Unveiling the relationships between diet composition and fermentation parameters response in dual-flow continuous culture system: a meta-analytical approach

Virginia L.N. Brandao and Antonio P. Faciola[1]

Department of Animal Sciences, University of Florida, Gainesville, FL 32611

ABSTRACT: The objective of this study was to investigate the functional form of the relationship between diet composition (dietary crude protein [CP] and neutral detergent fiber [NDF]) and amount of substrate (fermenter dry matter intake [DMI]) with microbial fermentation end products in a dual-flow continuous culture system. A meta-analysis was performed using data from 75 studies. To derive the linear models, the MIXED procedure was used, and for nonlinear models, the NLMIXED procedure was used. Significance levels to fit the model assumed for fixed and random effects were $P \leq 0.05$. Independent variables were dietary NDF, CP, and fermenter DMI, whereas dependent variables were total volatile fatty acids (VFA) concentration; molar proportions of acetate, propionate, and butyrate; true ruminal digestibilities of organic matter (OM), CP, and NDF; ammonia nitrogen (NH_3–N) concentration and flows of NH_3–N; non-ammonia nitrogen; bacterial-N; dietary-N; and efficiency of microbial protein synthesis (EMPS). Ruminal digestibilities of OM, NDF, and CP decreased as fermenter DMI increased ($P < 0.04$). Dietary NDF and CP digestibilities were quadratically associated ($P < 0.01$). Total VFA linearly increased as DMI increased ($P < 0.01$), exponentially decreased as dietary NDF increased ($P < 0.01$), and was quadratically associated with dietary CP ($P < 0.01$), in which total VFA concentration was maximized at 18% dietary CP. Molar proportion of acetate exponentially increased ($P < 0.01$) as dietary NDF increased. Molar proportion of propionate linearly increased and exponentially decreased as DMI and dietary NDF increased, respectively ($P < 0.01$). Bacterial-N quadratically increased and dietary-N exponentially increased as DMI increased ($P < 0.01$). Flows of bacterial-N and dietary-N linearly decreased as dietary NDF increased ($P < 0.02$), and dietary-N flow was maximized at 18% CP. The EMPS linearly increased as dietary CP increased ($P < 0.02$) and was not affected by DMI or dietary NDF ($P > 0.05$). In summary, increasing fermenter DMI increased total VFA concentration and molar proportion of propionate, whereas, dietary NDF increased the molar proportion of acetate. Dietary CP increased bacterial-N flow and was positively associated with NH_3–N concentration. Overall, the analysis of this dataset demonstrates evidences that the dual-flow continuous culture system provides valuable estimates of ruminal digestibility, VFA concentration, and nitrogen metabolism.

Key words: digestibility, in vitro, meta-analysis, microbial fermentation, volatile fatty acid

Transl. Anim. Sci. 2019.3:1064–1075
doi: 10.1093/tas/txz019

[1]Corresponding author: afaciola@ufl.edu
Received October 1, 2018.
Accepted February 18, 2019.

INTRODUCTION

The rumen is the main site of fiber digestion and protein degradation in ruminant animals. It is estimated that up to 97% of total ingested neutral detergent fiber (NDF) is digested in the rumen (Huhtanen et al., 2010), and 50% to 80% of ingested crude protein (CP) is degraded in the rumen (NRC, 2001). Digestion and passage rates are two competitive processes (Mertens, 1977) that are difficult to be studied separately in vivo. Therefore, studies to understand how dry matter intake (DMI), dietary CP, and NDF affect ruminal digestion independently of passage rate are warranted. In the dual-flow continuous culture system (DFCCS), liquid and solid passage rates are controlled, which allows evaluation of ruminal digestion under controlled conditions and independently from possible differences in animal passage rate and DMI. The DFCCS is a long-term fermentation system with tight variable control, which makes it suitable for evaluating digestion processes. It was developed by Hoover et al. (1976) and recently modified to test beef (Benedeti et al., 2015; Amaral et al., 2016; Silva et al., 2017) and dairy diets (Paula et al., 2017; Brandao, Dai et al. 2018; Brandao, Silva et al., 2018).

The end products of carbohydrate and protein fermentation are volatile fatty acids (VFA), microbial cells, CH_4, and CO_2 (NRC, 2016), in which VFA and microbial cells represent the majority of them. Organic acids, notably VFA, largely contribute to metabolizable energy supply for ruminants (Bergman, 1990; Aschenbach et al., 2011), and it is estimated that VFA produced in the rumen may account for up to 75% of the total metabolizable energy from the diet (Siciliano-Jones and Murphy, 1989). The major VFA produced in the rumen are acetate, propionate, and butyrate, which represent up to 95% of the total acid produced by fermentation (Bergman, 1990); moreover, VFA type may affect animal performance and milk and meat composition. In addition to VFA, microbial protein can also be used as gluconeogenic precursor (Lobley, 1992). In beef cattle, microbial protein can provide between 50% and 100% of total required metabolizable protein (NRC, 2016); however, depending on animal energy status and production level, they may rely more on energy provided by amino acids.

Therefore, the objective of this study was to investigate the functional form of the relationship between diet composition (dietary CP and NDF) and amount of substrate (fermenter DMI) with microbial fermentation end products in a DFCCS using a meta-analytical approach. We hypothesized that fermenter DMI, dietary CP, and NDF independently affect microbial fermentation end products. We acknowledge that there are other variables that affect ruminal fermentation, such as dietary fatty acids, non-fiber carbohydrates, and starch; however, these are not as frequently reported in others DFCCS and therefore, we focused this review on fermenter DMI, dietary CP, and NDF as independent variables.

MATERIALS AND METHODS

Data Collection and Preparation

Data used in this article were generated from 75 peer-reviewed published studies, and the dataset was comprised 523 treatment means. Articles were published in Journal of Dairy Science, Journal of Animal Science, and PLoS One from 1985 to 2018. Keywords used to search for relevant papers were "dual-flow," "continuous culture," "dual-flow continuous culture system," "microbial fermentation," and "in vitro". The first step of study selection was to ensure that the study under consideration used a dual DFCCS and not any other in vitro system. Second, the study must have used rumen inoculum from dairy or beef cattle, therefore, studies using sheep or goat inoculum were excluded. All studies included in the database reported the independent variables of interest (dietary CP, NDF, and fermenter DMI), and only studies that met all the earlier-cited criteria were included.

The passage rates had minimal variation among studies, in which average solid passage rate was 5%/h (SD = 0.7), whereas liquid passage rate averaged 10%/h (SD = 1.4). Most of the dual-flow studies used the same artificial saliva described by Weller and Pilgrim (1974). Therefore, artificial saliva and passage rate were not selection criterion in our study. In DFCCS studies, pH is often maintained constant (by infusion of either NaOH or HCl), and it is not used as a response variable. Of the 75 studies, 39% controlled pH, and 25% did not report it. Therefore, pH was not used as a variable, due to the fact that in the majority of the cases it does not reflect a response to treatments, or it was not reported.

