) proposed that when microbial protein is the major source of MP for the cow, His might not be supplied in sufficient quantities due to the relatively low concentration of His in microbial protein. Future research should be directed towards further understanding the relationship between the supply of AA and the supply of ME in pasture-fed cows.

**Table 7.1.** The predicted AA supply (Reparameterized CNCPS v7.0; Chapter 5) for the perennial ryegrass-only diet from Chapters 3 and 4, compared with the calculated optimal supply (g digested AA/Mcal ME; Higgs, 2014)

| g AA/Mcal ME | G[1] | Target |
|---|---|---|
| Arg | 1.81 | 2.04 |
| His | 0.64 | 0.91 |
| Ile | 2.03 | 2.16 |
| Leu | 2.87 | 3.42 |
| Lys | 2.92 | 3.03 |
| Met | 1.14 | 1.14 |
| Phe | 1.85 | 2.15 |
| Thr | 1.89 | 2.14 |
| Trp | 0.80 | 0.59 |
| Val | 2.02 | 2.48 |

[1]G = perennial ryegrass-only diet

The present research demonstrated that protozoa have the capability to grow and leave the rumen at much higher rates than previously assumed. This was demonstrated through the simultaneous direct measurement of protozoa rumen pool size and omasal flow. Omasal sampling was performed at 12 time points across the 24-h cycle of the day. However, due to logistical restrictions, rumen evacuations were performed two hours before and two hours after the main meal to estimate rumen pool size. As rumen protozoa, especially isotrichids, have been reported to follow a diurnal cycle (Potter and Dehority, 1973; Dehority, 2003) future research should investigate the effect of rumen evacuation frequency on the measurement of protozoal pool size. Finally, due to the varying preferences for substrate, chemotaxis abilities (Diaz et al., 2014), and involvement in bacterial degradation (Belanche et al., 2012) among the two main ciliated protozoa sub-groups found in the rumen (entodiniomorphs and isotrichids), future work should be directed towards understanding their respective densities and effects on host metabolism.

## 7.3. REFERENCES


Beever, D. E. and R. C. Siddons. 1986. Digestion and metabolism in the grazing ruminant. Pages 479–497 in Control of Digestion and Metabolism in Ruminants. L. P. Milligan, W. L. Grovum, and A. Dobson, ed. Prentice Hall, Englewood Cliffs, NJ.

Belanche, A., G. de la Fuente, J. M. Moorby, and C. J. Newbold. 2012. Bacterial protein degradation by different rumen protozoal groups. J. Anim. Sci. 90:4495–4504.

Cotanch, K. W., R. J. Grant, M. E. Van Amburgh, A. Zontini, M. Fustini, A. Palmonari, and A. Formigoni. 2014. Applications of uNDF in ration modeling and formulation. Pages 114-131 in Proc. Cornell Nutrition Conference, Syracuse, NY. Cornell Univ., Ithaca, NY.

Dehority, B. A. 2003. Rumen microbiology, Nottingham: Nottingham University Press.

Diaz, H. L., K. N. Barr, K. R. Godden, J. E. Plank, I. Zapata, A. N. Schappacher, M. P. Wick, and J. L. Firkins. 2014. Eukaryotic inhibitors or activators elicit responses to chemosensory compounds by ruminal isotrichid and entodiniomorphid protozoa. J. Dairy Sci. 97:2254–2269.

Higgs, R. 2014. Development of a dynamic rumen and gastro-intestinal model in the Cornell Net Carbohydrate and Protein System to predict the nutrient supply and requirements of dairy cattle. Ph.D. Dissertation, Cornell Univ., Ithaca, NY.

Keim, J. P. and R. Anrique. 2011. Nutritional strategies to improve nitrogen use efficiency by grazing dairy cows. Chilean J. Agri. Res. 71:623–633.

Khalili, H., and P. Huhtanen. 1991. Sucrose supplements in cattle given grass-silage based diet. 2. Digestion of cell wall carbohydrates. Anim. Feed Sci. Technol. 33:263–273.

Lee, C., A. N. Hristov, K. S. Heyler, T. W. Cassidy, H. Lapierre, G. A. Varga, and C. Parys. 2012. Effects of metabolizable protein supply and amino acid supplementation on nitrogen utilization, milk production, and ammonia emissions from manure in dairy cows. J. Dairy Sci. 95:5253-5268.

Mould, F. L., E. R. Ørskov, and S. O. Mann. 1983. Associative effects of mixed feeds. I. Effects of type and level of supplementation and the influence of the rumen fluid pH on cellulolysis in vivo and dry matter digestion of various roughages. Anim. Feed Sci. Technol. 10:15–30.

NorFor. 2011. The Nordic feed evaluation system. Wageningen Academic Publishers, The Netherlands.

Oba, M. and M. Allen. 2003. Effects of corn grain conservation method on ruminal digestion kinetics for lactating dairy cows at two dietary starch concentrations. J. Dairy Sci. 86:184-194.

Potter, E. L., and B. A. Dehority. 1973. Effects of changes in feed level, starvation, and level of feed after starvation upon the concentration of rumen protozoa in the ovine. Appl. Microbiol. 26:692–698.

Raffrenato, E., D. Ross, and M. E. Van Amburgh. 2018. Development of an in vitro method to determine rumen undigested aNDFom for use in feed evaluation. J. Dairy Sci. 101:9888-9900.

Raffrenato, E., C. Nicholson, and M. E. Van Amburgh. 2019. Development of a mathematical model to predict pool sizes and rates of digestion of 2 pools of digestible neutral detergent fiber and an undigested neutral detergent fiber fraction within various forages. J. Dairy Sci. 102:351-364.

Van Soest, P. J. 1994. Nutritional ecology of the ruminant. 2nd Ed. Cornell Univ. Press, Ithaca, NY.

Zontini, A., A. Foskolos, D. A. Ross, J. Metcalf, P. H. Doane, and M. E. Van Amburgh. 2015. Research Update: Formulating diets for lactating cattle using multiple pools of NDF digestibility. Pages 189–196 in Proc. Cornell Nutrition Conference, Syracuse, NY. Cornell Univ., Ithaca, NY.