Evaluation and development of the Cornell Net Carbohydrate and Protein System v.7 using a unique pasture-based data set

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Introduction

The Cornell Net Carbohydrate and Protein System (CNCPS) has been regularly updated since its first publication in 1992 (Fox et al., 2004, Tylutki et al., 2008, Van Amburgh et al., 2015) and is now widely used for diet formulation in the U.S. with growing usage across the world. Nutritional modelling allows the user to quantify the requirements of an animal and formulate diets, using the available resources, to meet the animal's demands. In the latest CNCPS v.7 (Higgs and Van Amburgh, 2016) key components such as carbohydrate and nitrogen (N) digestion, microbial N (MicN) flow and amino acid (AA) supply have been described in a more dynamic and mechanistic manner. While maintaining the functionality of previous versions, this update provides users with new capabilities and potentially increased precision in diet formulation.

The digestion of protein along the gastrointestinal tract is now calculated on a N basis. By incorporating compartmental analysis, reconciliation across the whole tract can occur, to ensure all N is accounted for throughout each compartment (e.g. rumen, omasum, abomasum, etc.). Within this new structure are mechanistic representations of growth of bacteria and protozoa, including interactions such as protozoal predation of bacteria. Rather than accounting for protozoa statically, by reducing the theoretical maximum growth yield of bacteria from 0.5 to 0.4 g cells per g carbohydrate fermented as in previous versions (Russell et al., 1992), the influence of protozoa on nutrient digestion and microbial flow is now described mechanistically and dynamically. This has the potential to predict a more precise quantification of metabolizable AA supply to the animal, as protozoa have been show to contribute 5-23% of MicN flow (Sylvester et al., 2005, Fessenden et al., 2019). Further, the composition of protozoa is different than bacteria, especially for certain AA such as lysine (Jensen et al., 2006; Fessenden et al, 2019). Finally, protozoa have also been implicated in altering the rumen environment such as ammonia N production and pH regulation (Jouany et al., 1988, Williams and Coleman, 1988, Hristov and Jouany, 2005).

Utilizing literature data sets, evaluations of v.7 indicated a strong ability to predict non-ammonia N (NAN) flow at the omasal canal (Higgs and Van Amburgh, 2016). However, within this NAN flow, biases were present where non-ammonia, non-microbial N (NANMN) flow was over predicted and the MicN flow was under predict compared to the observed literature values. While literature data sets are a powerful tool to evaluate models, in many of these studies protozoal flow was not directly measured due to the

difficulty of protozoal isolation. Thus, the NANMN fraction reported might have included protozoal N and conversely, the MicN pool measured might not be accounting for this protozoal N (Brito and Broderick, 2007). To more fully evaluate these constraints on model development, our laboratory conducted an omasal flow study, incorporating a rapid technique to isolate mixed protozoa in order to directly measure protozoal flow (Fessenden et al., 2019). While the total MicN flow was predicted accurately in the study, the model underestimated protozoal flow by approximately 43%. This evaluation suggested that more studies directly measuring protozoal N flow and its contribution to the total MicN flow are required in order to better describe the contributions of protozoa to total microbial flow and the interaction among protozoa and bacteria. Further, it was important to study this in a feeding management system different from the data sets used to develop the model and to have data outside what is available in the literature and Northeast U.S feeding systems.

The CNCPS was developed with data utilizing corn silage and alfalfa based diets with subsequent model evaluations being performed on similar data sets. In vitro and in vivo analysis suggests that fresh perennial ryegrass (PRG) swards, managed intensively, are rapidly degradable with a large proportion of the aNDFom in the potentially digestible pool (~90%); drastically different to conventional forages used in the U.S. Further, a large proportion of the feed N in this type of pasture is digested in the rumen (Sairanen et al., 2005) contributing poorly to metabolizable protein supply. These feed behaviors, that are distinctly different from typical U.S. diets, have the potential to provide a boundary test to challenge the robustness of the underlying biology and feed fractionation schemes of the CNCPS. Therefore, we designed an experiment incorporating pasture-based diets, rapid isolation of mixed protozoa, and the omasal sampling technique to generate a unique data set for model evaluation and development.