Nutrient digestibility (organic matter [OM], NDF, and CP) used in this study was true ruminal digestibility, and dietary NDF and CP are expressed as %DM. When molar proportion of individual VFA and efficiency of microbial synthesis

(EMPS) were not reported, they were calculated as follows:

$$\text{Molar proportion individual VFA} = \frac{\text{concentration of individual VFA}}{\text{Total VFA concentration}}$$

$$\text{Efficiency of microbial synthesis (EMPS)} = \frac{\text{g bacterial nitrogen}}{\text{kg organic matter truly digested}}$$

Model Derivation Procedure

An initial graphical examination of the data was performed to identify the relationships that were studied (Sauvant et al., 2008) and the meta-analysis was performed according to St-Pierre (2001). All statistical analyses were performed using SAS (SAS Institute Inc., 2004). To derive linear models, the MIXED procedure was used, and for nonlinear models, the NLMIXED procedure was used. Significance levels to fit the model assumed for fixed and random effects were $P \leq 0.05$. Independent variables used were dietary NDF, CP, and fermenter DMI. The dependent variables and descriptive statistics are presented in Table 1. The diets used in the data set ranged in CP from 4% to 28.7%, NDF from 15.1% to 74.22% (Table 1), and forage inclusion in the diets ranged from 9% to 100%. The fermenter DMI, dietary NDF, and CP (independent variables) effects on response variables were tested using linear, quadratic (linear models), exponential, and power models (nonlinear models). Random coefficients model was used considering study as a random effect and including the possibility of covariance between the slope and the intercept. The covariance parameter was considered non-zero when $P \leq 0.05$. Seventeen variance–covariance structures were tested and Akaike's information criteria was used to define the best fit. When Cook's distance was greater than 1, the study was removed from the database in each specific analysis. Outliers were removed when studentized residuals were greater than 2 or less than –2. It should be noted that our model derivation was not intended to generate prediction models, instead our objective was to understand and to evaluate the functional form of the relationship between diet composition (dietary CP and NDF) and amount of substrate (fermenter DMI) with microbial fermentation end products in a DFCCS using a meta-analytical approach.

Table 1. Descriptive statistics

Item	*n*	Max	Min	Average	SD
	Independent variables				
Diet CP, % DM	513	28.7	4.0	15.9	4.1
Diet NDF, % DM	504	74.2	15.1	37.1	11.8
DMI, g/d	511	120.0	12.8	72.6	18.4
	Dependent variables				
True digestibility, %					
OM	400	86.4	23.7	53.5	12.6
CP	373	98.3	15.5	61.5	17.5
NDF digestibility, %	404	94.0	15.3	53.5	17.4
VFA, %, otherwise stated[a]					
Total, mM	460	183.1	24.7	90.7	29.2
Acetate	479	82.2	24.7	59.0	11.5
Propionate	479	64.8	8.9	24.6	8.5
Butyrate	480	42.8	1.7	12.5	6.3
NH_3–N, mg/dL	420	28.1	1.2	12.6	6.2
N flow, g/d					
NH_3–N	365	0.8	0.003	0.3	0.2
NAN[b]	405	3.3	0.06	1.6	0.8
Bacterial-N	422	2.4	0.04	0.9	0.5
Dietary-N	367	2.0	0.003	0.7	0.5
EMPS[c]	417	74.4	4.3	27.5	10.5

[a]Volatile fatty acids.

[b]Non-ammonia nitrogen.

[c]Efficiency of microbial protein synthesis, calculated as g of bacterial nitrogen/kg of organic matter truly digested.

Table 2. Equations using fermenter DMI as independent variable

Dependent variables	Equation	AIC	MSE	R^2	*P* value[d]
VFA, %, otherwise stated[a]					
Total VFA, mM	$\hat{Y} = 33.5202 + 0.8296 \times DMI$	3,253	213.0	0.73	0.01
Acetate	$\hat{Y} = 59.1$	2,794	45.3	—	>0.05
Propionate	$\hat{Y} = 17.4964 + 0.09516 \times DMI$	2,674	52.3	0.81	0.01
Butyrate	$\hat{Y} = 11.41$	1,851	12.0	—	>0.05
True digestibility, %					
OM	$\hat{Y} = 86.4729 \times (1 - 0.005413)^{DMI}$	2,516	29.9	0.81	0.01
CP	$\hat{Y} = 112.11 - 0.8022 \times DMI + 0.001905 \times DMI^2$	2,679	210.0	0.63	0.04
NDF digestibility	$\hat{Y} = 90.0419 - 0.4956 \times DMI$	2,516	111.0	0.71	0.04
NH_3–N, mg/dL	$\hat{Y} = 28.0475 - 0.3098 \times DMI + 0.001337 \times DMI^2$	–115	13.0	0.08	0.02
N flow, g/d					
NH_3–N	$\hat{Y} = 0.3224$	–335	0.03	—	>0.05
NAN[b]	$\hat{Y} = 1.0213 - 0.02418 \times DMI + 0.000437 \times DMI^2$	16.5	0.06	0.88	0.01
Bacterial-N	$\hat{Y} = 0.3224 - 0.00857 \times DMI + 0.000228 \times DMI^2$	–1.03	0.05	0.84	0.01
Dietary-N	$\hat{Y} = 0.08241 \times \exp^{(0.02869 \times DMI)}$	–86.1	0.03	0.85	0.01
EMPS[c]	$\hat{Y} = 30.1485$	2,475	46.2	—	>0.05

AIC = Akaike information criteria; MSE = mean square error.

[a]Volatile fatty acids.

[b]Non-ammonia nitrogen.

[c]Efficiency of microbial protein synthesis, calculated as g of bacterial nitrogen/kg of organic matter truly digested.

[d]*P* value of the model.

EFFECTS ON TRUE RUMINAL DIGESTIBILITY

True organic matter digestibility (TOMD) decreased as DMI increased (Table 2), and a similar response to DMI was also observed for digestibilities of NDF and CP, suggesting that the decline in true NDF digestibility (NDFD) and true CP digestibility (TCPD) is associated with the decline in TOMD. Our results are in agreement with a meta-analysis that evaluated the effects of feeding level and diet composition on digestibility performed by Huhtanen et al. (2009). Similar to our results, these authors reported a negative association between DMI, OM digestibility, and NDFD in lactating dairy cows. Our results indicate that TOMD, NDFD, and TCPD measured in DFCCS may be comparable to in vivo response. As DMI increases, it is expected that the total amount of OM truly digested will increase; however, TOMD (expressed as percentage) is decreased. In a DFCCS, the passage rate is not affected by DMI or type of feed, which are the factors typically associated with this response in vivo (Huhtanen et al, 2006). However, increasing DMI in a DFCCS resulted in decreased TOMD, possibly because at greater DMI, the relationship between feed and fermenter volume decreases. This context of reduced feed to fermenter volume possibly limits the ability of the microbial population to colonize and ferment feed. Furthermore, in our dataset, TOMD was not affected by increasing dietary NDF (Table 3) and CP (Table 4).

