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ENERGY AND PROTEIN EFFICIENCY OF LACTATING DAIRY COWS FED GROUND PEAS, CANOLA MEAL AND RUMEN-PROTECTED AMINO ACIDS

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BY

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DISSERTATION

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LIST OF ABBREVIATIONS

This dissertation was written following the Instructions for authors from Journal of Dairy Science (2016). A list of abbreviations and standard units that does not require definition from the instructions for authors was used hereon per University of New Hampshire thesis manual policy. The instructions from Journal of Dairy Science can be found in: http://www.journalofdairyscience.org/pb/assets/raw/Health%20Advance/journals/jods/2016_SF.pdf

The following are a list of abbreviations not defined in the Journal of Dairy Science instructions for authors and that are defined at the first time they appear in each chapter: $Q$$CH_4$: Methane flux; $Q$$CO_2$: Carbon dioxide flux; $Q$$O_2$: Oxygen consumption; HP: Heat Production; AL: Ad libtum intake; RI: Restricted Intake; DE: Digestible energy; GE: Gross energy; SBM: Soybean meal; GFP: Ground field peas; CAM: Canola meal; DE: Digestible energy; RP Rumen protected; VFA: Volatile fatty acids; EAA: Essential amino acids; TUCAA: total urea cycle amino acids; TSAA: Total sulfur amino acids, TAA: Total amino acids; TNEAA: total non-essential amino acids; U: urea diet, CSAA: corn and soybean meal based diet supplemented with rumen protected amino acids; FP: ground field peas diet; FPAA: ground field peas supplemented with rumen protected amino acids; FPSB: ground field peas and soybean meal diet; FPCM: ground field peas and canola meal diet.

ABSTRACT

ENERGY AND PROTEIN EFFICIENCY OF LACTATING DAIRY COWS FED GROUND PEAS, CANOLA MEAL AND RUMEN-PROTECTED AMINO ACIDS

By

Andre de Barros Duarte Pereira

University of New Hampshire, September 2016

Forages as conserved silage or grass cannot supply enough nutrients and energy as required by lactating dairy cows. As a result, supplementation with grains is needed to provide animals with enough nutrients to be healthy and produce milk being profitable (NRC, 2001). High producing cows need protein supplementation from sources other than forages in order to maximize milk protein production, with emphasis on replenishing requirements for specific amino acids. Excessive protein in the diet or deficiency of an essential amino acid can reduce productivity and increase excretion of N to the environment, causing pollution. Research must be conducted to help dairy farmers make informed decisions about the use of alternative protein supplements as a way to improve farm profitability, optimize protein and energy utilization and increase knowledge about environmental pollution. Therefore, strategies to reduce feed costs through sourcing lower-cost, yet high nutritional value feed ingredients, may optimize milk production enhancing the economic and social sustainability of dairy farming in the Northeast U.S. Therefore, the 2 research areas identified as the main focuses of this dissertation were: 1) development of a proof of concept technique to determine dry matter intake (DMI) for animals on pasture, and 2) improvement of economic and nutrient use efficiencies when feeding ground field peas (GFP), an alternative feedstuff, in order to decrease costs of dairy rations.

In the first step, a proof of concept technique was developed to estimate energy requirements and DMI of lactating Holstein cows in a tie stall. The objective of this technique was to create a methodology to use spot short-term measurements of CH_4 (QCH_4) and CO_2 (QCO_2) integrated with backward dietary energy partition calculations to estimate DMI. Twelve multiparous cows averaging 173 ± 37 days in milk and 4 primiparous cows averaging 179 ± 27 days in milk were blocked by days in milk, parity, and DMI (as a percentage of body weight) and, within each block, randomly assigned to 1 of 2 treatments: ad libitum intake (AL) or restricted intake (RI = 90% DMI) according to a crossover design. Each experimental period lasted 22 d with 14 d for treatments adaptation and 8 d for data and sample collection. Diets contained (DM basis): 40% corn silage, 12% grass-legume haylage, and 48% concentrate. Spot short-term gas measurements were taken in 5-min sampling periods from 15 cows (1 cow refused sampling) using a portable automated open circuit gas quantification system (GreenFeed, C-Lock Inc., Rapid City, SD) with intervals of 12 h between the 2 daily samples. Sampling points were advanced 2 h from a day to the next to yield 16 gas samples/cow over 8 d to account for diurnal variation in QCH_4 and QCO_2. The following equations were used sequentially to estimate DMI: 1) Heat production (HP) (MJ/d) = (4.96 + 16.07 ÷ respiratory quotient) × QCO_2; respiratory quotient = 0.95; 2) Metabolizable energy intake (MJ/d) = (heat production + milk-energy) ± tissue energy balance; 3) Digestible energy (DE) intake (MJ/d) = metabolizable energy + CH_4-energy + urinary-energy; 4) Gross energy (GE) intake (MJ/d) = DE + [(DE ÷ in vitro true dry matter digestibility) – DE]; and 5) DMI (kg/d) = GE intake estimated ÷ diet GE concentration. Data were analyzed using the MIXED procedure of SAS and Fit Model procedure in JMP ($\alpha = 0.05$). Cows significantly differed in measured DMI (23.8 vs. 22.4 kg/d for AL and RI, respectively; $P < 0.01$). Dry matter intake estimated using QCH_4 and QCO_2 coupled with

dietary backward energy partition calculations (equations 1 to 5 above) was highest in cows fed for AL (22.5 vs. 20.2 kg/d). The resulting R^2 were 0.28 between measured DMI and estimated by gaseous measurements and 0.36 between measured and DMI predicted by the NRC (2001). Results showed that spot short-term measurements of $Q CH_4$ and $Q CO_2$ coupled with dietary backward estimations of energy partitions underestimated DMI by 7.8%. However, the approach proposed herein was able to significantly discriminate differences in DMI between cows fed for AL or RI.

The second focus of this dissertation was aimed to decrease feed costs while improving nutrient efficiency in dairy cows. Ground field peas are an adequate source of energy and protein compared to corn meal and soybean meal (SBM) that could be used as an alternative feedstuff in order to decrease feeding costs. Field peas are available for feed in the northern regions of the United States and Canada. Previous studies showed that diets with more than 25% GFP, DM basis) resulted in reduced milk and milk protein yield in dairy cows. Decreased yields may be caused by limited supplies of MP-Lys and MP-Met due to extensive degradation of GFP RDP in the rumen and we hypothesize that cows fed with GFP supplemented with RP Lys and RP Met will maintain performance when compared to a diet with corn meal and soybean meal supplemented with RP Lys and Met. The objective of this study was to compare a source of non-protein N (i.e. urea) vs. a source of soluble true protein (i.e. GFP) and evaluate diets with 25% of GFP supplemented with rumen-protected (RP) Lys (AjiPro-L, Ajinomoto, Japan) and Met (Smartamine-M, Adisseo, France) as a substitute for corn meal and SBM on animal performance and energy balance. Twelve multiparous and 4 primiparous lactating Holstein cows were blocked by days in milk, milk yield and parity, and randomly assigned to 1 of 4 diets in a replicated 4 × 4 Latin square design. Diets were 35.5% corn silage, 15.5% grass-legume haylage,

5.9% roasted soybean, and: (1) 36% corn meal and 1.3% urea (3.59:1 MP-Lys:MP-Met ratio; negative control (U), (2) 29.7% corn meal, 9.8% SBM, and RP-Lys RP-Met (3.07:1 MP-Lys:MP-Met ratio (CSAA), (3) 25% GFP, 12.3% corn meal, and 2.4% SBM (3.88:1 MP-Lys:MP-Met ratio; FP), and (4) 25% GFP, 12.2% corn meal, 2.4% SBM, and RP-Lys RP-Met (3.13:1 MP-Lys:MP-Met ratio; FPAA). Data were analyzed using the MIXED procedure of SAS with orthogonal contrasts for pairwise comparisons between treatments ($\alpha = 0.05$). Dietary treatments had 15.4%, 15.1%, 14.9% and 15.0% CP, respectively for U, CSAA, FP and FPAA. As expected, cows fed U had decreased DMI (23.3 kg vs. 24.6 kg/d, $P < 0.01$), milk protein yield (1.15 kg vs. 1.21 kg/d, $P < 0.001$), total concentration of ruminal VFA (103 m*M* vs. 112 m*M*, $P < 0.001$), HP (129 MJ/d vs. 141 MJ/d, $P < 0.001$), NDF digestibility (30.2% vs. 46.0%, $P < 0.01$), ADF digestibility (37.6% 50.4%, $P < 0.02$), total purines derivatives (343 mmol/d vs 414 mmol/d, $P < 0.01$), and highest excretion of MUN (9.85 mg/dL vs. 9.09 mg/dL, $P < 0.01$) when compared to cows fed FP. Cows fed FP had decreased plasma concentration of Met (19.6 m*M*). Feeding cows CSAA and FPAA mitigated these negative responses. Cows fed FPAA had positive tissue energy balance, higher HP and consequently higher metabolizable energy intake when compared to CSAA diet. In addition, increased milk yield was correlated to a decrease in HP ($R^2 = 0.329$, n = 16 observations). Results showed that feeding FPAA increased HP and milk protein yield to levels compared to cows fed CSAA. Results suggest that feeding diets with 25% GFP and RP-Lys and RP-Met will improve animal performance and energy efficiency.

When cows were fed FPAA, a decrease in plasma His concentration was found compared to CSAA. Cows fed FPAA could, then, be limiting in His, which could have caused a decrease in milk protein production. Results from the literature show that feeding RP-Met can cause a decrease in the plasma concentration of EAA for reasons that still need to be studied. Canola

meal is a good alternative to SBM that has potential to mitigate the effect on AA concentration in plasma. Previous studies reported increased plasma concentrations of most EAA when feeding CAM, mostly due to an increase in DMI, but research feeding GFP and CAM with RP Met have never been performed. The hypothesis of this study was that cows fed GFP with CAM and RP Met would have higher milk protein percentage and yield when compared to cows fed 25% GFP, SBM and RP Met due to an increase in DMI and consequent increase in plasma AA concentration. The objectives of this study were to compare lactating production responses of cows fed diets with (DM basis) 35.0% Corn silage, 14.0% grass-legume silage, 25% GFP, 1.5% citrus pulp, and corn meal, flaked corn and dry distillers grains in variable amounts with 1) SBM (11%) as the major source of supplemental protein, (FPSB diet), 2) CAM (13.5%) as the major source of supplemental protein (FPCM diet). For each experimental diet, RP Met was top dressed to half of the cows (27 g/d) to result in a total of 4 treatments: 1) FPSB diet with no RP Met supplemented, 2) FPSB diet with supplementation of RP Met, 3) FPCM diet with no RP Met supplemented and 4) FPCM diet with supplementation of RP Met. Twelve multiparous and 4 primiparous lactating Holstein cows were blocked by DIM, milk yield and parity, and randomly assigned to 1 of 4 diets in a replicated 4 × 4 Latin square design. Data were analyzed using the MIXED procedure of SAS and pairwise tests for protein source and supplementation or not of RP Met was performed ($\alpha = 0.05$). Cows fed FPCM had higher DMI and milk yield when compared to cows fed FPSB. No effect on DMI and milk yield was observed for supplementation of RP Met. Cows produced milk with higher concentration of protein when supplemented with RP Met, but RP Met had no effect on milk protein yield. On the other hand, cows fed FPCM had higher yield of milk protein when compared to cows fed FPSB. No difference was found for milk fat and lactose concentrations between diets and addition of RP

Met. Milk true N efficiency (Milk true N ÷ N intake) was higher and MUN was lower for cows fed FPCM compared to cows fed FPSB, showing that overall N efficiency of cows fed FPCM was better. Results show that CAM will increase N efficiency and increase milk and milk protein yield when fed to diets with 25% GFP, as a result of higher DMI.