Omasal Flow Experiment

In temperate regions, pasture-based diets are an important source of nutrients for the production of animal products and are an appropriate and beneficial use of the resource (Dijkstra et al., 2013). Whilst well-managed pasture is highly digestible, energy intake is typically reported as first limiting milk solids production. There is a large amount of research investigating the effect of providing energy dense supplements to grazing dairy cattle however, wide variation in milk response, dry matter intake (DMI) and substitution effects exist with little explanation of how or why different responses to these supplements occur (Bargo et al., 2003). In this experiment, we utilized rolled barley (RB) as a supplement and evaluated its effects on milk production, rumen metabolism, rumen digestion kinetics and omasal flow of nutrients in lactating dairy cattle fed fresh PRG indoors. We also quantified the rumen pool size and omasal flow of bacteria and protozoa. As RB is a source of rapidly degrading starch, we hypothesized that it would stimulate protozoal growth which would provide treatment effects for model parameter evaluation (Chamberlain et al., 1985, Jaakkola and Huhtanen, 1993, Ahvenjärvi et al., 2002).

This study was undertaken at Teagasc, Animal and Grassland Research and Innovation Centre, Moorepark, Fermoy Co. Cork, Ireland. Ten ruminally cannulated Holstein cows averaging 49±23 DIM and 513 ± 36 kg of BW were assigned to one of two treatments in a switchback design. Treatments were (on a DM basis) 100% PRG (**G**) or 80% PRG and 20% RB grain (**G+RB**). Swards of PRG were mechanically cut twice daily using a "zero grazer" process where the grass is cut at 4 cm above ground level with no additional processing so the forage is provided as is and can be 20 to 30 cm long. The forage was fed across 6 meals daily indoors with RB grain being fed at milking as 2 equal meals. Refusals of both PRG and RB were collected and weights recorded with feeding rate being adjusted daily to yield refusals of 5% to 10% of daily intake. Daily samples of PRG and RB were dried at 105 °C and analyzed for DM. Additional daily samples were either freeze dried or oven dried at 60 °C before being ground and analyzed for chemical composition using wet chemical methods (Table 1). The trial consisted of three 29 d periods where each period consisted of 21 d of diet adaptation/wash-out and 8 d of marker infusion and animal sampling. During this latter phase a double marker system utilizing CoEDTA (Udén et al., 1980) and undegraded NDFom (uNDFom; Raffrenato et al., 2018) was used to quantify liquid and particle flow at the omasal canal, respectively. Ytterbium was used as part of a triple-marker system, but was abandon upon analysis and will not be discussed. Ytterbium recovery in the feces was low indicating the Yb didn't bind at a high rate or concentration. This further indicates that due to the rate and extent of fiber digestion in the rumen and rate of passage out of the rumen of both the fiber and marker, there was low affinity and binding, which led to a failure of the marker. Additionally, double-labelled ammonia sulfite ($^{15}N^{15}N$-ammonia sulfite, 10% enriched $^{15}NH_4SO_4$) was continuously added to the rumen in order to quantify microbial flow and pool size.

Samples of whole omasal contents were collected from the omasal canal using the sampling technique developed by Huhtanen et al. (1997) and adapted by Reynal and Broderick (2005). The pattern of sampling was in three 8 hour intervals: at 16:00, 18:00, 20:00, and 22:00 h on day 24; at 00:00, 02:00, 04:00, and 06:00 h on day 26; and at 08:00, 10:00, 12:00, and 14:00 on day 27. Sample times were chosen to encompass every two hours of the average twenty-four hour cycle. Fecal grab samples were obtained following the same sampling pattern. At the end of each session, bacterial isolations were performed according to Whitehouse et al. (1994) with modifications. In tandem, an additional 250-mL sample was obtained and immediately processed to isolate protozoa using flocculation and filtration techniques, as described in Fessenden et al. (2019). On day 28 and 29 of each period, rumen contents were evacuated, weighed, mixed, and a representative sample was obtained and stored at −20°C. Rumen contents were returned to the cow via the rumen cannula within 30 min of evacuation. All data were analyzed with a mixed-effects model, using fixed effects of sequence, period, treatment, interaction of period and treatment and the random effect of cow within sequence. For the purposes of this paper, the fixed effect of treatment will be discussed.