Digestibility of NDF linearly decreased as DMI increased (Table 2), possibly due to similar effects observed on TOMD. This result is in agreement with Huhtanen et al. (2009), which also reported negative association between DMI and NDFD. Similar to the response observed for TOMD, NDFD was not affected by dietary CP (Table 4). The majority of fiber degradation occurs in the rumen (Van Soest, 1994), and according to Huhtanen et al. (2010), the rumen contribution to total tract NDFD can be up to 97%. This result suggests that the hindgut contribution to NDF digestion is marginal and that dietary treatment differences observed in the ruminal NDFD closely represent total tract NDFD.

The NDFD was quadratically associated with dietary NDF (Table 3) and NDFD was maximized at 58% of dietary NDF. Diets containing more than 58% NDF most likely have greater proportion of poorly digestible nutrients, which could result in lower NDFD as observed in our data. The NRC (2001) recommends a minimum of 25% NDF, and Zebeli et al. (2012) suggested that a high producing dairy cow diet should contain between 14% and 18% of physical effective NDF to avoid issues with low ruminal pH (subacute acidosis) without compromising DMI. Therefore, even though diets containing 58% NDF may not be adequate for

Table 3. Equations using dietary NDF as independent variable

Dependent variables	Equation	AIC	MSE	R^2	*P* value[d]
VFA, %, otherwise stated[a]					
Total VFA, mM	$\hat{Y} = 195 - 154 \times (1 - \exp^{(-0.03 \times NDF)})$	3,863	571	0.86	0.01
Acetate	$\hat{Y} = -40.06 + 102.86 \times (1 - \exp^{(-0.1151 \times NDF)})$	2,669	21.9	0.53	0.01
Propionate	$\hat{Y} = 189 - 167 \times (1 - \exp^{(-0.1368 \times NDF)})$	2,841	25.5	0.64	0.01
Butyrate	$\hat{Y} = 13.3716 - 0.05787 \times NDF$	2,168	6.00	0.58	0.03
True digestibility, %					
OM	$\hat{Y} = 58.24$	2,627	28.0	—	>0.05
CP	$\hat{Y} = 20.7049 + 1.9055 \times NDF - 0.01995 \times NDF^2$	2,976	127.0	0.04	0.01
NDF digestibility	$\hat{Y} = 28.4240 + 0.9311 \times NDF - 0.00781 \times NDF^2$	3,076	64.0	0.11	0.04
NH_3–N, mg/dL	$\hat{Y} = 3.3167 + 0.2849 \times NDF$	2,534	12.0	0.18	0.04
N flow, g/d					
NH_3–N	$\hat{Y} = 0.06133 + 0.007539 \times NDF$	–348	0.01	0.14	0.01
NAN[b]	$\hat{Y} = 2.2818 - 0.01503 \times NDF$	143	0.04	0.91	0.01
Bacterial-N	$\hat{Y} = 1.3215 - 0.00973 \times NDF$	–133	0.02	0.81	0.02
Dietary	$\hat{Y} = 1.0040 - 0.00625 \times NDF$	–31	0.03	0.95	0.01
EMPS[c]	$\hat{Y} = 29.69$	2,703	25.0	—	>0.05

AIC = Akaike information criteria; MSE = mean square error.

[a]Volatile fatty acids.

[b]Non-ammonia nitrogen.

[c]Efficiency of microbial protein synthesis, calculated as g of bacterial nitrogen/ kg of organic matter truly digested.

[d]*P*-value of the model.

Table 4. Equations using dietary CP as independent variable

Dependent variables	Equation	AIC	MSE	R^2	*P*-value[d]
VFA, %, otherwise stated[a]					
Total VFA, m*M*	$\hat{Y} = 29.2671 + 7.9317 \times CP - 0.2264 \times CP^2$	3,485	135.1	0.13	0.01
Acetate	$\hat{Y} = 54.56$	2,882	28.4	—	>0.05
Propionate	$\hat{Y} = 25.723 + 0.3937 \times CP - 0.02629 \times CP^2$	3,014	26.3	0.64	0.04
Butyrate	$\hat{Y} = 13.16$	2,636	11.0	—	>0.05
True digestibility, %					
OM	$\hat{Y} = 55.89$	2,966	29.2	—	>0.05
CP	$\hat{Y} = 57.69$	2,395	44.3	—	>0.05
NDF digestibility	$\hat{Y} = 45.45$	2,767	62.0	—	>0.05
NH_3–N, mg/dL	$\hat{Y} = 4.2717 \times \exp^{(0.0647 \times CP)}$	1,045	11.0	0.16	0.01
N flow, g/d					
NH_3–N	$\hat{Y} = 0.09499 \times \exp^{(0.07532 \times CP)}$	185	0.03	0.16	0.01
NAN[b]	$\hat{Y} = 1.0905 + 0.03793 \times CP$	172	0.04	0.79	0.04
Bacterial-N	$\hat{Y} = 0.9246 + 0.002654 \times CP$	–18	0.03	0.83	0.02
Dietary	$\hat{Y} = 0.7082$	24.7	0.13	—	>0.05
EMPS[c]	$\hat{Y} = 21.9473 + 0.3668 \times CP$	2,724	25.0	018	0.02

AIC = Akaike information criteria; MSE = mean square error.

[a]Volatile fatty acids.

[b]Non-ammonia nitrogen.

[c]Efficiency of microbial protein synthesis, calculated as g of bacterial nitrogen/ kg of organic matter truly digested.

[d]*P*-value of the model.

high-producing animals. Even though the *P* value for a quadratic model was significative, the R^2 of this model was considerably low, possibly due to its small slope.

TCPD quadratically decreased as DMI increased (Table 2); however, the minimum point of this curve was out of our data range (210 g/d) because our DMI data ranged from 12.8 to 120 g/d (Table 1). This result indicates that further investigation of this relationship using a wider range of DMI is warranted. Dietary NDF and TCPD were quadratically associated (Table 3), and TCPD was maximized at 48% dietary NDF. When dietary NDF was greater than 48%, TCPD was depressed,

possibly due to low energy availability for microbial growth. A similar trend was also observed on NDFD; when dietary NDF was greater than 58%, there was a decline on NDFD. Therefore, diets containing elevated NDF can compromise fiber and protein digestion.