CHAPTER I: REVIEW OF LITERATURE

Introduction

Forages as conserved silage or grass cannot supply enough nutrients and energy as required by lactating dairy cows. As a result, supplementation with grains is needed to provide animals with enough nutrients to be healthy and produce milk being profitable (NRC, 2001). A previous survey performed in 4 Northeastern US states shows that most farms in this region have less than 300 cows and are managed with confined animals, with the other 13% of farms using rotational grazing systems and the remaining 7% managing farms with more than 300 cows in modern confinement systems (Winsten et al., 2010). But recent prices in milk have increased the pressure on small conventional farmers, causing an 83% decrease in the amount of farms in the Northeast region, and a consequent reduction of dairy cows from 2,948,000 heads in 1960 to 1,480,100 heads until 2010 (Winsten et al., 2010). During this period, the average number of milking cows per farm has increased by almost 50% (Blayney, 2002), and animal productivity has more than doubled over this period (Winsten et al., 2010).

Larger herd sizes occur as a result of the increasing pressure caused by lower cash margins per unit of milk sold (MacDonald et al., 2007). In the Northeast US, milk prices are higher when compared to other regions of the country due to greater fluid milk consumption (Winsten et al., 2010), and increased productivity either with more cows or cows that produce more milk per unit of feed can improve the cash margin and relief the industry pressure (MacDonald et al, 2007). Greater farms can also purchase feeds with lower cost due to buying feed in bulk sizes, reducing shipping and handling costs. Purchased feeds, including conserved forage and grains, accounted for an average of 36% of the total cash expenses of organic dairy farms located in New England (Maine and Vermont) in a study conducted from 2004 to 2006 (Dalton et al., 2008). According to Pereira et al. (2013), 73% of organically certified dairy

farmers in the Northeast (88% of organic farmers in New England) grow and harvest their own forage but purchase all grain used in the farm. Northeast producers cited the high costs of production as one of the most challenging aspects of sustaining organically certified dairying in the region (McBride and Greene, 2009) and that ensuring a steady, fair price for milk is one of the greatest challenges in the northeast milk agriculture (Pereira et al., 2013), the same probably being true to non-organically certified dairy farmers.

Research must be conducted to help dairy farmers make informed decisions about the use of alternative protein supplements as a way to improve farm profitability, optimize protein and energy utilization and increase knowledge about environmental pollution. Therefore, strategies to reduce feed costs through sourcing lower-cost, yet high nutritional value feed ingredients, may optimize milk production enhancing the economic and social sustainability of organic dairy farming in the Northeast. For developing mechanisms to reduce feed costs, the following critical needs must be addressed: 1) design protein and energy supplementation strategies to enhance feed conversion efficiency and minimize grain purchase; 2) enhance diet quality and nutrient utilization with the most efficient protein supplements; 3) evaluate strategies to minimize environmental impact while being sustainable.

An alternative feed supplement, ground field peas, (*Pisum sativum*, **GFP**) was identified as a source of carbohydrates with a low to moderate content of crude protein (22%) (Petit et al., 1997) that could decrease feeding costs and improve income over feed cost of conventional or organic dairy farmers (Price per tonne: $720.00 for organic feed peas and $200.00 for conventional feed peas). Ground field peas have a high content of RDP (NRC, 2001) and low percentage of Met in the crude protein (1.17%, Vander Pol et al., 2008) and could be fed supplemented with a good source of protein that can deliver proper amounts of amino acids (**AA**)

to the duodenum such as canola meal (**CAM**). Canola meal is a low cost ($315.00/ton for conventional feed) protein supplement relative to soybean meal (US$ 400.00/ton) that improved plasma concentration of essential AA when fed as a substitute to other protein sources (Martineau et al., 2014).

A general concern about protein supplements rich in rumen degradable protein (**RDP**), such as SBM, GFP, and CAM is excessive release of ammonia in the rumen resulting in inefficient N utilization by the animal, which increases urea-N waste to the environment. The release of ammonia to atmosphere and runoff of N to water bodies as a result of soil ureases are the most pressing environmental concerns in response to excess concentration of N in dairy rations or unbalanced supplies of RDP and fermentable energy (Hristov et al., 2013). Ammonia in the atmosphere can react with sulfuric and nitric acids to form dangerous air pollutants including ammonium sulfate, ammonium bisulfate or ammonium nitrite. Runoff to water bodies can cause eutrophication and consequent decrease in water quality for human consumption (Hristov et al., 2011). In addition, excess dietary N intake unnecessarily increases overall production costs. Increasing the energy density of dairy diets with feeds high in non-fiber carbohydrates, such as GFP, holds strong potential to maximize microbial protein production in the rumen as a result of enhanced supply of fermentable energy via starch. However, feeding GFP can cause an increase in N release in the environment, as a result of higher RDP content.

Feeding GFP to dairy cows reduced milk yield and milk protein concentration in dairy cows. Albrecht (2012) evaluated the production response of dairy cows fed incremental concentrations of GFP (DM basis, 0%, 12%, 24% and 36%). When cows were fed 24% and 36% of GFP in the diet, cows had decreased milk and milk protein yields. One hypothesis for low performance is overall low duodenal availability of Met when cows are fed diets with high

concentration of GFP. Vander Pol et al. (2008) discussed that concentration of Met in GFP protein is lower than other grains, such as SBM, arguing that supplementation of Met could be necessary for high producing cows fed pea protein.

Most nutritional models use energetic estimates and equations in order to improve energy balance of dairy cows in order to prevent weight loss and low milk yield due to low availability of energy (NRC, 2001; Tylutki et al., 2008). Carbohydrates and protein in the diet provide the substrates for VFA production. Acetate and Butyrate are lipogenic products, as propionate is considered a glucogenic product. Lipogenic products will result in Acetyl-CoA production and glucogenic products will yield oxaloacetate production, both which will enter the Krebs cycle and generate energy in the body (Van Knegsel et al., 2007). Comparison of dietary energy sources on energy partitioning and overall energy efficiency has potential to improve nutritional models and help improve energy balance of dairy cows. High content of starch in GFP holds potential to improve energetic balance because GFP can cause high production of propionate in the rumen. A major portion of the metabolizable energy consumed by ruminants is dissipated as heat or equivalently HP. Heat production of cattle is influenced by a series of interrelated factors such as diet supply and quality, environmental conditions, and animal behavior (Brosh et al., 2010). Most of the methods used for determining EE of farm animals are based on O_2 consumption (QO_2) measurements under controlled, confined conditions. Both acute tracheal intubation for measurement of QO_2 (Young and Webster, 1963) and infusion of ^{14}C-labeled bicarbonate (Corbett et al., 1971; Young and Corbett, 1972) interfere with normal behavior, have limited field applicability, and are expensive to use on large animals. According to Nagy (1989), using doubly labeled water to predict CO_2 turnover is the preferred method to estimate the metabolic rate of free-ranging animals. However, the doubly labeled water method are prone to

errors in addition to its high analytical and labeling costs (Schoeller and van Santen, 1982). Kaufmann et al. (2011) determined the EE of grazing and confined dairy cows using the ^{13}C-labeled bicarbonate dilution technique combined with an automated blood sampling system. However, their measurements were limited to six hours in a single day to minimize the technique interference on grazing behavior. One additional limitation is that determining HP during a single six-hour period may not be representative of the diurnal variation in HP. The use of calorimetric chambers to determine heat is expensive and restrict animal locomotion, Therefore, alternative reliable methodologies with minimal interference on normal intake behavior are needed to determine HP in ruminants.

In addition, QCH_4 and the consequent carbon footprint as a result of GFP addition to diets were never directly measured. Ground field peas are highly degraded by microbes inside the rumen but did not cause a difference in acetate to propionate ratio when replacing SBM at 15% of the diet, which could result in similar production of CH_4 (Vander Pol et al., 2009).

In order to fill these gaps in knowledge about use of GFP, a study needs to be done with the objective to evaluate if high-producing dairy cows receiving diets with 25% GFP (dry matter basis) and addition of RP AA as Lys and Met would maintain milk and milk protein production compared to cows that did not receive AA supplements. The study should be done with the following objectives: 1) Evaluate the effects of feeding GFP and RP AA on milk yield and composition and energy balance using a portable gas quantification system [GreenFeed, C-Lock Inc., Rapid City, SD]. 2) Evaluate the effects of feeding GFP as a replacement for corn and SBM mix on greenhouse-gas emissions [CH_4 and carbon dioxide (CO_2)] using the GreenFeed system.

No studies have been conducted to date investigating the effects of feeding 25% of GFP in dairy cows' diets supplemented with RP AA on N and energy use efficiency. It was

hypothesized that GFP-based diets with addition of supplements with high potential of duodenal AA delivery, such as RP AA or CAM, would improve animal productivity. It was hypothesized that feeding GFP with CAM can improve absorption of EAA, increase milk protein yield and, consequently, increase premiums received for milk due to higher protein yield despite the high content of RDP in a diet with GFP and CAM.

Canola meal has been identified previously as a good source of AA for lactating Holstein cows (Huhtanen et al., 2011; Martineau et al., 2013). When fed, this supplement resulted in cows with higher DMI when compared to a diet with SBM (Brito and Broderick, 2007), higher duodenal flow of RUP (Brito et al., 2007) and higher overall plasma concentrations of AA (Martineau et al., 2014). Cows fed diets supplemented with RP Lys and Met have been shown to have an abrupt drop in plasma His concentration with no apparent reasons (Patton et al., 2015). This drop could have been caused by either 2 reasons: 1) His became the third limiting AA in the mammary gland after Lys and Met were replenished, which can be potentiated when cows are fed diets with high RDP content, or 2) there was a competition in the intestinal wall for AA absorption, in which diets with high concentration of Lys and Met caused a drop in absorption rates of His. Patton et al. (2015) performed a meta-analysis about flow of individual AA in the duodenum (using a nutritional model) and found that diets which rely on 70% or more of microbial crude protein production may find His as one of the most limiting AA. In addition, although His causes inhibition of feed intake in rats through formation of histamine compounds, increased concentration of His in the diet causes an increase in feed intake in some studies (Lee et al., 2012; Giallongo et al., 2016). The trend of an increase in plasma His found in Martineau et al. (2014) could either be caused by higher absorption of His when diets have CAM, or by higher DMI observed in most studies used in the meta-analysis.

The Greenfeed system

The GreenFeed (**GF**) system has been used recently to obtain spot short-term measurements of methane emissions (***Q*CH$_4$**) and carbon dioxide emissions (***Q*CO$_2$**) in near real-time mode and with minimal disturbance to the cow's natural behavior (Hammond et al., 2015; Dorich et al., 2015; Huhtanen et al., 2015). The GF consists of an air sampling and gas quantification module powered by solar energy (if on pasture following a grazing system) or regular alternating current electricity (when used inside a free-stall barn, a tie-stall barn as in Figure 1 (a). The GF uses radio frequency identification to individualize animals visiting the system. When the animal is inside, the system delivers feed automatically every 45 seconds (or any other interval chosen by the user) and pulls air from the muzzle region using a fan inside the system. Airflow is measured using a sensor close to where a subsample is acquired and pumped to a non-dispersive near-infrared sensor for CH_4 and CO_2. Infrared light in frequencies between 2 and 15 micrometers is used inside the sensor. The frequency was chosen as the closest to the rotating frequency of light absorption within orbitals of molecules according to the Beer-Lambert law. Methane absorbs light at 3 and 7.8 micrometers. Carbon dioxide absorbs light at 4.2 micrometers. Water vapor absorbs light around 2.5 micrometers. When a subsample passes through a channel, the near infrared light passes through the gas inside the channel and then is read through pyro-electric detectors (made of a lithium tantalite crystal, $LiTaO_3$) at the end of the light channel. The change in temperature in the crystal causes an electric current flow that is read (Personal communication, Sensors Europe GmbH, Erkrath, Germany). The remaining air is released away from the system through an exhaust pipe to avoid recirculation. Data are read only if the position of the muzzle is relatively close to the sampling area (< 30 cm from the front of

the feed area). Although each user can configure head position in the system, it is recommended that points read with head position greater than 30 cm from the sampling area are not used. As described by Huhtanen et al. (2015), when head position is read far from the sampling area inside the system, CH_4 and CO_2 flux emissions estimations will have larger variation and lower repeatability.