Results and Discussion

Diet nutrient composition

Crude protein content of the harvested PRG was slightly lower than anticipated, averaging 16.3% across the three experimental periods (Table 1). Typically, mid-season pastures are approximately 18% crude protein but this can be extremely variable depending on factors such as climatic conditions and N fertilizer application (Peyraud and Astigarraga, 1998). A 12-hour in vitro fermentation time point was included in the analysis of the PRG aNDFom digestibility, along with the 30, 120 and 240 h time points as described by Raffrenato et al. (2019). Given the rapid digestion of the PRG, the 30 h measurement misses a significant portion of the rapidly digestible aNDFom, therefore, to analyze this grass, we needed to include a 12-hour time point to better describe the degradation curve of intensively managed PRG swards (Dineen et al., unpublished). Output from the rate calculations of Raffrenato et al. (2019) partitioned 80%, 20% and 9% of the aNDFom into the fast, slow and indigestible pools with rates of 14% h^{-1}, 3% h^{-1} and 0% h^{-1}, respectively. Crude protein content, water-soluble carbohydrate (WSC) and aNDFom content were all numerically lower in G+RB diets compared to G diets (Table 1). Starch content, as was intended in diet formulation, was greater with supplementation. This resulted in an increase of non-fiber carbohydrate (NFC) for the G+RB diets however, the high WSC content of PRG prevented a drastic difference in the NFC content between diets. The content of indigestible aNDFom in the RB supplement was increased compared to PRG (33.0 vs. 9.9% uNDFom, % of aNDFom) which seems to be due to the hull material being included in the barley supplement.

Animal performance and rumen characteristics

During the milk sampling phase (day 21-23; Table 2), total DMI tended to increase in G+RB diets compared with G ($P = 0.11$). This was achieved through consumption of the RB offered in substitution for 0.88 kg of pasture DM per kg of RB DM consumed. This is similar to the results observed by Delagarde and Peyraud (1995) who fed comparable diets. The inclusion of RB had no effect on daily milk yield, ECM or milk solids (kg fat + protein). However, this study was not specifically designed to assess effects on milk production. Milk fat content decreased in cows fed the G+RB diet, whereas milk protein content increased which are similar to the results observed in the review by Bargo et al. (2003) of studies providing energy dense supplements to pasture-based diets. Milk urea N was lower in G+RB diets compared with G (12.7 vs. 16.5 mg/dL; $P < 0.01$) which might be explained by reduced ruminal ammonia pool sizes and concentration in G+RB cows (Table 3). This might have occurred due to the increased incorporation of feed N into MicN in G+RB cows as indicated by the higher MicN flow, discussed further below (Table 6). Feed efficiency (ECM/DMI) was reduced in G+RB diets compared with G (1.36 vs. 1.45; $P < 0.05$) and this seems surprising given the added fermentable carbohydrate. Concentrations of total VFA, propionate, valerate and isovalerate all increased due to RB supplementation (Table 3). Reticulum pH, measured using eCow® boluses (Devon, U.K) were not different among treatments, averaging 6.36 and 6.37 for diets G and G+RB,

respectively. These means were slightly higher than the mean reported by Kolver and deVeth (2002) of 6.15 for a number of pasture-based treatments.