EFFECTS ON VFA

As fermenter DMI increased, total VFA concentration linearly increased (Table 2), and as dietary NDF increased, total VFA concentration exponentially decreased (Table 3). Dietary CP was quadratically associated with total VFA concentration, which was maximized at 18% dietary CP. Diets containing more than 18% CP may result in greater ruminal NH_3–N accumulation, which can compromise fermentation and result in a decrease in total VFA concentration. As DMI increased, we observed a linear increase in total VFA concentration, and this response was associated with a greater OM intake that can be potentially digested in the rumen. The TOMD in this study was expressed as percentage; therefore, even though the percentage of TOMD decreased as DMI increased, the total amount of OM digested increased and this can be confirmed by the increased total VFA concentration observed in this study. Dietary composition and DMI affect VFA concentration and the molar proportion of individual VFA. In a meta-analysis conducted by Loncke et al. (2009), it was reported that total VFA concentration was significantly related to digestible OM and ruminal fermented OM intake. In the same study, authors estimated that an increase of 1 g/d/kg of BW^{-1} of ruminal fermented OM represented an increase in VFA net portal appearance of 5.93 mmol/d × kg of BW^{-1}, demonstrating that changes in ruminal fermentation directly affect energy supply to the animal.

Although in a DFCCS passage rate is controlled, the response of total VFA concentration to dietary NDF content was similar to what is typically observed in vivo: as dietary NDF increases, total VFA concentration decreases (Figure 1A). In

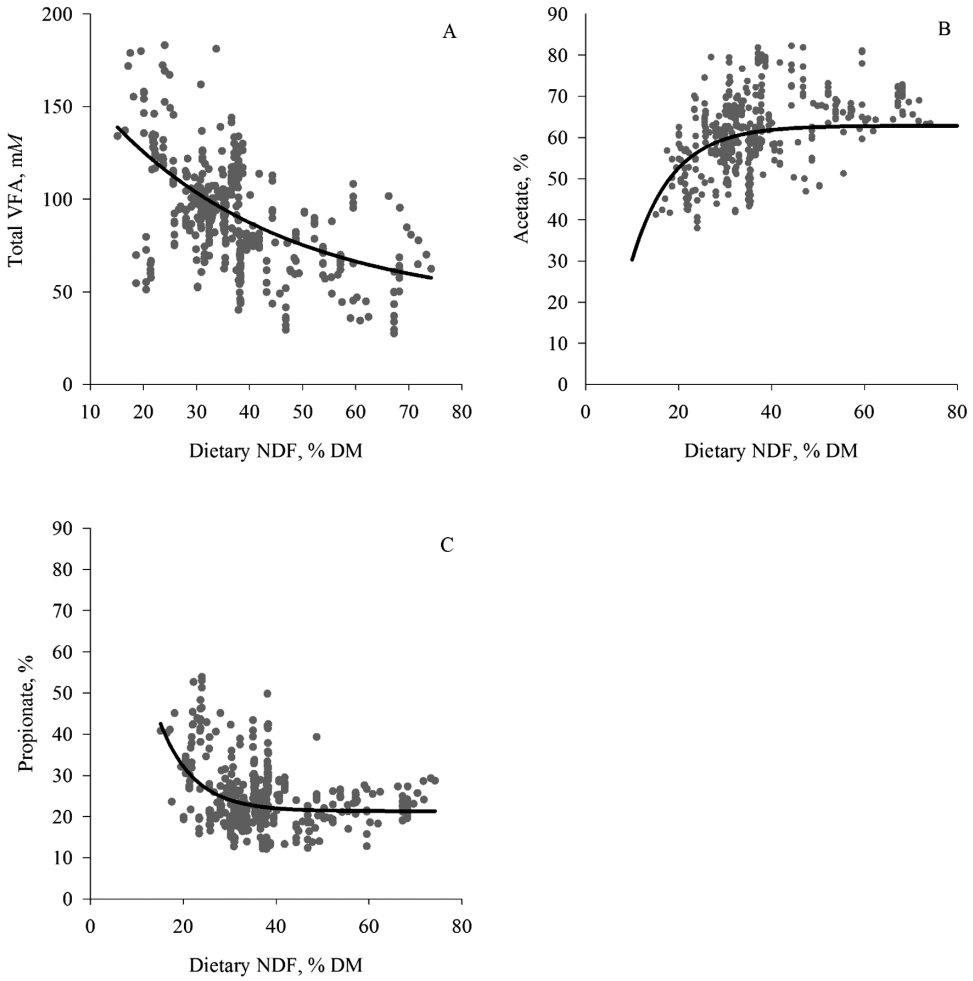

Figure 1. Concentration of total VFA (A; AIC = 3,863; MSE = 571), and molar proportion of acetate (B; AIC = 2,669; MSE = 21.9), and propionate (C; AIC = 2,841; MSE = 25.5) using dietary NDF as independent variable.

vivo, this response is attributed to two main factors: 1) reduction of passage rate due to large quantity of fiber intake, resulting in reduced DMI (Zebeli et al., 2012), and 2) as dietary NDF increases, the concentration of rapidly fermentable carbohydrates decreases, resulting in a reduction in total VFA concentration. However, due to fixed and controlled DMI in DFCCS, this response can be attributed mainly to a reduction in rapidly fermentable carbohydrates due to increasing dietary NDF. Furthermore, there was a quadratic response of total VFA to dietary CP. This result suggests that at low dietary CP, there is a low availability of nitrogen (N) to ensure adequate microbial growth, which compromises fermentation end products. Then, as dietary CP increases, the rumen environmental conditions are improved, favoring microbial growth and resulting in increased total VFA. According to our dataset, at 18% dietary CP, total VFA concentration was maximized.

Although the overall pattern and response of the data reported in DFCCS experiments are similar to those in vivo, the individual values can be slightly different. In a meta-analysis comparing variability of ruminal fermentation data from in vivo and continuous culture studies, Hristov et al. (2012) reported a mean total VFA concentration in vivo of 116.9 mM and from continuous culture system (including a wide variety of in vitro systems) as 93.8 mM, whereas we found a least squared means of 95 mM. The differences between in vivo and data from continuous culture studies can be attributed to two main factors: 1) the way in which VFA are absorbed or removed from the system, and 2) the ratio of fermenter DMI per ruminal fluid volume. In vivo, VFA removal is mainly accomplished through absorption across the rumen wall (Gäbel et al., 2002), and on average, disappearance (presumably absorption) of acetate across rumen wall is 65% (Peters et al., 1992) and 66% for propionate (Peters et al., 1990). Although the amount of VFA that is absorbed across the rumen wall increases as production rate increases, the molar proportion rate typically remain constant (Peters et al., 1990, 1992). The remaining VFA are washed out with the liquid rumen digesta and absorbed in the lower gut. In a DFCCS, the digesta flow is continuous and VFA removal occurs through outflow. The second factor that explains part of the differences in VFA concentration between in vivo and DFCCS studies is the average DMI:ruminal fluid volume ratio. For a dairy cow, considering a DMI of 20 kg and 80 L of rumen volume, this ratio is 250 g/L (Hristov et al., 2012), whereas in a DFCCS we observed a range from 18.3 to 79.6 g/L. In our laboratory, the DMI:ruminal fluid volume ratio of the fermenters is approximately 58 g/L (Benedeti et al., 2015; Silva et al., 2016; Brandao, Dai et al., 2018).