Methane emissions from lactating dairy cows housed in a poor ventilated tie stall barn and measured with the GF was less variable than QCH_4 measured with SF_6 (Dorich et al., 2015). It is noteworthy that both the GF and SF_6 can sample large number of animals particularly in outdoor settings (Hegarty, 2013; Storm et al., 2012). The GF estimated spot CO_2 and CH_4 flux emissions with less variability than a non-flux spot sampling of emitted carbon in growing heifers (Huhtanen et al., 2015). Compared with calorimetric chambers, the GF yielded similar results of emitted carbon (Hammond et al., 2015), although correlations between the 2 techniques were weak. Calorimetric chambers are the gold standard for gaseous measurements, as they can measure a complete diurnal pattern (compared to spot short-term measurements done by the GF). In order to reduce variability of results and improve measurements using the GF, high rate of visitation to the system daily and a constant visitation pattern throughout long periods have to be done. As discussed in Hammond et al. (2015), the low correlation between calorimetric chambers and the GF system were caused by low visitation to the spot short term GF system. On the other hand, use of chambers may disrupt natural animal behavior and normal feeding patterns, causing the chamber method to be biased (Storm et al., 2012). Repeatability of gaseous measurements averaged 0.75 and 0.86 for QCH_4 and QCO_2, respectively in an experiment with 75 lactating dairy cows in a free-stall barn with access to a GF and 118 lactating dairy cows using a robotic milking system fitted with a GF (Huhtanen et al., 2015). In general,

the GF system can be used with up to 40 cows inside a free-stall barn, a tie-stall facility or on pasture in grazing systems.

As described by Gill et al. (2010), a total biophysical potential of reduction in 5.5 to 6.0 Gt of CO2 equivalents (**CO_2e**)/year can be achieved by improvements in the world agricultural system. Within the improvements identified, cropland and grazing land management as direct reduction of greenhouse gases (**GHG**) emissions from ruminants could help countries achieve this goal. On the other hand, a direct reduction of GHG emissions from ruminants may negatively affect animal production and reduce feed efficiency (Hristov et al., 2013; Knapp et al., 2014). As a result, an increase in GHG emissions efficiency, which is the amount of CO_2 equivalents emitted related to how much product per kg of CO_2e is produced has been recommended (Yan et al., 2010; Hristov et al., 2015; Hammond et al., 2015). A comparison between the environmental impact of dairy production between 1944 and 2007 showed that dairy cows produced twice as much CO_2e daily in 2007 (27.8 kg/cow) when compared to 1944 (13.5 kg/cow), but almost 3 × less CO_2e/kg of milk produced when compared to the middle of last century (1.35 vs. 3.66 CO_2e/kg of milk for 2007 and 1944, respectively; Capper et al., 2009). According to the authors, the reduction of GHG emissions relative to the amount of product is caused by improvements in animal genetics, reduced dairy population size with higher efficiency of milk production, improved nutritive value of feedstuffs that provides more nutrient dense rations and use of ration balancing software, which makes diet formulation more precise (Capper et al., 2009).

Energy estimation in dairy cattle

Energy can be partitioned for feeding dairy cows as follows (Moe et al., 1972; Moe and Tyrrell, 1975; Moe, 1981; NRC, 2001; Reynolds et al., 2010): Gross energy (**GE**) is the total energy intake measured in the feed using adiabatic calorimetric bombs. Gross energy can be converted to digestible energy (**DE**) based on how much energy is excreted in feces. The problem of this approach is that it does not account for endogenous energy loss, and, for this reason, DE is considered an apparent digestibility (NRC, 2001; Reynolds et al., 2010). After subtracting energy losses from urine and CH_4, the remaining energy available can be fully used by the body and is called metabolizable energy (**ME**). Although excreted urea is part of the metabolism, it is not considered to be available for metabolic purposes on a net basis and is not part of ME (Reynolds et al., 2010). High protein intake will increase the amount of urea excreted and consequently reduce the proportion of ME available from total GE intake (Colmenero and Broderick, 2006; Calsamiglia et al., 2010; Reynolds et al., 2010). The remaining energy retained for milk production is called net energy for lactation (NE_L) and is achieved by subtracting HP from ME. Direct separation of energy used by the mammary gland and energy stored or released from metabolic processes can be done using the NRC (2001) model, in which for each kg of body weight gained or lost (for a body condition score of 3.0), 31.8 MJ of energy is stored or 29.2 MJ lost, respectively. Energy utilization values from the body vary based on the body condition score of the cow (NRC, 2001, Table 2-4).

Estimations of the energy required for milk production are expressed in NE_L as recommended by the NRC model (NRC, 2001). Calculations using this approach are performed using digestibility of CP, NDF, fat and non-fiber carbohydrates data from published literature multiplied by constant factors, which have generated doubts about the numbers, as described by the model (NRC, 2001). The problem pointed out by the model is that equations to estimate DE

and ME take into account a value for maintenance intake of 1 ×. Doubts about the model equation were discussed by Huhtanen et al. (2009) which describes that for a DMI value of 3 × maintenance, an 8% reduction in digestibility as a result of higher intake and passage rate must be accounted for (Huhtanen et al., 2009). Animal intake variation in the field, from 2 × to 4 × maintenance, can result in biased estimates of feed digestibility and efficiency (Huhtanen et al., 2009).

Compared with poultry (19%), swine (21%) and beef cattle (7%), dairy cattle are more efficient in converting energy (25%) into product, probably as a result of genetic selection, development of new technologies and processing of feed (Reynolds et al., 2010). Early studies performed at the USDA-ARS Ruminant Nutrition Laboratory tried to identify the sources of variation for energetic efficiency and implement the concept of NE_L efficiency, which has a linear relationship with DE intake (Moe and Tyrrell, 1975). The 5 major processes identified as factors affecting energy efficiency in cattle are DMI, digestion of feed and related energy costs, body metabolism, animal activity and thermoregulation (Herd and Arthur, 2009). In the beef industry, the use of residual feed intake is done in order to separate individual more efficient animals and thus improve the genetic pool of farms (Kennedy et al., 1993; Herd and Arthur, 2009; Hafla et al., 2013). Connor et al., (2013) obtained data from 453 lactations from 292 individual cows and measured residual feed intake as the difference between actual energy intake and predicted intake based on a linear model from the NRC (2001). The authors used fixed effects of parity, metabolic body weight, ADG and ECM and found a heritability of 36% for energetic efficiency and 56% repeatability for RFI calculations.

Energy requirements of dairy cows and relationships with DMI were examined by a review paper (Ferrell and Oltjen, 2008). The most common approach has been the use of

individual or small groups of cows in enclosed chamber systems, which allows for precise measurements of utilization and excretion of energy using gaseous emissions (Blaxter and Clapperton, 1965). Alternative techniques to respiratory chambers are enabling scientists to collect or record gas measurements from cattle in their own production settings (e.g., grazing, free-stall). Specific examples include quantifications of: 1) heat production from O_2 consumption per heartbeat (Brosh et al., 1998; Aharoni et al., 2006; Brosh et al., 2010), 2) energy expenditure using the ^{13}C bicarbonate technique coupled with O_2 consumption and respiratory quotient (**RQ**) (Junghans et al., 2007; Kaufmann et al., 2011), 3) carbon emissions using tracer techniques (Stewart et al., 2008; Madsen et al., 2010), and 4) CO_2 flux and QCH_4 in animal breath (Dorich et al., 2015; Huhtanen et al., 2015; Watt et al., 2015). Respiration chambers remain the gold standard technique, but require that animals stay confined with restricted movement and without access to feed during the measurement (Hristov et al., 2013; Knapp et al., 2014).

In order to estimate energy requirements, measurement of HP must be done. Direct measurements of HP from dairy cows were done by Brouwer (1965) and improved by Nicol and Young (1990), Brosh et al. (1998), and Aharoni et al. (2003). Brouwer (1965) used data from respiratory chambers to estimate oxygen consumption ($\boldsymbol{QO_2}$) and release of QCO_2 and other gases such as CH_4 to build an equation to estimate HP. Nicol and Young (1990), performed a study reducing body heat and increasing metabolic rate of sheep in metabolic chambers. Researchers infused cold water in the rumen and measured increase in heat production by the animals until normal body temperature was achieved and found that sheep produce 20.47 kJ/L of O_2 consumed. Brosh et al. (1998) improved HP measurements technique, and was able to measure animals on their own production setting (i.e. grazing) with low interference to animal behavior and feed intake. The authors used heart rate monitors and an oxygen sensor coupled

with a facemask open-circuit respiratory system in order to estimate QO_2 per heartbeat. Animals were fed a high and a low energy diets and energy expenditure using this technique was compared. The authors found that animals on low energy diets had lower heartbeat rate but higher utilization of O_2 per heartbeat, resulting in low energy diets being more energetically efficient. Animals had to be held inside a portable chute for 5 min intervals while measurements were made and, although researchers were careful about not disturbing normal behavior, it is possible that higher respiratory rate and higher heart rate due to the stress of being confined in the chute occurred. Aharoni et al. (2003) performed a study with the objective to estimate variability of O_2 pulse in cattle, with varying environmental conditions. The authors found that QO_2 was different and varied between high producing cows exposed to different heat load conditions, showing that individual measurements of QO_2 should be performed from all animals instead of measuring only a few animals and drawing conclusions for all herd. In a further study trying to evaluate differences in energy efficiency of Holstein cows or F1 Montbeliarde × Holstein cows, Aharoni et al. (2006) found that the F1 cows were less efficient relative to milk production as a function of metabolizable energy intake as Holstein cows produced more milk overall.

Dong et al. (2015) performed a study to investigate the utilization of energy in the body of Holstein-Friesian cows. The authors calculated maintenance energy requirement from HP minus the energy losses from inefficiencies of ME use for lactation, energy retention and pregnancy. The authors found no difference in maintenance energy between Holstein-Friesian cows, but found that maintenance energy requirement increases with increasing feed intake (Dong et al., 2015). As DMI is one of the main factors that correlates to milk yield, increase in

productivity can cause an increase in metabolizable energy requirements, which is not taken into account in the majority of energy feeding system around the world (Dong et al., 2015).

Another factor that can influence individual efficiency of milk production is HP (Herd and Arthur, 2009). Individual animals that produce less heat may derive more energy towards milk production and be more energy efficient overall. Individual differences in HP can be caused by differences in digestion physiology, efficiency in which feed is digested in the gut, physical activity, body composition, protein turnover and metabolism, use of recombinant grown hormone, insulin and glucagon status, and other metabolic factors and are mostly genetically or phenotypically related (Herd and Arthur, 2009; Reynolds et al., 2010).

Tissues of the splanchnic bed including the gastrointestinal tract, liver, spleen, pancreas and mesenteric fat deposits comprise around 20% of total body mass in ruminants, and require between 35 and 60% of total O_2 consumption (Herd and Arthur, 2009). According to the authors, an increase in liver and gut oxygen use occurs when excess protein is fed to cattle. Reynolds et al. (2010) attribute this effect mostly to AA catabolism and not to urea synthesis in the urea cycle.