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

	Diet[2]		
Nutrient composition[4]	G	G+RB	RB[3]
DM, %	21.0 ± 3.0	34.7 ± 3.6	86.9 ± 0.8
CP, % of DM	16.3 ± 3.1	15.4 ± 2.7	11.6 ± 0.4
Soluble protein, % of CP	35.3 ± 3.0	31.5 ± 2.9	17.1 ± 1.9
Starch, % of DM	2.2 ± 0.5	14.4 ± 1.5	60.7 ± 0.7
Sugars (water soluble), % of DM	23.9 ± 1.6	19.3 ± 1.1	1.9 ± 0.2
NFC, % of DM	37.7 ± 3.8	43.5 ± 3.0	65.6 ± 2.7
aNDFom, % of DM	36.3 ± 1.2	32.7 ± 1.5	19.2 ± 1.0
12-h uNDFom, % of aNDFom	50.9 ± 8.5	-	71.0 ± 0.3
30-h uNDFom, % of aNDFom	20.9 ± 2.8	-	-
72-h uNDFom, % of aNDFom	-	-	38.5 ± 1.4
120-h uNDFom, % of aNDFom	11.8 ± 0.3	-	33.0 ± 0.6
240-h uNDFom, % of aNDFom	9.9 ± 0.4	-	-
ADF, % of DM	20.7 ± 1.7	17.5 ± 1.9	5.0 ± 0.6
ADL, % of NDF	4.2 ± 0.8	5.8 ± 0.9	11.8 ± 2.7
Ether extract, % of DM	3.1 ± 0.5	2.9 ± 0.4	1.7 ± 0.2
Ash, % of DM	6.6 ± 0.5	5.6 ± 0.4	2.6 ± 0.6

[1]Analyzed values from 12 samples (4 day x 3 period).
[2]G = 100% (DM basis) perennial ryegrass; G+RB = 80% perennial ryegrass and 20% rolled barley grain.
[3]RB = rolled barley grain.
[4]NFC = non fiber carbohydrate; aNDFom = amylase- and sodium sulfite-treated NDF corrected for ash residue; uNDFom = undigested amylase- and sodium sulfite treated NDF corrected for ash residue; ADF = acid detergent fiber; ADL = acid detergent lignin.

Table 2. Effect of rolled barley inclusion on dry matter intake (DMI), milk production, and animal performance of pasture-fed lactating dairy cattle

	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, kg/d	24.6	24.1	0.8	0.70
Milk solids[3], kg/d	1.68	1.65	0.05	0.64
Milk fat, %	4.52	4.28	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[4], mg/dL	16.5	12.7	0.9	<0.01
Feed efficiency[5]	1.45	1.36	0.05	<0.05
BW change, kg/d	7.8	6.6	4.2	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]Milk solids = kg fat + protein
[4]MUN = milk urea nitrogen.
[5]ECM/DMI.

Digestion of DM, OM and aNDFom

As the experimental animals were exposed to increased human contact during the omasal sampling procedure, which might have slightly reduced their DMI, separate intakes are reported for the milk production data versus the omasal sampling data (Tables 2 and 4, respectively). During the sampling phase (day 24-28), the inclusion of RB increased DM and OM intake in comparison to G diets, while flow of DM and OM measured at the omasal canal were also increased ($P < 0.01$). The amount of DM truly degraded in the rumen tended to be greater in G+RB diets ($P = 0.13$). Organic matter truly degraded in the rumen increased ($P < 0.01$) in cows fed the G+RB diet. Compared with G diets, the inclusion of RB reduced the total tract digestibility of DM and OM that was consumed ($P < 0.01$).

Table 3. Effect of rolled barley inclusion on rumen concentration and pool size[1] of ammonia N, VFA and reticulum pH

	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] 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
Reticulum pH	6.36	6.37	0.2	0.78

[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

The intake of aNDFom was reduced in cows fed the G+RB diets; however, aNDFom flow at the omasal canal was increased, relative to cows fed the G diet. Accordingly, aNDFom digestibility decreased, both ruminally and total tract, in cows fed the G+RB diet. In addition, rumen pool size of aNDFom and uNDFom increased ($P < 0.05$; data not presented) due to supplementation of RB. Low rumen pH is typically cited as the cause for reduced aNDFom digestibility. However, in the present study low ruminal pH cannot be linked to the decreased aNDFom digestibility, as the rumen pH was not different among treatments, averaging 6.36. Further, de Veth and Kolver (2001) reported that in vivo digestibility of pasture might not be compromised by low ruminal pH (< 6.0) for dairy cows fed diets of high quality pasture. Reduced aNDFom digestibility can be a