Quantifying in vivo VFA production can be challenging due to the constant absorption through the rumen wall. For accurate quantification of VFA concentration, total rumen volume needs to be measured, which can be assessed indirectly with ruminal liquid markers or directly by using the rumen emptying technique. In a comprehensive study, Hall et al. (2015) investigated the relationships among ruminal VFA concentration, pool size, and amount of ruminal liquid digesta, and pointed out that differences found between VFA concentration and pool size are likely associated with amount of ruminal liquid digesta. In vivo, large differences among animals can be observed for ruminal liquid digesta and ruminal dry matter content, on the other hand, in DFCCS, both liquid digesta and fermenter dry matter content are similar across treatments, which controls variation and potentially eliminates confounding effects of these traits and VFA concentration. Therefore, VFA concentration data need to be cautiously evaluated in in vivo studies when used to explain differences among dietary treatments. On the other hand, due to lack of absorption, data from continuous culture studies are more closely related to VFA production (Calsamiglia et al., 2002).

Molar proportion of acetate was not affected by DMI or dietary CP (Tables 2 and 4, respectively), and the least squared means for molar proportion of acetate was 59.1%. However, as dietary NDF increased, acetate exponentially increased (Figure 1B). This result is in agreement with the NDFD data, demonstrating that the increase in NDFD (up to the maximum point on the curve) resulted in an increase of acetate molar proportion. The molar proportion of acetate increased with greater magnitude when dietary NDF was between 30% and 40% than when dietary NDF concentration was greater than 40% (Figure 1B).

Acetate molar proportion typically increases when high forage diets are fed, which is also associated with greater ruminal pH and NDFD. Therefore, it is difficult in in vivo studies to isolate effects of diet and pH on ruminal fermentation. In an experiment aiming to evaluate the contribution of ruminal pH and different diet compositions on end products of fermentation, while isolating pH effect, Calsamiglia et al. (2008) fed eight fermenters with diets containing 60:40 and 10:90 forage:concentrate and maintained pH at 8 different levels, ranging from 4.9 to

7.0. The authors reported that acetate was affected by varying pH but not by varying forage:concentrate. Whereas propionate concentration was greater on the high concentrate diet and increased as pH decreased, demonstrating that acetate is more responsive to ruminal pH than to dietary NDF. Similarly, Calsamiglia et al. (2002) reported that concentration of acetate was decreased and propionate increased in fermenters kept at low pH, regardless of diet composition. The acidic condition decreased digestibilities of NDF and acid detergent fiber, which is most likely responsible for the decreased acetate. These results demonstrate that the composition of fermentation end products is a result of a combination between substrate type and ruminal pH.

Propionate linearly increased as DMI increased (Table 2), exponentially decreased as dietary NDF increased (Table 3), and quadratically increased as dietary CP increased (Table 4). As expected, molar proportions of acetate and propionate had opposite responses to dietary NDF (Figures 1B and 2C). The same dietary NDF range that resulted in larger increases in acetate (30% to 40% NDF), corresponds to the range of greater decrease in propionate, and after this point, increases in dietary NDF resulted in lower decline in molar proportion of propionate (Figure 1C). Propionate production is mainly associated with fermentation of rapidly fermentable carbohydrates. As NDF concentration in the diet increases, typically dietary NFC decreases, which could explain the response observed in this study. This response of propionate to increased dietary NDF has been widely reported in vivo (Batajoo and Shaver, 1994; Schwab et al., 2006) and in DFCCS studies (Bas et al., 1989; Benedeti et al., 2015; Silva et al., 2017). Therefore, according to the present data, propionate and acetate response to dietary NDF found in DFCCS studies follows a similar pattern as in vivo.

In our dataset, molar proportion of butyrate ranged from 1.7 to 42.8 (Table 1), mean of 12.5 (± 6.5), and it was not affected by DMI (Table 2) or CP (Table 4). The wider range of molar proportion of butyrate observed in DFCCS, when compared to in vivo, may be explained by the lack of absorption by the rumen wall. Rémond et al. (1995) estimated that 18% to 30% acetate, 30% to 70% propionate, and 74% to 90% butyrate produced during ruminal fermentation are used by the rumen wall. The large butyrate use by the rumen wall suggests butyrate values found in vivo may be lower than those in DFCCS studies, due to lack of absorption.

Molar proportion of butyrate linearly decreased as dietary NDF increases (Table 3), potentially due to a reduction in NFC (notably starch) as dietary NDF increased. Butyrate accumulation in the rumen has been reported in animals with greater amounts of ruminal lactate (Nagaraja et al., 1985; Coe et al., 1999), which indicates that diets high in starch and low in NDF can result in increased proportion of butyrate and propionate. Butyrate can be formed from acetate (Nagaraja and Titgemeyer, 2007); however, it has the opposite response, when compared to acetate, with regard to increasing dietary NDF. Therefore, strengthening the hypothesis that as dietary NDF increases, the formation of acetate is preferred over propionate or butyrate (Nagaraja and Titgemeyer, 2007).

EFFECTS ON N METABOLISM

Ammonia N concentration (mg/dL; NH_3–N) quadratically decreased as DMI increased (Table 2), whereas ammonia N flow (NH_3–Nf) was not affected

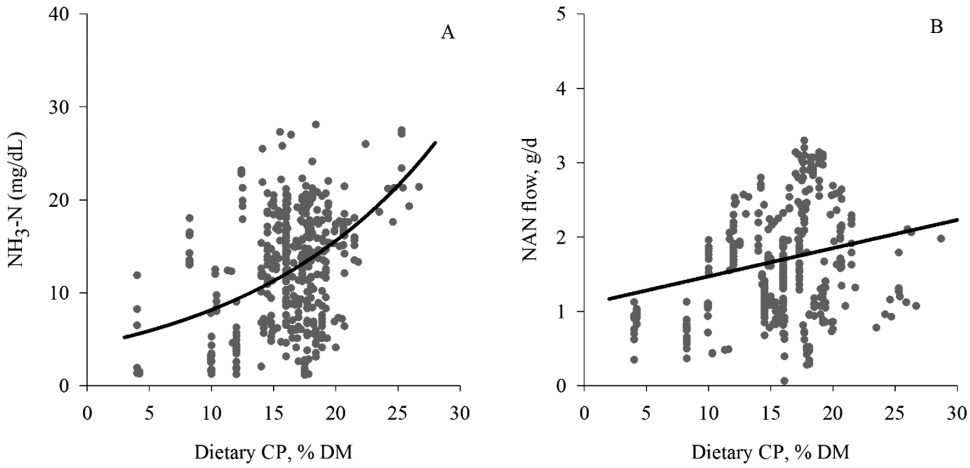

Figure 2. Ammonia nitrogen concentration (mg/dL; A; AIC = 1,045; MSE = 11) and non-ammonia nitrogen flow (NAN g/d; B; AIC = 172; MSE = 0.04) using dietary CP as independent variable.