Increasing feed and nutrient efficiency in dairy cattle

The first studies performed in order to determine the most limiting AA in dairy diets were done by Schwab et al. (1976). In this earlier study, the authors performed 5 trials infusing AA into the abomasum of lactating dairy cows. The experiments were set up as Latin square designs with 9-day periods and diets were based on corn silage and alfalfa hay with a grain mix mostly composed of shelled corn. According to Schwab et al. (1976), Lys and Met were found to be the first 2 limiting AA. Results of the exxperiments showed that infusion of Lys accounted for 16%

of the total response in milk yield and a combination of Lys and Met infused accounted for 43% of the milk yield response in cows fed low CP diets. In trial 1, a significant increase in milk protein yield occurred when cows were infused with Lys and Met. In trial 3, infusion of Lys and Met did not increase milk protein yield to values of casein infusion, but when cows were infused with Lys, Met and Val, protein yield increased to values similar to casein infusion.

After 1986, the whole herd buyout caused a destabilization milk prices started to become volatile because the demand for processed products and byproducts, such as cheese and yogurt, increased, resulting in the demand for utilization of components to produce byproducts, resulting in a destabilization of the milk market, which became volatile to consumer demand and supply of specific products. Also, different regions in the country had different demands for fluid milk or byproducts, and this would lead to high difference in prices and destabilize the economy (Federal Milk Marketing Orders, 1990).

Milk market is based only on supply and demand of products (and also the stock markets) and when solids had higher demand values, manufacturing companies started to increase the incentive for farmers to increase the solids yield in order to be more competitive. Also, some companies increased their premiums above the federal market order in order to stimulate an increase in milk solids production from their farmers and, in consequence, increase in profits by supplying the demand. It is important to emphasize that milk companies and co-ops pay for yield and not percentage of solids (USDA, 2016) milk market orders, https://www.ams.usda.gov/rules-regulations/moa/dairy).

At this point, the motivation to perform studies to determine limiting AA was coming from the milk market and from improvements in nutrient efficiency (Schwab et al., 1976). Schwab et al. (1992) used 4 ruminally cannulated cows on all stages of lactation (peak at 4

weeks, early lactation at 8 to 12 weeks, mid lactation at 17 to 21 weeks and late lactation at 27 to 31 weeks) to determine which AA, Lys or Met, was first limiting in these different stages of lactation. Infusion of both Met and Lys resulted in similar milk and milk protein production when compared to casein infusion. The authors found that Lys was first limiting AA for early lactation cows and Met could not be distinguished between second limiting or co-limiting. In mid-lactation cows, infusion of Lys or Met alone did not increase milk protein yield, but a combination of both resulted in yields similar to when cows were infused with casein. The authors suggested that the extent of AA limitation decreased as lactation progressed from early to late, reaching a point in which late lactation cows requirements caused Lys and Met to not be limiting (Schwab et al., 1992). It is also important to observe that His was, for several times, found to be limiting in diets based on grass or grass silage (Huhtanen et al., 2002). Most His comes from microbial synthesis (NRC, 2001) and when the diet is deficient in metabolizable protein (**MP**) and when the MP provides low microbial crude protein content, His could be limiting for milk protein production (Lee et al., 2012).

After the regulation of milk market orders paying for milk solids production, feed companies started to specialize in production of AA products that could be protected from the rumen in order to be able to increase the amount of AA that could be delivered in the duodenum. Some research was focused on heat treated oilseed and oilseed meals, which have good protection from the rumen microbes, but could affect microbial health due to oxidative potential. Other research focused on treating protein supplements with chemical agents that act by creating interactions between protein and carbohydrate, known as Maillard reactions, or protein cross-links that will make the portion of protein unavailable for microbes in the rumen, but partially available in the duodenum after denatured in the abomasum by low pH (NRC, 2001).

Overheating proteins could cause, on the other hand, damage to the molecules causing a decrease in AA bioavailability (NRC, 2001). For example, Lys is the most sensitive protein and will completely be degraded or unavailable if too much heat is applied (NRC, 2001).

Protection of purified AA is a methodology that allow for feeding a precise amount of AA to the cow, especially if the diet needs only one AA to be balanced for maximum productivity. Current technologies used are described in the NRC (2001): coating of the surface with fat or pH sensitive polymer mixture, coating of AA micelles with fat or saturated fatty acids. Another technology for Met delivery is using liquid sources of Met hydroxyl analog (DL-2-hydroxy-4-methylthiobutanoic acid, or HMB). The problem with protection technologies is that the AA is not completely protected from ruminal metabolism, and techniques for bioavailability measurements were then implemented to assess quality of product protection (Ordway et al., 2009; Ji et al., 2016).

Colmenero and Broderick (2006) performed a study feeding 5 concentrations of CP to lactating dairy cows (13.5%, 15.0%, 16.5%, 17.9% and 19.4%). According to the authors, feeding cows 16.5% CP was the best concentration of CP, which yielded numerically higher milk and milk protein compared to the other CP concentrations. A linear decrease in milk N efficiency was found as CP in the diet increased in the study, showing that lower CP diets improved milk N efficiency in the cows, probably due to an increase in recycling mechanisms within the body (Lapierre and Lobley, 2001) or higher shift of feed N to milk production. Leonardi et al. (2003) fed 16 mid-lactation Holstein cows 2 concentrations of CP (18.8% vs. 16.1%) with or without addition of RP-Met and investigated N efficiency and production responses. The authors found that milk protein concentration increased when cows were fed RP-Met. On the other hand, milk protein concentration, but not yield, was lower for cows fed 18.8%

CP diets compared to 16.1% CP diets. Lower protein diets resulted in increased efficiency of N utilization (Leonardi et al., 2003).

In order to study AA limitation in late lactation cows fed low protein diets, Pereira et al. (2015) conducted a study feeding dry distillers grain as main source of protein compared to diets with SBM. The comparison was done in diets based on corn silage or ryegrass silage. The objective of this study was to assess if late-lactation cows fed diets with low percentage of CP based on dry distillers' grain corrected with RP-Lys and RP-Met to balance for AA availability in the duodenum would maintain milk production and overall performance. The authors found no difference for milk and milk protein yield between recommended CP (16.5% or 15.5%) and low CP (13.5%) diets for late lactation cows. Values of MUN found were the lowest in literature without affecting production, of 6.5 mg/dL, which according to the authors may have been caused by high efficiency of AA utilization coupled with high recycling rate of N and low requirements of late-lactation cows. The authors also discussed how low AA requirements of late lactation dairy cows could have caused the increase in N efficiency. Cows in late-lactation produce less milk with higher concentration of true protein and have lower demands for AA when compared to cows in early lactation (Schwab et al., 1976). Short term studies also can cause bias in AA requirements as body reserves can replenish requirements not met by feed. In the case of Pereira et al. (2015), the 2 studies conducted were crossover designs with 21 day periods. The authors acknowledge that a study with longer periods could cause cows to become deficient in AA feeding 13.5% CP diets with RP AA.

Ordway et al. (2009) performed a study comparing 2 technologies of AA delivery for periparturient cows: use of HMB or coating with a pH sensitive polymer (Metasmart and Smartamine-M, respectively, Adisseo, Antony, France). Diets were fed to reach a 3.0:1 ratio Lys

and Met in MP and had 13.8% CP prepartum and 16.4% CP postpartum. For the prepartum diets, no difference was found between treatments and protection technology used. For postpartum cows, milk protein concentration was higher for cows fed both RP-Met when compared to control treatments, but plasma concentration of Met was higher for cows fed Smartamine-M when compared to the other treatments.

Lee et al. (2012) performed a study feeding diets negative in MP balance with RP-Lys, RP-Met and RP-His to early lactation (94 DIM and 54 DIM at the beginning of the experiment) dairy Holstein cows. There were 4 diets, a control with adequate MP balance diet contained 15.7% CP, and the other 3 diets were negative in MP balance and contained 13.5%, 15.7% and 13.6% CP. A control diet negative in MP (- 317 g/d) and without any RP AA was fed as a negative control and the other 2 diets (-370 g/d and -385 g/d) were supplemented with RP AA. The MP deficient diet decreased DMI, which did not happen to the adequate MP diet and the MP deficient diets with added RP AA. Milk yield followed the trend in DMI and was highest for adequate MP diet and for deficient MP diets with RP AA added. No difference was found for animal performance except for an increase in DMI when RP-His was added, compared to the other diets with RP-Lys and RP-Met. Although feeding His can cause a decrease in intake for non-ruminant animals, several studies in literature that fed RP-His or infused His to the abomasum found it caused an increase in DMI when compared to other diets (Lee et al., 2012; Giallongo et al., 2016; Patton et al., 2015) without affecting animal productivity, although a decrease in DMI was found in other studies. More studies providing His to the duodenum are necessary in order for conclusions to be drawn.

In a long-term study (12 wks) with 72 cows fed diets based on SBM and corn meal, deficient in MP, and supplemented with RP-Met or RP-His, feeding RP-His together with RP-

Met increased DMI by 5.1% when compared to a diet with RP-Met only (Giallongo et al., 2016). In addition, milk protein content and yield increased when cows were fed RP-His and RP-Met, although the increase cannot be linked with feeding RP-His alone (Giallongo et al., 2016)

In order to study requirements of His for milk production, Lapierre et al. (2014) conducted a study with 4 multiparous Holstein cows in a Latin square design. The objective of this study was to understand if His requirements could be inferred from the diet or if other factors, such as endogenous metabolism, could interfere with His requirements. Cows were fed a deficient MP diet (72% MP requirements) and infused in the abomasum with His at 0, 7.6, 15.2 or 22.8 g/d in addition to an AA mixture, representing 1.60, 1.95, 2.30 and 2.65% of MP supply, respectively. The researchers found that milk yield was high for cows infused with either 2.30% or 2.65% of His on total dietary MP, and milk protein yield reached a plateau at 1.95% of infused His in total supplied MP. The authors concluded that muscular metabolism, specifically from Carnosine and Anserine, could bias required estimations of His from duodenal flow (Lapierre et al., 2014) as these other 2 molecules can be precursors of His in the body.

Using the AA oxidation indicator technique, Ouellet et al. (2014), evaluated His requirements using 6 lactating Holstein cows in a 6 × 6 Latin square fed 75% of total MP requirements. Cows were infused into the abomasum with a mixture of all AA and His. Histidine was infused in rates of 0, 7.6, 15.2, 22.8, 30.4, and 38.0 g/d, which is relative to 1.5, 1.83, 2.15, 2.46, 2.78 and 3.09% of total MP fed. From the results, authors identified that infusion of 7.6 g/d was sufficient to meet the requirements. At 2.46% of MP, plasma concentration of His and milk protein yield reached a plateau. (Ouellet et al., 2014).

Ground Field Peas

Ground field peas (*Pisum sativum*) is one of the most studied plants, and it was the first one to be scientifically studied genetically, in order to improve crop productivity, decrease loss in productivity from poor genetics or diseases and implement the classic scientific method in genetics (Mendel, 1866). Some GFP varieties will flower for long periods and cold conditions can prolong ripening, resulting in a pulse crop that can be harvested before the weather is appropriate for planting other crops such as corn and sorghum (McKay et al., 2003). The major producers of GFP are Russia, China, Canada, Europe, Australia and the United States. This feed is grown as green manure or a pulse crop around the globe as is sold as a dry, shelled product. Human food industry competes for utilization of FP with the dairy industry and the majority (70%) of the produce from the United States is exported (McKay et al., 2003).

Ground field peas are commonly used as a protein supplement for organically certified dairy farmers in the northern regions of United States and in Canada. Although not yet a problem in these countries, Europe's ban of genetically modified organisms' crops has stimulated European Union countries to grow their own feedstuffs and decrease source of origin problems (European council, 2007). Ground field peas is an alternative crop that can be grown at the beginning and end of the growing season, increasing land productivity and possibly reducing costs. Germination can occur at soil temperature of 3 to 4^{o} C and this plant is tolerant to frosts to - 6^{o} C (Cousin, 1997).