multifaceted issue. The concentration of uNDF was higher in the RB grain compared with PRG, as discussed earlier, due to the grain containing hull material. This might have contributed to the reduction in aNDFom digestibility, as reported in other studies (Van Vuuren et al., 1993, Sairanen et al., 2005). Additionally, in this experiment, barley starch altered rumen metabolism with higher propionate concentrations being observed and this change in the type of carbohydrate digested might have created a potential deficiency. In a review by Hoover (1986), the author suggested that rumen ammonia N concentrations 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. Other authors speculated that the rumen ammonia N concentration required by the particulate associated microbes digesting fiber might be greater than that of the fluid 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 5.0 mg/dL in cows fed the G+RB diet, potentially explaining a further portion of the reduced aNDFom digestibility. This suggests that on a dynamic basis, with the rumen ammonia levels most likely variable throughout the day, at times the NFC bacteria outcompete the fiber bacteria for ammonia, decreasing aNDFom digestion.

Fiber digestion is predicted in v.7 of the CNCPS utilizing 1) the uNDFom240 assay (Raffrenato et al., 2018) to determine aNDFom available for microbial degradation 2) fractionation of this calculated pdNDFom into two digestible pools that degrade concurrently but at differing rates (Raffrenato et al., 2019). In the present study, ruminal aNDFom digestion was predicted well in comparison to observed for both the G diet (4,326 v 4,218 g day^{-1}, respectively) and the G+RB diet (3,965 v 3,540 g day^{-1}, respectively). The means of period 2 and 3 for the G diet can be used to remove the variation caused by low ruminal ammonia N concentration due to low forage N content and the associative effect of RB in Period 1. Accordingly, the difference between predicted and observed for ruminal aNDFom digestion was 1.6% (Period 2) and 1.1% (Period 3) above the measured amount. These results indicate that the in vitro approach used to calculate pools and rates, in combination with model predicted passage rates, accurately describe in vivo observations of ruminal aNDFom digestion in animals fed high quality pasture.

Table 4. Effect of rolled barley inclusion on digestibility of DM, OM, and aNDFom

Item[1]	Treatment[2] G	Treatment[2] G+RB	SEM	*P*-Value
DM				
Intake, kg/d	16.1	17.1	0.4	<0.01
Flow at omasal canal, kg/d	10.5	11.3	0.5	<0.01
Apparently digested in the rumen, kg/d	5.6	5.8	0.2	0.41
Truly digested in the rumen,[3] kg/d	12.1	12.7	0.3	0.13
% of DMI	74.3	74.2	2.1	0.94
Total-tract apparent digestibility, %	82.8	79.7	0.3	<0.01
OM				
Intake, kg/d	15.1	16.1	0.4	<0.01
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	0.27
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				
Intake, kg/d	5.8	5.6	0.2	<0.05
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[4] 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

[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]pdNDFom = potentially digestible aNDFom.

Nitrogen Flow

Nitrogen intake was similar across treatments (Table 5). However, compared with G diets the inclusion of RB increased the flow of NAN at the omasal canal ($P < 0.01$). 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. While both Younge et al. (2004) and O'Mara et al. (1997) reported no difference in NAN flow, values observed in both studies were increased for supplemented diets compared to non-supplemented diets. In the current experiment, the increase in NAN can be attributed to the increased flow of MicN in G+RB diets compared with G ($P < 0.01$). There was no difference in NANMN flow between the treatments however, the contribution of NANMN to the total NAN flow was relatively low (12%) compared to 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. A key difference between studies was that in Younge et al. (2004)