by DMI (Table 2). The NH_3–N and NH_3–Nf linearly increased as dietary NDF increased (Table 3), and similar results using DFCCS were previously reported by Castillejos et al. (2005). When dietary CP ranged from approximately 15% to 20%, the slope of the regression was greater, indicating that NH_3–N accumulation within this range was accentuated (Figure 2A). Both NH_3–N and NH_3–Nf also increased as dietary CP increased (Table 4). Satter and Roffler (1975) reported a similar response of ruminal NH_3–N to dietary CP; however, they found a quadratic response whereas the model that best fit our data was exponential.

As dietary NDF is increased, the concentration of rapidly fermentable carbohydrates is typically reduced, consequently resulting in less energy available for microbial growth, and greater NH_3–N accumulation. Another potential effect associated with this response is the lack of protein and energy synchronization, which can also result in NH_3–N accumulation. Rumen-degraded protein is broken down into peptides and amino acids by microorganisms and are either deaminated to NH_3 or incorporated into microbial protein (Bach et al., 2005). Microbial protein synthesis is dependent on carbohydrate supply to provide energy for microbial metabolism and if the rate of carbohydrate degradation exceeds microbial assimilation, microbial protein synthesis is compromised. Similarly, when the protein degradation rate exceeds the carbohydrate degradation rate, then N can be lost in the form of NH_3 (Bach et al., 2005). When there is surplus of rumen-degraded protein or lack of energy, NH_3 release rate (from feed) exceeds microbial NH_3 uptake NRC (2001), resulting in NH_3 accumulation in the rumen. In vivo, this can lead to increased N excretion (Broderick et al., 2015).

In a continuous culture study, Satter and Slyter (1974) reported that once NH_3 starts to accumulate inside the fermenters, the growth of NH_3-using bacteria is not enhanced and suggested that 5 mg NH_3–N/dL is sufficient to support adequate microbial growth rates. In the ruminal environment, a minimal NH_3 concentration is required to ensure adequate microbial growth. Data from studies using ^{15}N suggested that at least 50% microbial protein produced in the rumen uses N from NH_3, and the remaining microbial protein is derived from peptides and amino acids (Leng and Nolan, 1984). However, that proportion can vary depending on the availability and sources of N within the rumen. In addition, fibrolytic bacteria preferably uses NH_3–N as N sources, instead of amino acids and peptides (Russell et al., 1992, 2002). Therefore, the findings of Griswold et al. (1996) illustrate the importance of maintaining a minimum concentration of ruminal NH_3 not only for adequate microbial fermentation but also for adequate fiber digestion.

Low-producing animals, and animals fed low CP levels, rely more on N coming from recycled N and on rumen-degraded protein than high-producing animals. However, in a DFCCS, N recycling is simulated through addition of urea in the saliva, thus urea is continuously added in the system independently of the physiological state that is being simulated in the study. It is possible that when feeding diets with greater CP to fermenters, the urea continuously added via saliva will contribute to greater NH_3–N accumulation in the fermenters. This can result in slightly greater NH_3–N concentration when high-protein diets are fed to fermenters compared to values observed in vivo under similar dietary CP. For instance, Broderick et al. (2008) fed lactating cows a control diet containing 16.5% CP and found NH_3–N concentration of 13.9 mg/dL. Although Brandao, Silva et al. (2018) fed continuous culture fermenters diets containing 16% CP and reported NH_3–N ranging from 16.2 to 18.2 mg/dL.

Non-ammonia nitrogen (NAN) flow quadratically increased as DMI increased (Table 2), whereas it linearly decreased as dietary NDF increased (Table 3). It was expected that increasing dietary CP would result in increased NAN flow (Figure 2B), as well as flows of NH_3–N and bacterial-N, due to greater N input into the system. Flow of bacterial-N quadratically increased and dietary-N flow exponentially increased as DMI increased. Flows of bacterial-N and dietary-N linearly decreased as dietary NDF increased (Table 3), whereas as DMI increases, bacterial-N quadratically increased and dietary-N exponentially increased (Table 4). According to our dataset, dietary-N flow was maximized at 18% CP. Low dietary CP limits N available for digestion and microbial growth. In addition, diets containing low CP may limit microbial growth due to the lack of N in the form of amino acids and peptides, which are required for maximum microbial growth (Griswold et al., 1996). These findings are in agreement with our data on bacterial-N flow and VFA response to dietary CP, where at low dietary CP we observed low bacterial-N flow and total VFA concentration, suggesting that at low dietary CP overall fermentation is compromised.

Efficiency of microbial protein synthesis linearly increased as dietary CP increased (Table 4); however, it was not affected by DMI and dietary

NDF (Tables 2 and 3, respectively). Microbial efficiency is a combination of ruminal ATP yield (Stouthamer, 1973) or amount of OM truly digested, and the efficiency in which ruminal microbial population use this energy to convert into bacterial-N (Bach et al., 2005). The TOMD was not affected by dietary CP; however, total VFA, molar proportion of propionate, and bacterial-N flow were positively associated with dietary CP. The fermentation end products can affect bacterial efficiency, as the metabolic routes to produce acetate result in greater energy loss than to produce propionate due to greater gas production (Van Kessel and Russell, 1996). Therefore, we speculate that the positive association of dietary CP with molar proportion of propionate and bacterial-N resulted in increased EMPS. Even though increasing dietary CP increased EMPS, it is not recommended, for dairy animals, to feed diets above 18% CP. At this protein level, total VFA concentration and molar proportion of propionate were maximized; however, after this point, increases in dietary CP resulted in a decline of these parameters. Furthermore, feeding CP in excess to cattle results in ruminal NH_3–N accumulation, ultimately increasing urea excretion in milk and urine (Reynolds and Kristensen, 2008).

SUMMARY AND IMPLICATIONS

Increases in dietary CP resulted in increased microbial efficiency and bacterial-N flow. However, as dietary CP and NDF increased, we observed that NH_3–N also increased. Fermenter DMI was mostly associated with greater substrate availability, resulting in increases on VFA concentration and molar proportion of propionate, whereas dietary NDF increased the molar proportion of acetate, NDFD and CPD. However, it was also positively associated with NH_3–N concentration. Overall, the analysis of this dataset composed of 523 treatment means from 75 peer-reviewed published studies from 1985 to 2018 demonstrates strong evidences that the DFCCS technique provides valuable estimates of ruminal digestibility, VFA concentration and nitrogen metabolism, which were closely related to the expected in vivo response. However, further studies comparing in vivo ruminal fermentation with DFCCS data are warranted. In addition, our data demonstrate that this technique can be a valuable tool for testing a large variety of dietary treatments in a short period of time, with continuous removal of fermentation end products and for longer period of time than other in vitro systems.