Ground field peas are known as a good, low cost, substitute for protein and energy sources in dairy cattle diets (Vander Pol et al., 2008) in addition to being widely utilized in organic dairy farmers in the Northeast. However, little research to date has been conducted to investigate the impact of FP on milk production, N utilization and carbon footprint in dairy cows. In an experiment comparing diets in which FP replaced SBM and corn, no significant differences

were found for DMI, milk yield, milk components, milk organoleptic characteristics, and N efficiency (Vander Pol et al., 2008). However, a study comparing differing grain processing (i.e., rolled vs. ground peas) showed that field peas should be ground to avoid depression in total tract digestibility of nutrients (Vander Pol et al., 2009). Corbett et al. (1995) comparing diets containing mixtures of SBM plus CAM or field peas reported that concentration of milk fat was higher for diets with field peas as major source of protein compared to the control counterpart.

Ground field peas can replace a mix of 58% corn meal and 42% SBM (Anderson et al., 2006), and with organic corn meal priced as US$440 per metric ton, and organic SBM and GFP priced as US$1160 and US$640/metric ton, respectively (USDA-AMS, 2014), the 58:42% corn-SBM mix would cost US$100 more per metric ton than the proportional amount of GFP (prices from 2014). Feed costs are responsible for 40 to 50% of total costs in an organic dairy farm (Dalton et al., 2008), meaning that feeding GFP may decrease a good portion of the expenses of organic dairy farms and improve their long-term economic sustainability.

Several studies showed that the addition of up to 15% of GFP in dairy cow diets maintain animal performance (Vander Pol et al., 2008) and can potentially increase farm profitability. Addition of more than 15% may affect performance negatively causing a decrease in milk yield and solids production (Albrecht, 2012). Albrecht (2012) fed increasing concentrations of GFP (0%, 12%, 24%, and 36%) to lactating dairy cows. Experimental diets had between 16.5 and 17.3% of CP and had increasing levels of RDP due to GFP high ruminal degradability of 65.3% on protein fraction B_1 (as described in the Cornell Net Carbohydrate and Protein System model). Albrecht (2012) reported decreasing linear levels of DMI, milk yield and milk protein yield as amounts of GFP increased in the diet ($P < 0.05$). Authors also reported increasing levels of MUN which they related to high solubility of protein in the rumen from GFP.

Ground field peas have a low concentration of Met (1.0% or less of CP, NRC 2001 and this could result in reduction of milk production and milk protein yield when cows are fed this supplement, as Met is the first limiting AA for dairy cows in typical North American diets (Schwab et al., 1992; NRC, 2001). Several studies have been designed supplementing animals with 15% of FP or less in the diet without harm for the animal's health and productive performance (Corbett et al., 1995; Masoero et al., 2006; Vander Pol et al., 2008, 2009; Volpelli et al., 2012). When GFP are added as more than 20% of the diet, production of milk and milk protein decline, and the probable cause may be unbalanced duodenal AA availability (Albrecht, 2012). In addition, high levels of RDP can cause a decrease in flow of His to the duodenum as a result low His supply by microbial protein.

Supplementation of RP AA could minimize the problem of low Lys and Met profile in GFP. Ground field peas is deficient in Met with 1.17% as a % of CP (Vander Pol et al., 2008), meaning supplementation with RP AA could replenish animal requirements for specific AA (NRC, 2001). No studies were done with GFP and addition of RP AA.

Canola Meal

In an effort to reduce the amount of glucosinolate concentration in rapeseed meals, cultivars such as the Tower, Reagent, Candle and Altex are used instead of high glucosinolate rapeseed with no more than 3 mg/g of the goitrogenic component (Sánchez and Claypool, 1983). Glucosinolates are hydrolyzed in the gut or rumen into either isothiocyanates, oxazolidine-2-thione, nitriles or thiocyanates. These compounds can cause inhibition of thyroid function by suppressing synthesis of triiodothyronine, in the pathway of iodine incorporation (Cornell University communication: http://poisonousplants.ansci.cornell.edu/toxicagents/glucosin.html).

Canola meal is the name given to low glucosinolate (less than 3 mg/g) cultivars of rapeseed meal that can be safely fed in dairy cows' diets. Sánchez and Claypool, (1983) performed a study feeding diets with CAM (11.7%, DM basis) to 30 lactating Holstein cows, for 4 months, in comparison with diets based on SBM (8.6% DM basis) and cottonseed meal (10.4% DM basis). The authors identified higher feed intake for cows fed CAM but with no statistical difference between diets for production responses. Ruminal parameters were also similar between diets and no effects on goitrogenic hormone concentrations in plasma, which resulted in CAM being a safe supplement to be added to dairy cows' diets (Sánchez and Claypool, 1983).

According to the NRC (2001), CAM has high degradability in the rumen, with 23.2% of the CP in the A fraction and 70.4% in the B fraction with a degradation rate of 10.4% per hour. Harstad and Prestløkken (2001) performed a study comparing in situ ruminal digestibility of CP and total intestinal digestibility of CAM to corn gluten meal and 2 types of fish meal. The main objective of this study was to observe if prediction of total AA from the diet could be inferred from rumen degradability of CP in diets with CAM. The authors found a high degradability of CAM in the rumen and showed that availability of AA could be predicted from rumen degradability of CP, being feed specific. The authors found a CP rumen degradation of CAM of 70.6% after 16 hours of in situ incubation and 83.5% after 24 hours, higher than corn gluten meal. The degradability of all AA followed the same trend of high degradability of CP. (Harstad and Prestløkken, 2001). Results of this study should be carefully analyzed. The in situ technique can have some bias such as low access of microbes to feeds, feedstuffs ground to 1 mm are not the same particle size as real feed (NRC, 2001). Considerations such as passage and degradation rate should be done as considerations that acid detergent insoluble nitrogen can be partially degraded in the rumen in non-forage plant protein that has gone through heat treatment (Weiss et

al., 1989; NRC, 2001). In addition, if the particle size of RUP protein is smaller than the bag pore size, some protein can be lost (NRC, 2001).

In order to compare urea with true protein sources (SBM, CAM and cottonseed meal), Brito and Broderick (2007) performed a study with 24 lactating Holstein cows with the objective to assess N efficiency. The authors compared 4 diets based on alfalfa and corn silage in a 4 × 4 Latin square with: 1) 2% urea, 2) 12% SBM, 3) 14% cottonseed meal and 4) 16% CAM as main protein sources. The authors found that cows fed CAM had higher intake compared to SBM, but similar to cottonseed meal. Milk yield and milk fat yield did not differ among the 3 true protein sources. Milk protein yield did not differ between CAM and SBM, and was lower with cottonseed meal. Overall, urea had the poorest production values. In addition, the authors found that urinary excretion of N of cows fed CAM was similar to excretion of cows fed SBM, and lower in cows fed cottonseed meal. Concentration of NH_3-N in the rumen was similar among CAM, SBM and cottonseed meal based diets, and lowest in cows fed urea. In conclusion, although N efficiency (milk N as a % of N intake) was highest for cows fed SBM based diets, no other significant differences were found between CAM and SBM based diets (Brito and Broderick, 2007).

To assess ruminal outflow of nutrients, Brito et al. (2007) performed omasal sampling of using 3 markers to quantify large, small particles, and liquid passage rate. For the 8 ruminally cannulated cows, no difference was found for DMI and N intake among diets with true protein sources (SBM, cottonseed meal, and CAM). The authors found that cottonseed meal had the highest omasal flow of His, followed by CAM and SBM-based diets. The values of AA flow, specifically for Lys (underestimated), Met (overestimated) were wrongly estimated by the NRC (2001) model according to the values presented when cows were fed diets with true protein

sources. Cows fed cottonseed meal had higher omasal flow of non-ammonia-non-microbial N [(or rumen undegraded protein (**RUP**)] than those fed SBM and CAM (Brito et al., 2007).

Three recent meta-analyses were performed comparing several protein supplements vs. CAM (Huhtanen et al., 2011; Martineau et al., 2013, 2014). Huhtanen et al. (2011) compared SBM- with CAM-based diets either raw or heat treated and sunflower meal. A total of 122 studies were used in the dataset, with the prerequisite that at least one of the protein supplements was used (CAM, SBM, heat treated CAM, and sunflower meal) in 2 different levels. Huhtanen et al. (2011) reported that cows fed CAM had higher DMI, N intake, and milk and milk protein yields when compared to cows fed SBM. In addition, cows fed CAM had a higher slope for organic matter and CP total tract apparent digestibility. In conclusion, the authors stated that dietary models overestimated the MP value of SBM when compared to CAM. In addition, treating CAM with heat reduced its feed value because of Maillard reactions and decreased bioavailability of CP (Huhtanen et al., 2011).

Martineau et al. (2013) performed a meta-analysis comparing diets with CAM or other protein source in the same experiment using 49 isonitrogenous treatments reported in 27 experiments since 1975. The authors found that milk and milk protein yield were increased when cows were fed CAM, showing that the substitution of a specific protein source (e.g., SBM, cottonseed meal or sunflower meal) for CAM improved production responses (Martineau et al., 2013). It was questionable if the increase in production was caused by an increase in DMI or by higher plasma concentrations of essential AA. To answer this questions, Martineau et al. (2014) used the same dataset of Martineau et al. (2013) removing studies that did not report plasma AA values (n = 21). Total DMI, milk and milk protein yield, and milk lactose yield were higher for cows fed CAM when compared to other protein sources. In addition, cows fed CAM had

increased concentrations of plasma Arg, Met, Lys, and other EAA. His and Phe had a tendency ($0.05 < P < 0.10$) to increase in cows fed CAM.

In summary, diets that are heavily based in GFP can have low availability of MP for the cow and low Met delivered in the duodenum. Diets with GFP could, on the other hand, improve microbial protein production when compared to a basic non-protein N source such as urea, but no studies have compared both. High RDP values found in GFP diets can cause low MP, which can be mitigated by addition of RP Lys and RP Met. A comparison of a diet with corn and soybean meal supplemented with RP Lys and RP Met vs. a diet with GFP supplemented with the same RP products could show if GFP can replace corn meal and soybean meal without being detrimental to milk production. As explained before, CAM increases DMI and, as a consequence, increases plasma EAA concentrations, milk yield and milk protein yield. This supplement holds potential to improve DMI and production values in diets based on GFP when replacing SBM, but direct comparisons have never been performed in literature.

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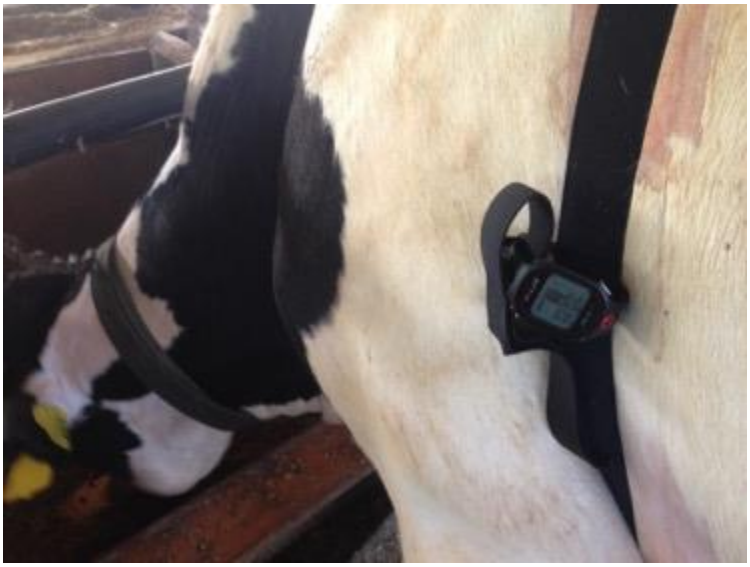
Figure 1. The Greenfeed system mounted on a cart inside the tie stall barn (a) and the use of a heart-rate sensor in cows to determine O_2 pulse (b).