and O'Mara et al. (1997), purine derivatives were utilized to determine MicN which have been shown to have lower precision and accuracy compared with techniques using ^{15}N, used in the current study, while also underestimating MicN flow (Klopfenstein et al., 2001, Firkins and Reynolds, 2005, Reynal et al., 2005, Ipharraguerre et al., 2007). Further, Sairanen et al. (2005) reported that the purine derivative method underestimated MicN flow in the pasture only treatment by 15% and thus, over predicting the NANMN flow by the same amount. This inaccuracy has further implications in regards to the determination of true ruminal digestible N, as an underestimated MicN flow will underestimate true digestibility. In the present study, the average true ruminal digestible N was 88%, was not different between treatments and was comparable to the 85% reported by Sairanen et al. (2005). Of the total MicN flow, protozoal N contributed on average 22% and was not different between treatments. There are few data describing protozoal flow in pasture-fed cows however, this average was within the range of that proposed by Dijkstra et al. (1998; 10.7 – 26.1%) in computer simulations of animals consuming similar DMI. In contrast to our hypothesis, supplementation with RB did not increase protozoal N flow. It is difficult to ascertain the reason for this however; the high WSC content of the fresh temperate PRG might have provided ample sugar to sustain high protozoal growth (Clarke, 1965, Williams and Coleman, 1988). Further, recent studies have clearly demonstrated that mixed protozoa can sequester sugar away from bacteria, giving protozoa a competitive advantage and stabilizing fermentation in the rumen (Denton et al., 2015). As the majority of N in high quality pasture is ruminally digestible (> 80%), this data describes the significant dependence, of animals grazing such swards, for MicN as their main source of metabolizabe AA. Thus, it is essential to maintain optimum rumen environments with ample supply of fermentable material to achieve desired animal performance from high forage diets.

Microbial dynamics

In the CNCPS, microbial growth is described based on the amount and type of carbohydrate fermented, as this is the main source of energy for microorganisms (Russell et al., 1992). Models designed to calculate microbial yield based on organic matter digestion, ignores the fact that most ruminal bacteria are unable to utilize protein, fat or lipid as an energy source for growth (Nocek and Russell, 1988). Compared with G diets, the inclusion of RB increased both the pool of rumen fermentable carbohydrates ($P < 0.01$) and the true ruminal carbohydrate digestion rates ($P < 0.01$; Table 6). This is consistent with the observed increase in MicN flow for G+RB diets (Table 5). Rumen microbial OM pool was not different among treatments, and averaged 24% of the rumen OM pool which is similar to results previously reported (Craig et al., 1987, Fessenden et al., 2019). Rumen protozoal N pool similarly was not affected by treatment, however; protozoa contributed considerably less to the total MicN pool in the rumen (6%) in comparison to at the omasal canal (22%). Sylvester et al. (2005) reported similar protozoal proportions in the rumen (9%) using a real-time polymerase chain reaction assay.

Table 5. Effect of rolled barley inclusion on the flow of nitrogen in pasture-fed lactating dairy cattle

Item[1]	Treatment[2]		SEM	*P*-Value
	GO	G+RB		
N intake, g/d	429	424	11	0.53
Flow at omasal canal				
Total N, g/d	394	438	18	<0.01
Ammonia N, g/d	21	14	1	<0.01
NAN				
g/d	373	422	18	<0.01
% of N intake	90.8	99.3	2.8	<0.05
NANMN				
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	0.23
Protozoa NAN				
g/d	79	73	11	0.55
% of microbial NAN flow	23.5	20.0	3.2	0.23

[1]N = nitrogen; NAN = non-ammonia nitrogen; NANMN = non-ammonia, non-microbial nitrogen.
[2]G = 100% (DM basis) perennial ryegrass; G+RB = 80% perennial ryegrass and 20% rolled barley grain.