ACKNOWLEDGMENTS

We would like to thank Dr. Marcos Marcondes for providing valuable insights and assisting with data analysis and interpretation, and to our laboratory group members for assisting with data interpretation.

Conflict of interest statement. The authors declared that there are no conflicts of interest.

LITERATURE CITED

Amaral, P. M., L. D. Mariz, P. D. Benedeti, L. G. Silva, E. M. Paula, H. F. Monteiro, T. Shenkoru, S. A. Santos, S. R. Poulson, and A. P. Faciola. 2016. Effects of static or oscillating dietary crude protein levels on fermentation dynamics of beef cattle diets using a dual-flow continuous culture system. PLoS One 11:e0169170. doi:10.1371/journal.pone.0169170

Aschenbach, J. R., G. B. Penner, F. Stumpff, and G. Gäbel. 2011. Ruminant nutrition symposium: role of fermentation acid absorption in the regulation of ruminal ph. J. Anim. Sci. 89:1092–1107. doi:10.2527/jas.2010-3301

Bach, A., S. Calsamiglia, and M. D. Stern. 2005. Nitrogen metabolism in the rumen. J. Dairy Sci. 88:E9–E21. doi:10.3168/jds.S0022-0302(05)73133–7

Bas, F. J., M. D. Stern, and N. R. Merchen. 1989. Influence of protein supplementation of alkaline hydrogen peroxide-treated wheat straw on ruminal microbial fermentation. J. Dairy Sci. 72:1217–1227. doi:10.3168/jds.S0022-0302(89)79226-2

Batajoo, K. K., and R. D. Shaver. 1994. Impact of nonfiber carbohydrate on intake, digestion, and milk production by dairy cows. J. Dairy Sci. 77:1580–1588. doi:10.3168/jds.S0022-0302(94)77100-9

Benedeti, P. D. B., L. Galoro da Silva, E. Marostegan de Paula, T. Shenkoru, M. I. Marcondes, H. F. Monteiro, B. Amorati, Y. Yeh, S. R. Poulson, and A. P. Faciola. 2015. Effects of partial replacement of corn with glycerin on ruminal fermentation in a dual-flow continuous culture system. PLoS One 10:e0143201.

Bergman, E. N. 1990. Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. Physiol. Rev. 70:567–590. doi:10.1152/physrev.1990.70.2.567

Brandao, V. L. N., X. Dai, E. M. Paula, L. G. Silva, M. I. Marcondes, T. Shenkoru, S. R. Poulson, and A. P. Faciola. 2018a. Effect of replacing calcium salts of palm oil with camelina seed at 2 dietary ether extract levels on digestion, ruminal fermentation, and nutrient flow in a dual-flow continuous culture system. J. Dairy Sci. 101:5046–5059. doi:10.3168/jds.2017–13558

Brandao, V. L. N., L. G. Silva, E. M. Paula, H. F. Monteiro, X. Dai, A. L. J. Lelis, A. Faccenda, S. R. Poulson, and A. P. Faciola. 2018b. Effects of replacing canola meal with solvent-extracted camelina meal on microbial fermentation in a dual-flow continuous culture system. J. Dairy Sci. 101:9028–9040. doi:10.3168/jds.2018-14826

Broderick, G. A., A. P. Faciola, and L. E. Armentano. 2015. Replacing dietary soybean meal with canola meal improves production and efficiency of lactating dairy cows. J. Dairy Sci. 98:5672–5687. doi:10.3168/jds.2015-9563

Broderick, G. A., N. D. Luchini, S. M. Reynal, G. A. Varga, and V. A. Ishler. 2008. Effect on production of replacing dietary starch with sucrose in lactating dairy cows. J. Dairy Sci. 91:4801–4810. doi:10.3168/jds.2008-1480

Calsamiglia, S., P. W. Cardozo, A. Ferret, and A. Bach. 2008. Changes in rumen microbial fermentation are due to a combined effect of type of diet and ph. J. Anim. Sci. 86:702–711. doi:10.2527/jas.2007-0146

Calsamiglia, S., A. Ferret, and M. Devant. 2002. Effects of ph and ph fluctuations on microbial fermentation and nutrient flow from a dual-flow continuous culture system. J. Dairy Sci. 85:574–579. doi:10.3168/jds.S0022-0302(02)74111-8

Castillejos, L., S. Calsamiglia, A. Ferret, and R. Losa. 2005. Effects of a specific blend of essential oil compounds and the type of diet on rumen microbial fermentation and nutrient flow from a continuous culture system. Anim. Feed Sci. Technol. 119:29–41. doi:10.1016/j.anifeedsci.2004.12.008

Coe, M. L., T. G. Nagaraja, Y. D. Sun, N. Wallace, E. G. Towne, K. E. Kemp, and J. P. Hutcheson. 1999. Effect of virginiamycin on ruminal fermentation in cattle during adaptation to a high concentrate diet and during an induced acidosis. J. Anim. Sci. 77:2259–2268.

Gäbel, G., J. R. Aschenbach, and F. Müller. 2002. Transfer of energy substrates across the ruminal epithelium: implications and limitations. Anim. Health Res. Rev. 3:15–30. doi:10.1079/AHRR200237

Griswold, K. E., W. H. Hoover, T. K. Miller, and W. V. Thayne. 1996. Effect of form of nitrogen on growth of ruminal microbes in continuous culture. J. Anim. Sci. 74:483–491.

Hall, M. B., T. D. Nennich, P. H. Doane, and G. E. Brink. 2015. Total volatile fatty acid concentrations are unreliable estimators of treatment effects on ruminal fermentation in vivo. J. Dairy Sci. 98:3988–3999. doi:10.3168/jds.2014-8854

Hoover, W. H., B. A. Crooker, and C. J. Sniffen. 1976. Effects of differential solid-liquid removal rates on protozoa numbers in continous cultures of rumen contents. J. Anim. Sci. 43:528–534. doi:10.2527/jas1976.432528x

Hristov, A. N., C. Lee, R. Hristova, P. Huhtanen, and J. L. Firkins. 2012. A meta-analysis of variability in continuous-culture ruminal fermentation and digestibility data. J. Dairy Sci. 95:5299–5307. doi:10.3168/jds.2012-5533

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. doi:10.3168/jds.2009-2988

Huhtanen, P., S. Ahvenjärvi, M. R. Weisbjerg, and P. Nørgaard. 2006. No title. In: Nielsen, K. S. T. H. M. O., editor. Wageningen: Wageningen Academic Publishers.