CHAPTER II: INTEGRATING SPOT SHORT-TERM MEASUREMENTS OF CARBON EMISSIONS AND BACKWARD DIETARY ENERGY PARTITION CALCULATIONS TO ESTIMATE INTAKE IN LACTATING DAIRY COWS FED AD LIBITUM OR RESTRICTED

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INTRODUCTION

A new methodology was developed in this dissertation that could be applied for DMI and energy intake measurements in grazing dairy cows using a backward energy estimation method coupled with a gas quantification system [GreenFeed (**GQS**) system, C-Lock Inc., Rapid City, SD] fitted with sensors for CH_4, CO_2 and O_2 as a portable open circuit metabolic chamber in order to measure heat production (**HP**) and energy release from CH_4. This methodology was developed as a proof of concept in which measurement of gaseous emissions from dairy cattle will give an estimate of HP from cows, which, coupled with energetic content of feeds, excreta, milk, body tissue, and CH_4 release from the rumen, allow for possible use of model estimates on energetic balance of dairy cows and, consequently, DMI requirement.

Alternative noninvasive and nonrestrictive methods for accurate estimation of DMI in grazing animals are needed to advance pasture-based research. Herbage intake for grazing animals has been generally estimated by the equation: fecal output ÷ (1 – diet digestibility). Although this equation is simple in principle, accurate determination of fecal output and nutrient digestibility for grazing animals is challenging. Fecal collection bags have been used to measure total fecal output in grazing animals (Sankhyan et al., 1999; Storeheier et al., 2003). However, this technique is laborious and may have limited application for large ruminants such as cattle

because it can negatively affect grazing behavior (Mayes and Dove, 2000). An alternative approach is to use external and internal markers to estimate fecal output and diet digestibility, but marker methodology has its own set of limitations including cyclic changes in fecal marker concentration or poor recovery of markers in feces (Malossini et al., 1996; Dove, 2010). While increasing the number of samples or animals could improve the accuracy of spot sampling techniques used for feces and urine (Malossini et al., 1996), they are not practical to be applied in commercial farming settings. In addition, there are several currently available equations [e.g., Agricultural and Food Research Council (1993); NRC (2001); Cornell Net Carbohydrate and Protein System (Fox et al., 2004; Tylutki et al., 2008)] that use empirical approaches to predict DMI with animal-related variables such as BW, milk yield and composition, and DIM entered as fixed effects in the models.

Energy requirements of dairy cows and relationships with DMI were examined extensively by indirect calorimetry experiments (Ferrell and Oltjen, 2008). The most common approach has been the use of individual or small groups of cows in enclosed chamber systems, which allows for precise measurements of utilization and excretion of energy using gaseous emissions (Blaxter and Clapperton, 1965). Alternative techniques to respiratory chambers are enabling scientists to collect or record gas measurements from cattle in their own production settings (e.g., grazing, free-stall). Specific examples include quantifications of: 1) heat production (**HP**) from O_2 consumption per heartbeat (Brosh, 1998; Aharoni et al., 2006), 2) energy expenditure using the ^{13}C bicarbonate technique coupled with O_2 consumption and respiratory quotient (**RQ**) (Junghans et al., 2007; Kaufmann et al., 2011), 3) carbon emissions using tracer techniques (Stewart et al., 2008; Madsen et al., 2010), and 4) CO_2 flux ($\boldsymbol{QCO_2}$) and

CH_4 flux (***Q*CH$_4$**) in animal breath (Dorich et al., 2015; Huhtanen et al., 2015; Branco et al., 2015).

A portable automated open circuit gas quantification system (**GQS**; GreenFeed; C-Lock, Inc., Rapid City, SD) has been used recently to obtain spot short-term measurements of *Q*CH_4 and *Q*CO_2 in near real-time mode and with minimal disturbance to the cow's natural behavior (Dorich et al., 2015; Huhtanen et al., 2015; Branco et al., 2015). Methane flux from lactating dairy cows housed in a poor ventilated tie stall barn and measured with the GQS was less variable than *Q*CH_4 measured with sulfur hexafluoride (Dorich et al., 2015). It is noteworthy that both the GQS and sulfur hexafluoride can sample large number of animals particularly in outdoor settings (Hegarty, 2013). The GQS measured spot *Q*CO_2 and *Q*CH_4 with less variability than a non-flux spot sampling of emitted carbon in lactating dairy cows (Huhtanen et al., 2015). Compared with calorimetric chambers, the GQS yielded similar results of emitted carbon (Hammond et al., 2013). The objective of the current study was to use the GQS technique as a proof-of-concept methodology to estimate DMI and differences in intake levels in lactating dairy cows fed for ad libitum (**AL**) or restricted intake (**RI**). Specifically, we aimed to determine HP through spot short-term measurements of *Q*CH_4 and *Q*CO_2 and coupled these gas measurements with backward dietary energy calculations to estimate gross energy (**GE**) intake and DMI (i.e. **DMI-Energy**). We then compared DMI-Energy with measured DMI (i.e. **DMI-Measured**), intake predictions from the NRC (i.e. **DMI-NRC**) and estimations from an empirical gas-based technique (i.e. **DMI-CM**, Casper and Mertens, 2010). Our overarching goal is to refine this methodology to estimate DMI from large number of animals in pasture-based and other farm conditions (e.g., free-stall, bedded pack barns). We hypothesized that *Q*CH_4 and *Q*CO_2 could be

used as biomarkers for accurate estimation of DMI in lactating dairy cows when integrated with backward dietary energy partition calculations.

MATERIALS AND METHODS

Experiment Date and Location

The experiment was conducted from March 4th to April 18th, 2013 at the University of New Hampshire Fairchild Dairy Teaching and Research Center, Durham, NH. Samples were processed at the Keener Dairy Research Building, Durham, NH. Care and handling of the animals was reviewed, approved, and conducted according to the University of New Hampshire Institutional Animal Care and Use Committee (IACUC no. 121203).

Animals and Diets

Twelve multiparous cows averaging (mean ± SD) 703 ± 41 kg of BW, 46.2 ± 4.1 kg of milk yield, and 173 ± 37 DIM and 4 primiparous cows averaging 629 ± 16 kg of BW, 34 ± 3.7 kg of milk yield, and 179 ± 27 DIM at the beginning of the study were used. Cows were housed in a tie stall barn equipped with individual feed tubs. Baseline DMI data were recorded using a Super Data Ranger (Calan Inc., Northwood, NH) for 2 wk prior to the beginning of the experiment and averaged (mean ± SD) 24.5 ± 2.6 kg/d during the last 7d. Body weight was recorded for 3 consecutive days before the beginning of the experiment and averaged (mean ± SD) 685 ± 49 kg. Animals were then randomly assigned to 2 groups balanced according to the proportion of DMI relative to BW, which averaged (mean ± SD) 3.94 ± 0.35%. Within each group, the following treatments were randomly assigned to cows in a crossover design: 1) Ad libitum intake adjusted daily to yield 10% orts (i.e., AL treatment), or 2) Restricted intake set to

restrict feed consumption by 10% of baseline DMI (i.e., RI treatment). Each experimental period lasted 22 d and consisted of 14 d for treatments adaptation and 8 d for data and sample collection.

Diets contained (DM basis) 52% forage as corn silage and grass-legume mix haylage, both grown and harvested during the 2012 growing season, and 48% concentrate mix. Diets were mixed twice daily using a Super Data Ranger and fed at 0500 and 1400 h. Approximately 40% of the total daily ration was fed in the morning and the remaining 60% in the afternoon to account for the difference in feeding time intervals. Orts, if present, were collected and weighed daily before the afternoon feeding using the Super Data Ranger. The ingredients and nutrient composition of the experimental diet are presented in Table 1.

Animal Performance and Milk Sampling and Analyses

Body weights were measured during 3 consecutive days before the beginning of the study and during the last 3 d of each data and sampling collection period immediately after the afternoon milking to determine ADG. Body condition scoring was performed by 3 experienced individuals in the first day of the experiment and in the last day of each experimental period according to Wildman (1982).

Cows were milked twice daily at approximately 0500 and 1600 h and milk yield was recorded at each milking throughout the experiment. Milk samples were collected on d 15, 16 and 17 of each experimental period during 6 consecutive milking events. Milk samples were preserved in tubes containing 2-bromo-2-nitropropan-1,3 diol, pooled by cow according to morning and afternoon milk yield, and kept at 4°C until shipped for determination of fat, protein, lactose, and MUN by mid-infrared reflectance spectroscopy (Dairy One Cooperative Inc., Ithaca,

NY). Concentrations and yields of milk components and MUN were computed as the weighted means from morning and afternoon milk yields using the results of the pooled milk samples. Energy corrected milk and 4% FCM were estimated according to Tyrrell and Reid (1965) and the NRC (2001), respectively. Milk efficiency was assessed as the ratio between DMI-Measured and milk yield, DMI-Measured and ECM, and DMI-Measured and 4% FCM.

Feed Sampling and Analyses

Samples of TMR were collected weekly and dried in an air forced oven (VWR Scientific, Radnor, PA) at 55ºC for 48 h for adjustments of dietary ingredients on a DM basis. Offered TMR, orts (when available), and individual dietary ingredients were collected daily during the sampling period, pooled to a weekly sample, and kept frozen in a − 20°C freezer until analysis. Total mixed ration, alfalfa pellets (Forage Extender; Poulin Grain, Newport, VT), and orts samples were dried in an air forced oven (55°C, for 48 h), ground through a 1-mm screen in a Wiley mill (Thomas Scientific, Swedesboro, NJ), and sent to analysis (Dairy One Forage Laboratory, Ithaca, NY). Total mixed ration and orts samples were analyzed for absolute DM (method 930.15; AOAC International, 2006), ash (method 942.05; AOAC International, 2006), total N using a combustion method (LECO TruMac N Macro Determinator; LECO Corp., St. Joseph, MI), NDF (NDF in feeds-filter bag technique for A200 method 6; Ankom Technology, Fairport, NY); solutions as in Van Soest et al., 1991), and ADF [ADF in feeds-filter bag technique for A200 method 5; Ankom Technology; solutions as in method 973.18 (AOAC International, 1998)]. Minerals (Ca and P) were analyzed using a Thermo ICAP 6300 inductively coupled plasma radial spectrophotometer after microwave digestion (CEM application notes for acid digestion; CEM, Matthews, NC). In vitro true DM digestibility (**IVTDMD**) was done for

TMR as 30 h ruminal digestibility plus 24 h enzymatic (ANALAB; Agri-King Inc., Fulton, IL). Samples were placed in F57 Ankom bags and incubated in Daisy II jars (Ankom Technology) with 1.6 L of phosphate buffer at 6.78 pH and 0.4 L of ruminal fluid for 24 h. A 6 *N* HCl solution with 10% pepsin was added to the jars for additional 30 h of incubation and weighting of the remaining residue in each bag assessed IVTDMD. The TMR was additionally analyzed for gross energy using an IKA C2000 basic calorimeter system (Dairy One Forage Laboratory).

Carbon Emissions Sampling and Analyses

Emissions of CH_4 and CO_2 were measured using the GQS. Operating procedures, sampling, and calculations used herein were done as reported previously (Dorich et al., 2015; Huhtanen et al., 2015; Watt et al. 2015). Sampling times started at 0700 and 1900 h on d 1 of the sampling period with the GQS moved sequentially from cow to cow with at least a 1-min interval between samplings for determination of background gas concentration. Sampling was advanced 2 h daily to account for diurnal variations in QCH_4 and QCO_2, thus allowing for 16 measurements/cow per period. The total time of QCH_4 and QCO_2 measurements was 14 h and 57 min during the entire experiment with an average of 4 min and 33 s per individual measurement. Cows were trained with the GQS for 10 d preceding the beginning of the study. Background concentrations **($[BC]$)** of CH_4 and CO_2 was calculated by linearly interpolating the $[_{BC}]$ between starting and ending background points. Data were considered accurate if the background concentration slope had not significantly changed between background points.