Fractional growth rate of bacteria tended to increase in cows feed the G+RB diet, with a number of studies reporting similar effects (P = 0.07; Nocek and Russell, 1988). While fractional growth rate of protozoa was similar between diets, the average observed (0.35 h^{-1}) is extremely high in comparison to current assumptions of the theoretical maximal fractional growth rate. Unfortunately, data in this area are lacking also (Firkins and Yu, 2006), as many in vivo studies investigating specific microbial outflows do not measure the rumen pool size and hence, cannot directly determine fractional growth rate. This limits our ability to compare the current observed result to previous literature. However, as noted by Wells and Russell (1996), the observed growth rate of rumen microbes does not address turnover. The true growth rate can be calculated as; observed growth rate/(1-turnover). Microbial turnover constants as high as 90% have been reported (Firkins et al., 1992) with ruminal dilution rate cited as a key factor influencing this variable (Wells and Russell, 1996). In the present study, the fluid passage rate averaged 0.21 h^{-1}, and because protozoa have predominantly been shown to associate with the liquid phase (Hungate, 1966, Dehority, 1998), this provides a mechanism to explain protozoal growth efficiency. Sylvester et al. (2009) demonstrated that rumen ciliated protozoa can decrease generation time in response to increasing dilution rate; Harrison et al. (1976) reported a similar effect. Further, it should be recognized that 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). The reciprocal of dilution rate determines the fluid retention time, which averaged

5 h in this experiment. Thus, for the protozoa to be associated with the fluid, a generation time of less than 5 h is required to maintain viable rumen populations (Dehority, 2003). Protozoal generation time was not affected by treatment and averaged 4 h in the current experiment. To the author's knowledge, a generation time this short in the rumen has only once been previously reported (Warner, 1962). The fresh PRG has a high digestion rate, and in the case of this study, particle size was supplied to the cattle at 20 to 30 cm, and the rumen turnover was also quite high averaging about 0.125 per h. That means the carbohydrate turns over about every 8 hours in these pasture fed cattle, thus even if a portion of the protozoa are "particle" associated, they still need to have a generation interval that is faster than previously characterized other than that reported by Warner (1962). Further studies are required to confirm the protozoal growth rate and efficiency observed in this study.

The observed Yg (yield of microbial DM per gram of carbohydrate degraded) increased in cows fed the G+RB diets compared with G (0.65 vs. 0.54, respectively). Variable Yg values, in vitro, have previously been reported due to differing carbohydrate sources (Nocek, 1988) however, values greater than 0.5, the theoretical maximum (Isaacson et al., 1975) are rare. This maximum calculated by Isaacson et al. (1975) and those measured for individual species (Russell and Baldwin, 1979, Theodorou and France, 2005) are often determined in pure cultures or in vitro environments. Due to the complexity of replicating in vivo conditions, it is possible that microbial yields reported in vitro might be depressed. Stouthamer (1973) reported, using a biochemical approach, a maximal Yg of approximately 0.8 g/g of glucose, indicating the potential for higher yields to be achieved in vivo. Again, these pasture diets are providing readily available and highly digestible carbohydrates that support the concept of faster growth rates as the whole of rumen contents turn over much faster than any traditional North eastern U.S. diet.

CNCPS v.7 predicted versus observed nitrogen flows

To evaluate the capacity of the CNCPS v.7 to predict N flows at the omasal canal, in pasture-fed dairy cows, model predicted estimates were compared against that of measured in the current experiment. The NAN flow predicted was in good agreement with observed (363 vs. 397 g N day^{-1}, respectively), a 9% underestimation. However, the biases reported in both the evaluations of Higgs and Van Amburgh (2016) and that of Van Amburgh et al. (2015; CNCPS v. 6.5) were present in the current evaluation. The MicN flow was under predicted compared to observed (246 vs. 349 g N day^{-1}, respectively) while NANMN flow was considerably over predicted (117 vs. 48 g N day^{-1}, respectively). The under prediction of MicN flow seems to particularly stem from a reduced bacterial N flow. The underestimation of protozoal flow in the current evaluation was less severe than that of Fessenden et al. (2019; 22% vs. 43%), potentially due to the high WSC content of the diet driving protozoal growth. Consequently, this large protozoal population increases the quantity of bacterial N predated by the protozoa, contributing to the reduced MicN flow. This provides further justification to update the growth rate and passage of protozoa, which are currently associated in the particle phase, to be in the liquid phase, within the structure of v.7. Further, the assumptions that protozoa retain only 50% of the N

consumed (Williams and Coleman, 1988) and a growth rate of half the fractional carbohydrate degradation rate seems too drastic especially under the current experimental condition of rapid protozoal generation times. There are a few potential offsets around all of these predictions of protozoal predation of bacteria, feed protein degradation and the high rate of passage of the liquid phase that all interact to provide part of the MP supply. For example, the current rate of degradation of the B1 protein pool is 15%/h for pasture, which might be too slow given the microbial growth rates and the degradation rate of the fast pool of aNDFom. However, accurate in vivo rates of N degradation are very difficult to quantify in vitro, thus further omasal flow measurements might be required.