Huhtanen, P., M. Rinne, and J. Nousiainen. 2009. A meta-analysis of feed digestion in dairy cows. 2. The effects of feeding level and diet composition on digestibility. J. Dairy Sci. 92:5031–5042. doi:10.3168/jds.2008-1834

Leng, R. A., and J. V. Nolan. 1984. Nitrogen metabolism in the rumen. J. Dairy Sci. 67:1072–1089. doi:10.3168/jds.S0022-0302(84)81409-5

Lobley, G. E. 1992. Control of the metabolic fate of amino acids in ruminants: a review. J. Anim. Sci. 70:3264–3275.

Loncke, C., I. Ortigues-Marty, J. Vernet, H. Lapierre, D. Sauvant, and P. Nozière. 2009. Empirical prediction of net portal appearance of volatile fatty acids, glucose, and their secondary metabolites (beta-hydroxybutyrate, lactate) from dietary characteristics in ruminants: a meta-analysis approach. J. Anim. Sci. 87:253–268. doi:10.2527/jas.2008-0939

Mertens, D. R. 1977. Dietary fiber components: relationship to the rate and extent of ruminal digestion. Fed. Proc. 36:187–192.

Nagaraja, T. G., T. B. Avery, S. J. Galitzer, and D. L. Harmon. 1985. Effect of ionophore antibiotics on experimentally induced lactic acidosis in cattle. Am. J. Vet. Res. 46:2444–2452.

Nagaraja, T. G., and E. C. Titgemeyer. 2007. Ruminal acidosis in beef cattle: the current microbiological and nutritional outlook. J. Dairy Sci. 90 (Suppl 1):E17–E38. doi:10.3168/jds.2006-478

NRC. 2001. Nutrient requirements of dairy cattle, 7th rev. ed. Washington, DC: The National Academies Press.

NRC. 2016. Nutrient requirements of beef cattle, 8th rev. ed. Washington, DC: The National Academies Press.

Paula, E. M., H. F. Monteiro, L. G. Silva, P. D. B. Benedeti, J. L. P. Daniel, T. Shenkoru, G. A. Broderick, and A. P. Faciola. 2017. Effects of replacing soybean meal with canola meal differing in rumen-undegradable protein content on ruminal fermentation and gas production kinetics using 2 in vitro systems. J. Dairy Sci. 100:5281–5292. doi:10.3168/jds.2016-12301

Peters, J. P., R. Y. Shen, and J. A. Robinson. 1992. Disappearance of acetic acid from the bovine reticulorumen at basal and elevated concentrations of acetic acid. J. Anim. Sci. 70:1509–1517.

Peters, J. P., R. Y. Shen, J. A. Robinson, and S. T. Chester. 1990. Disappearance and passage of propionic acid from the rumen of the beef steer. J. Anim. Sci. 68:3337–3349.

Rémond, D., I. Ortigues, and J. P. Jouany. 1995. Energy substrates for the rumen epithelium. Proc. Nutr. Soc. 54:95–105. doi:10.1079/PNS19950040

Reynolds, C. K., and N. B. Kristensen. 2008. Nitrogen recycling through the gut and the nitrogen economy of ruminants: an asynchronous symbiosis. J. Anim. Sci. 86 (Suppl 14):E293–E305. doi:10.2527/jas.2007-0475

Russell, J. B., J. D. O'Connor, D. G. Fox, P. J. Van Soest, and C. J. Sniffen. 1992. A net carbohydrate and protein system for evaluating cattle diets: I. Ruminal fermentation. J. Anim. Sci. 70:3551–3561.

Russell, J. B. 2002. Rumen microbiology and its role in ruminant nutrition. Ithaca (NY): Dept. of Microbiology, Cornell University.

SAS Institute Inc. 2004. Version 9.1 ed. SAS Institute Inc., Cary, NC.

Satter, L. D., and R. E. Roffler. 1975. Nitrogen requirement and utilization in dairy cattle. J. Dairy Sci. 58:1219–1237. doi:10.3168/jds.S0022-0302(75)84698-4

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

Sauvant, D., P. Schmidely, J. J. Daudin, and N. R. St-Pierre. 2008. Meta-analyses of experimental data in animal nutrition. Animal. 8:1203–1214. doi:10.1017/S1751731108002280

Schwab, E. C., C. G. Schwab, R. D. Shaver, C. L. Girard, D. E. Putnam, and N. L. Whitehouse. 2006. Dietary forage and nonfiber carbohydrate contents influence

B-vitamin intake, duodenal flow, and apparent ruminal synthesis in lactating dairy cows. J. Dairy Sci. 89:174–187. doi:10.3168/jds.S0022-0302(06)72082-3

Siciliano-Jones, J., and M. R. Murphy. 1989. Production of volatile fatty acids in the rumen and cecum-colon of steers as affected by forage:concentrate and forage physical form. J. Dairy Sci. 72:485–492. doi:10.3168/jds.S0022-0302(89)79130-X

Silva, L. G., P. D. B. Benedeti, E. M. Paula, F. Malekjahani, P. M. Amaral, L. D. S. Mariz, T. Shenkoru, and A. P. Faciola. 2017. Effects of carbohydrate and nitrogen supplementation on fermentation of cheatgrass (*Bromus tectorum*) in a dual-flow continuous culture system. J. Anim. Sci. 95:1335–1344.

Silva, L. G., J. Bunkers, E. M. Paula, T. Shenkoru, Y. Yeh, B. Amorati, D. Holcombe, and A. P. Faciola. 2016. Effects of flaxseed and chia seed on ruminal fermentation, nutrient digestibility, and long-chain fatty acid flow in a dual-flow continuous culture system. J. Anim. Sci. 94:1600–1609. doi:10.2527/jas.2015-9750

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. doi:10.1007/BF02578899

St-Pierre, N. R. 2001. Invited review: integrating quantitative findings from multiple studies using mixed model methodology. J. Dairy Sci. 84:741–755. doi:10.3168/jds.S0022-0302(01)74530–4

Van Kessel, J. A. S., and J. B. Russell. 1996. The effect of pH on ruminal methanogenesis. FEMS Microbiol. Ecol. 20:205–210.

Van Soest, P. J. 1994. Nutritional ecology of the ruminant, 2nd ed. Ithaca (NY): Cornell University Press.

Weller, R. A., and A. F. Pilgrim. 1974. Passage of protozoa and volatile fatty acids from the rumen of the sheep and from a continuous in vitro fermentation system. Br. J. Nutr. 32:341–351.

Zebeli, Q., J. R. Aschenbach, M. Tafaj, J. Boguhn, B. N. Ametaj, and W. Drochner. 2012. Invited review: role of physically effective fiber and estimation of dietary fiber adequacy in high-producing dairy cattle. J. Dairy Sci. 95:1041–1056. doi:10.3168/jds.2011–4421