The CH_4 sensor was calibrated weekly first by cleaning the sensors from any other gas using N_2 and then using a pre-established concentration of CH_4 (1,000 ppm) injected directly into the sensors. The concentration of gas in µL/L **($[C]$)** was then calculated from sensor voltage

output response compared with the known concentration of gas injected when calibration was performed. At the beginning of each experimental period, the air flux sensor was calibrated gravimetrically using CO_2 cylinders (prefilled 90 g CO_2 cylinders; KEE Action Sports LLC, Sewell, NJ). Carbon dioxide was released for 2 min followed by 2 minutes without any gas release into the feed trough of the GQS at the same place where the animal's nostril should be positioned. Five to 6 gas releases were done per calibration in order to decrease possible within cylinder variation, thus achieving 99.96% mean recovery of CO_2 with a variation of 3.5%.

Calculations of carbon emissions from the GQS were completed using the McLean and Tobin (1987) equation adapted from chamber systems and calculated on a per second basis. The volumetric flow rate in L/min at any given time ($\boldsymbol{Q_{C(i)}}$) equals the capture rate of air (**Cp**) times the difference between $[_C]$ and $[_{BC}]$ or atmospheric concentration of this gas multiplied by the volumetric air flow ($\boldsymbol{Q_{air}}$, L/min) corrected for 1 atm. The capture rate of air was considered to be 1.0 for indoor farm conditions without wind (Huhtanen et al., 2015). The equation is presented below:

$$Q_{C(i)} = C_{p(i)} \times ([C]_{(i)} - [BC]_{(i)}) \times Q_{air(i)} \div 10^6 \quad [1]$$

The mass flux of gas ($\boldsymbol{Q_{m(i)}}$) in grams was calculated using the ideal gas law, in which flux (***Q***) of any given gas (**m**) at any given time (**i**) equals $\boldsymbol{Q_C}$ times 273.15 K divided by the sum of 273.15 K and air temperature (T_{air}) in °C multiplied by the gas density (**ρ**) (0.717 g/L and 1.977 g/L for CH_4 and CO_2 respectively):

$$Q_{m(i)} = Q_{C(i)} \times 273.15 \div (273.15 + T_{air(i)}) \times \rho \quad [2]$$

The equilibrium time of the CH_4 and CO_2 concentration measurement was 5 s. Over a 5-min animal visit, the equilibrium time represented less than 1.5% of the visit and was disregarded. However, the response of the CH_4 and CO_2 sensors lags by 4 to 5 s, time necessary for gas to flow from the animal's muzzle to the sensors in the system. To account for lag time of the $[_C]$ and $[_{BC}]$ measurements, they were offset by 5 s, so that real-time sensor information (such as ear tag readings and head position data) were synchronized with the concentration sensor data.

Energy Balance and DMI Estimation

The NRC (2001) model equation for lactating Holstein cows was used to predict DMI-NRC. The prediction formula is as described in the NRC (2001): "DMI-NRC = {(0.372 × fat corrected milk + 0.0968 × body weight$^{0.75}$) × (1 – e$^{[(-0.192) \times (\text{week of lactation} + 3.67)]}$}". In addition, a second estimation of DMI was added for comparison, using the model of Casper and Mertens (2010, "DMI (kg/d) = {821.3 + [(0.27 × QCO$_2$ (g/d)] + [(1.18 × milk yield (kg/d)]} ÷ 126"), which is based on linear relationships between DMI and QCO$_2$ adjusted for milk yield. This model was previously used by the Cornell Net Carbohydrate and Protein System v 6.1 (CNCPS v 6.1; Fox et al., 2004) for estimation of DMI and comparison of gaseous emissions from forage-based diets (Higgs et al., 2013; Russomano et al., 2013) and grazed pasture (Watt et al., Accepted).

The DMI-Energy procedure was based on the Net Energy System to account for the loss of chemical energy in feces, urine, CH_4, and HP (Moe et al., 1972). Energy loss from CH_4 in the rumen was measured by multiplying volumetric QCH$_4$ by 2.17 MJ/L.

Heat production was calculated based on QO_2, which, in turn, was estimated based on the respiratory quotient (RQ = CO_2/O_2 consumption). According to Ferris et al. (1999) and Madsen et al. (2010), RQ ranges from 0.90 to 1.1. A RQ of 0.95 was used in the current study following Madsen et al. (2010). Heat production was calculated using the equation of Brouwer (1965) modified (i.e., no adjustment for excreted urinary N) by Kaufmann et al. (2011):

$$HP\ (MJ/d) = [(4.96 + 16.07 \div RQ) \times QCO_2\ (L/d)] \div 1{,}000 \quad [3]$$

where RQ = 0.95.

Intake of ME was corrected with energy retained in the body by using measurements of tissue energy balance:

$$ME\ intake\ (MJ/d) = [HP\ (MJ/d) + milk\text{-}energy\ (MJ/d)] + tissue\ energy\ balance\ (MJ/d) \quad [4]$$

where tissue energy balance was calculated according to the empty BW (**EBW**) energy needed to vary 1 kg with an efficiency of 0.85 for weight gain (31.8 MJ/kg) and 0.82 for weight loss (29.2 MJ/kg; NRC, 2001) according to actual BCS of cows when measurements were performed. Milk-energy output (MJ/d) was calculated according to the NRC (2001): milk energy (MJ/d) = {[0.384 × fat (%)] + [0.223 × protein (%)] + [0.199 × lactose (%)]} × milk yield (kg/d).

Digestible energy (**DE**) intake was estimated as the sum of ME intake and energy excreted as CH_4 and urine:

DE intake (MJ/d) = ME intake (MJ/d) + CH_4-energy output (MJ/d) + urinary-energy output (MJ/d) [5]

Urinary-energy was not measured in the present study, but it was calculated as 6.5% of estimated ME according to Ferris et al. (1999) and Ferrell and Oltjen (2008).

Gross energy intake was estimated as the sum of DE intake and fecal-energy output as follows:

GE intake estimated (MJ/d) = DE intake (MJ/d) + fecal-energy output (MJ/d) [6]

where fecal-energy was calculated as [DE intake (MJ/d) ÷ TMR IVTDMD (%)] – DE intake (MJ/d)

Dry matter intake was estimated as follows:

DMI-Energy = GE intake estimated (MJ/d) ÷ diet GE concentration (MJ/kg) [7]

where diet GE concentration averaged 19.2 and 19.1 MJ/kg for data and sampling periods 1 and 2, respectively.

Statistical and Sensitive Analyses

All response variables were analyzed using the MIXED procedure of SAS (SAS 9.4; SAS Institute, Cary, NC) and the Fit Model procedure of JMP (JMP Pro 10.0; SAS institute, Cary,

NC). Results are presented as least square means. The following model was used for milk yield, milk composition, DMI-Measured, ADG, BCS, $Q\text{CH}_4$, $Q\text{CO}_2$, $Q\text{O}_2$, DMI-Energy, DMI-NRC and DMI-CM:

$$Y_{ijkl} = \mu + S_i + P_j + C_k(S)_i + T_l + E_{ijkl}$$

where Y_{ijkl} is the dependable variable, μ is the overall mean, S_i is the effect of sequence i, P_j is the effect of period j, $C_k(S)_i$ is the effect of cow k within sequence i, T_l is the effect of treatment l, and E_{ijkl} is the overall error. Individual 5-min measurements of $Q\text{CH}_4$ and $Q\text{CO}_2$ were averaged in each sampling period to yield 1 measurement/cow. Averaged values were used for carbon flux results, compare DMI models (i.e. DMI-Measured, DMI-Energy, DMI-NRC and DMI-CM), and sensitivity analysis. Individual gas 5-min measurements from all animals were used to calculate repeatability (variance of cow within sequence divided by total variance; Huhtanen et al., 2015) and spot measurements, SD and CV.

The root mean square prediction error (**RMSPE**) was used to compare the DMI models and was calculated as: $\{\sqrt{[\Sigma (\text{Observed value i} - \text{Predicted value i})^2 \div \text{number of observations})]}\}$ as described in Kohn et al. (1998).

In order to assess the variation that could modify the estimation of DMI-Energy, a sensitivity analysis was conducted for HP by varying RQ in 1.05, 1.0, 0.95 0.90, and 0.85. Values were chosen according to the range of RQ reported in the literature (Madsen et al., 2010), as well as preliminary values from our laboratory using an O_2 electrochemical sensor (Pereira et al., 2015). We also conducted a sensitivity analysis to estimate DMI by reducing the IVDMD by

4, 8, and 12% from measured 76.1% value, in which for each multiple of maintenance intake, a digestibility depression of 4% should be taken into account (NRC, 2001).

RESULTS AND DISCUSSION

Nutrient Intake and Milk Yield and Composition

One cow and related data were removed from the statistical and sensitivity analyses because she consistently refused to use the GQS. Intake of nutrients is presented in Table 2. As expected, cows fed for AL had higher DMI-Measured ($P = 0.001$; mean = 23.8 kg/d) than those fed for RI (mean = 22.4 kg/d). Increased DMI-Measured with the AL treatment resulted in increased ($P \leq 0.001$) intakes of N, NDF, ADF, OM, TDN, and GE.

Milk yield and milk composition results are presented in Table 3. Although DMI-Measured was highest in cows fed for AL, milk yield did not differ significantly between treatments. Therefore, it appears that mobilization of body reserves was the metabolic strategy used by cows on the RI treatment to maintain milk yield. In fact, cows fed for AL gained weight (mean ADG = + 0.41 kg/d; $P = 0.002$), whereas those fed for RI lost weight (mean ADG = − 0.36 kg/d; Table 2). Concentrations of milk fat (mean = 3.82%), lactose (mean = 4.82%), and TS (mean = 11.8%) were not affected by treatments, whereas those of milk protein ($P < 0.001$) and milk SNF ($P < 0.001$) were highest in cows fed for AL. Cows fed for AL yielded more milk protein (1.26 vs. 1.18 kg/d; $P = 0.001$) and milk SNF (3.20 vs. 3.04; $P = 0.003$) than those fed for RI as a result of the highest concentrations of milk protein and milk SNF. In addition, cows fed for AL yielded highest milk lactose ($P = 0.006$) and milk TS ($P = 0.01$), which are explained by a numerical increase in milk yield and a significant increase in milk protein yield. The

concentration of MUN was highest in cows fed for AL (17.1 vs. 15.9 mg/dL; $P = 0.002$), and it is partially explained by highest N intake (674 vs. 625 g/d; Table 2), which agrees with previous research (Colmenero and Broderick, 2006). Both ECM and 4% FCM were highest in cows fed for AL because of higher yields of milk protein (+0.08 g/d; $P < 0.001$) and milk fat (+0.08 g/d; $P = 0.11$) compared with cows fed for RI. Feed efficiency, expressed as milk yield/DMI-Measured tended ($P = 0.10$) to be higher in cows fed for RI, and did not differ significantly between treatments when expressed as ECM/DMI or 4% FCM/DMI (Table 3).