Table 6. Effect of rolled barley inclusion on rumen pool sizes, fractional rates of microbial growth and nutrient digestion and generation time

Item	Treatment[1]		SEM	*P*-Value
	GO	G+RB		
Rumen pool size				
Digestible OM,[2] kg	4.75	5.22	0.35	0.07
Total fermentable CHO,[3] kg	3.58	4.19	0.30	<0.01
Total NAN, g	276	289	12	0.15
Microbial NAN, g	199	208	9	0.29
Microbial OM proportion of rumen OM pool, %	24.5	23.8	0.7	0.37
Bacteria NAN,[4] g	186	196	10	0.23
Protozoa NAN, g	13	12	3	0.73
Protozoa NAN pool, % total microbial NAN pool	6.6	5.9	1.4	0.69
Rumen kinetics				
Fractional growth rate of bacteria,[5] h^{-1}	0.056	0.064	0.003	0.07
Fractional growth rate of protozoa,[5] h^{-1}	0.412	0.286	0.084	0.22
Fractional growth rate of all microbes, h^{-1}	0.069	0.076	0.003	0.09
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,[6] h^{-1}	0.120	0.115	0.006	0.33
Fractional rate of CHO digestion,[6] h^{-1}	0.133	0.121	0.008	<0.05
Observed Yg,[7] g of cells / g of CHO degraded	0.54	0.64	0.04	<0.01
Generation time of bacteria,[8] h	18.8	16.6	0.9	0.10
Generation time of protozoa,[8] h	3.8	4.1	0.5	0.65
Generation time of microbes,[8] h	15.2	13.9	0.7	0.14
Fluid retention time,[9] 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 content).
[4]Microbial NAN pool – protozoal NAN pool

[5]Bacterial or protozoal daily flow (g/h)/bacterial or protozoal pool size (g)
[6]Organic matter or carbohydrate degraded (g/h)/ organic matter or carbohydrate rumen pool size (g)
[7]Fractional microbial growth rate/fractional rate of CHO digestion.
[8]Reciprocal of fractional growth rate of bacteria, protozoa or all microbes
[9]Reciprocal of fluid dilution rate

Conclusions

The inclusion of RB into pasture-based diets in the current study increased DMI, rumen pool size of fermentable carbohydrate and the rate of carbohydrate degradation. However, G+RB diets decreased total tract digestibility of DM, OM and aNDFom. Additionally, the NAN flow at the omasal canal increased because of increased MicN flow (50 g), in G+RB diets compared to G. The average contribution of MicN to the total flow of NAN together with high ruminal digestibility of feed protein portrays the large dependence of pasture-fed cattle on microbial protein supply. Although animals grazing pasture-based diets are often cited as being energy first limited, the increased performance typically achieved by supplying energy dense supplements might be through the mechanism of a rise in MicN flow and hence increased metabolizable AA supply – provided adequate rumen N is available. Further research is required to disentangle the mechanisms of increased milk solid production when energy dense supplements are fed as the responses are variable suggesting other limitations under certain conditions.

Evaluation of the capacity of CNCPS v7 to predict NDF degradation in vivo, from in vitro analysis and mathematical modeling, indicates the high precision of this approach. Further refinement is required, to capture the interacting effects of NFC and low rumen ammonia N concentrations on in vivo aNDFom digestion in pasture-fed animals. Finally, although NAN flow at the omasal canal was predicted well, potential modifications have been described to reduce the biases in MicN and NANMN flow predictions.

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