Carbon Fluxes, Dietary Energy Calculations, and DMI Estimation

Carbon fluxes, dietary energy calculations, and GE intake, DMI-Energy, DMI-NRC and DMI-CM are presented in Table 4. There was no significant difference between treatments for $Q\text{CH}_4$ and $Q\text{CO}_2$. Regression of $Q\text{CO}_2$ against $Q\text{CH}_4$ (Figure 1; $Q\text{CO}_2 = 4{,}961 + 18.3 \times Q\text{CH}_4$, $P < 0.001$) resulted in an r^2 of 0.73 indicating a strong relationship between these 2 variables, which agrees with previous research (Madsen et al., 2010; Huhtanen et al., 2015). In addition, the repeatability of gas measurements averaged 0.88 and 0.87 for $Q\text{CH}_4$ and $Q\text{CO}_2$, respectively. Huhtanen et al. (2015) also found high repeatability for $Q\text{CH}_4$ (mean = 0.75) and $Q\text{CO}_2$ (mean = 0.86) in an experiment with 75 lactating dairy cows in a free-stall barn with access to a GQS (Swedish experiment) and 118 lactating dairy cows using a robotic milking system fitted with a GQS (United States experiment). In general, the between cow and within cow variability of gaseous data measured was low in the current study. For instance, the spot measurements SD and CV for $Q\text{CO}_2$ were 1,176 L/d and 8.72%, respectively (measured using individual 5-min spot measurements of all cows). Variation in spot short-term measurements of carbon gases may be smaller by increasing sampling frequency and using the GQS for more days, as hypothesized by

Hammond et al. (2013) and Dorich et al. (2015). The SD of 8.72% observed for QCO_2 (L/d) resulted in a difference of ± 0.28 MJ in the estimations of HP and in an absolute difference of 6.83% (or 19.9 to 22.9 kg/d) of DMI-Energy. Therefore, this variation should be taken into account when using the proposed methodology for assessment of DMI-Energy, and, as indicated above, may be decreased with more samples of gaseous measurements for longer periods of time.

Heat production, estimated using equation 3, was not significantly different between treatments and averaged 149 MJ/d (Table 4). The lack of treatment effect on HP may be explained by the relatively small differences in milk yield, DMI, and ADG across treatments. In addition, cows' exercise (i.e., roundtrip from the stall to the milking parlor) was similar and diet composition did not differ for cows fed for AL or RI. According to Brosh et al. (1998), HP is affected by animal metabolic rate, which varies according to milk yield, BW, feed composition, and rate of exercise. Milk-energy output did not differ and averaged 118 MJ/d across treatments (Table 4). On the other hand, EBW change was positive (13.3 MJ/d; $P = 0.002$) for cows fed for AL and negative (− 10.6 MJ/d) for those fed for RI. Intake of ME, calculated using equation 4, was highest with the AL treatment (282 vs. 253 MJ/d; $P = 0.01$) primarily because of positive EBW change and ADG. Positive ADG results in retention of body energy mostly as protein and fat, and contains 29.2 MJ/kg of BW, whereas tissue loss releases 31.8 MJ/kg of BW as energy available for other body functions (NRC, 2001). The efficiency in which the body utilizes body tissue as a source of energy is 0.85 for anabolism and 0.82 for catabolism (NRC, 2001).

Intake of DE estimated by the sum of ME intake, CH_4-energy, and urinary-energy (i.e., equation 5) was highest in cows fed for AL (327 vs. 295 MJ/d; $P = 0.01$; Table 4). Similarly, GE intake estimated (equation 6) was highest with the AL treatment (430 vs. 388 MJ/d; $P = 0.01$;

Table 4). However, mean GE intake estimated (409 MJ/d) was lower than mean GE intake measured (442 MJ/d) by 43 MJ/d. We assumed a RQ of 0.95 to calculate HP in cows fed for AL or RI as previous studies showed that the RQ can vary from 0.9 to 1.1 (Ferris et al., 1999; Madsen et al., 2010). The use of a common RQ to estimate HP may not have captured individual animal variation, explaining, to a certain extent, the underestimation of GE intake in the current study. In addition to the RQ, the sampling schedule used herein (every 2 h for 8 d) may had not being entirely representative of the daily $Q\mathrm{CH_4}$ and $Q\mathrm{CO_2}$ bouts, ultimately impacting our estimations of HP.

Using GE intake measurements, DMI-Energy was calculated using equation 7, and was highest for cows fed for AL (22.5 vs. 20.2 kg/d; $P = 0.01$; Table 4). It is important to note, however, that mean DMI-Energy across treatments (21.4 kg/d) was lower than mean DMI-Measured (23.1 kg/d) by 1.7 kg/d, and this underestimation was more pronounced with RI (− 2.11 kg/d) than AL (− 1.34 kg/d). Coupling of $Q\mathrm{CO_2}$ and $Q\mathrm{CH_4}$ with backward dietary energy partition calculations were used for DMI-Energy estimations, and cumulative estimation errors likely contributed for the difference between mean DMI-Energy and DMI-Measured. On the other hand, DMI-NRC averaged 25.7 kg/d across treatments (Table 4), thereby overestimating DMI-Measured by 2.6 kg/d. The equation of Casper and Mertens (2010) was also used to estimate DMI resulting in a mean value of 22.9kg/d for AL and 22.4 kg/d for RI, and thus underestimated DMI-Measured only by 0.9 kg/d for AL treatment, and had the same value for RI (22.4 kg/d). This equation had less cumulative errors when compared to backward estimation measurements and used fewer variables.

Regressions between DMI-Measured, DMI-Energy, DMI-NRC and DMI-CM are presented in Table 5. The regression between DMI-Measured and DMI-Energy resulted in

moderate (r^2 = 0.39, RMSPE = 2.88; AL cows) and poor (r^2 = 0.07, RMSPE = 3.47; RI cows) relationships (Figure 2). Greater values of RMSPE indicate that observed values are less correlated with the predicted model. All regressions had a larger RMSPE for the RI treatment than for the AL counterpart. We identified 3 cows in the AL treatment that, compared with the remaining animals, emitted lower amounts of CO_2 (mean = 6,686 vs. 6,818 L/d), but had higher milk production (mean = 44.0 vs. 38.9 kg/d) and DMI-Measured (mean = 26.7 vs. 22.7 kg/d). Consequently, these animals had HP values lower than expected. When these 3 cows were removed from the dataset, the r^2 improved to 0.66 (DMI-Energy = 1.22 + 0.92 × DMI-Measured; data not shown), which was similar to the r^2 between DMI-Measured and DMI-NRC for the AL treatment (r^2 = 0.66; Table 5; Figure 2). The difference in QCH_4 between the outlier cows (mean = 478 ± 18 g/d) and the remaining animals in the same treatment (474 ± 13 g/d) was very small. In general, higher DMI lead to higher QCH_4 (Hristov et al., 2013; Watt et al., Accepted). Therefore, our data did appear to indicate that outlier cows had both lower QCH_4 and QCO_2 than expected, but gross feed conversion efficiency (mean = 1.64 ± 0.10) was very similar compared to the non-outlier animals (1.67 ± 0.04). An alternative explanation is that the 3 highest milk producers may have generated less HP than their counterparts because HP was the only variable that was lower for these cows when compared to all the other cows. Less HP with higher milk yield would be an indicative of cows with greater efficiency to partition more energy towards milk yield rather than HP (i.e. greater net feed efficiency). It can also be hypothesized that the outlier animals did not keep their heads inside the GQS feed trough during each 5-min sampling period. It is noteworthy that the GQS only records data when the muzzle is properly positioned.

The poor relationship (r^2 = 0.07) between DMI-Measured and DMI-Energy for cows fed for RI may be related to biased measurements of QCO_2 when animals are losing BW. For

instance, 10 out of 15 observations for cows fed for RI resulted in negative ADG and these animals were likely using body tissues as a source of energy for gluconeogenesis and milk production (Velez and Donkin, 2005). Although the ADG data were sparse, the fact that most ADG values for RI fed cows were negative resulted in larger differences between DMI-Measured and DMI-Energy (Figure 3). The use of body tissue for energy is more efficient than the use of feed for the same metabolic processes (NRC, 2001). As a result, less O_2 is consumed and less heat is produced (Velez and Donkin, 2005). In addition, Sahlu et al. (1988) reported that fasted sheep (negative ADG) had RQ values lower than those fed ad libitum (positive ADG), ultimately resulting in lower than expected HP due to biased QCO_2 measurements. It could also be possible that the baseline period used for feed intake determination was not long enough or that intake demands for individual cows changed during each experimental period of the study. These potential sources of variation may have increased model errors and biased DMI-Energy values against DMI-Measured.

The relationship between DMI-Measured and DMI-CM was relatively low (r^2 = 0.29 and 0.31 for AL and RI, respectively). On the other hand, a better relationship (r^2 = 0.49) between DMI-Energy and DMI-CM was observed, which was expected, as both methodologies rely on QCO_2 measurements. It is important to notice that the range of DMI-Measured (17.8 to 27.6 kg/d, average of 23.1 kg/d) and milk yield (29.0 to 47.8 kg/d, average of 39.4 kg/d) in our study was narrower when compared to the work from Casper and Mertens (2010; 5.1 to 56.6 kg/d, average of 23.3 kg/d). Our experiment was designed to have 2 levels of intake (AL and RI), and this can lead to insufficient variability in the data to adjust for regressions. A design with wider range of DMI could possibly improve regression's correlations and our proposed methodology.

Sensitivity analysis

Sensitivity analysis results are shown in Table 6. The analysis showed that DMI-Energy was closest to DMI-Measured at 68.1% IVDMD and RQ values of 0.95, 0.90 and 0.85, according to the percentage difference between the data (Table 6). For instance, Cows in the AL treatment with a RQ of 0.95 and IVDMD of 68.1% and cows in the RI treatment with a RQ of 1.00 and IVDMD of 64.1% had exactly the same DMI-Measured and DMI-Energy averages (RMSPE = 2.25 and r^2 of 0.48 for AL, and RMSPE = 2.42 and r^2 of 0.43 for RI; data not shown). In the current experiment, DMI-Measured was between 3 to 4 times the maintenance intake, which could considerably decrease the feed digestibility by 8 to 16% (NRC, 2001; Huhtanen et al., 2009), thereby validating our sensitivity analysis approach.

SUMMARY AND CONCLUSIONS

The methodology proposed herein, which integrated spot short-term measurements of QCO_2 and QCH_4, obtained with a portable automated open circuit gas quantification system (i.e., GreenFeed; C-Lock, Inc.), and backward dietary energy partition calculations was able to discriminate differences in DMI between cows fed for AL or RI despite the low to moderate relationships between DMI-Measured and DMI-Energy (i.e., estimated). However, the proposed methodology underestimated DMI particularly when cows were in negative ADG (i.e., RI treatment). Caution is required when interpreting results from this preliminary, proof-of-concept work because it was assumed constant urinary-energy losses, DM digestibility (done as IVTDMD), and RQ across cows. Variations in RQ between animals can occur as a result of different individual tissue energy balances. Further work should incorporate individual spot short term measurements of O_2 consumption while including a larger number of animals in the

experiments. In addition, further studies should focus on testing different sampling protocols (e.g., number of daily samplings and time of sampling relative to feeding) and length of sampling period (e.g., >8 d) to better understand how diurnal variation in enteric carbon emissions impact overall data accuracy and precision.

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Table 1. Ingredient and nutrient composition of the experimental TMR offered to lactating dairy cows fed for ad libitum (AL) or restricted intake (RI)[1]

Ingredient composition (% of diet DM)	AL + RI
Corn silage	40.4
Grass-legume haylage	11.2
Steam flaked corn	3.61
Corn grain, ground, dry	9.86
Citrus pulp	7.33
Soybean hulls	2.93
Molasses	0.71
Dry distillers' grain	1.07
Soybean meal (48% CP)	11.4
Canola meal	3.50
Urea	0.18
Minerals and vitamins premix[2]	3.30
ProvAAl ELITE[3]	1.59
BergaFat F100[4]	0.81
Alfalfa pellets[5]	2.11
Nutrient composition (DM basis)	
DM, % of fresh matter	43.3
OM, % of DM	92.3
Gross energy, MJ/kg	19.2
CP, % of DM	16.8
NDF, % of DM	34.7
ADF, % of DM	24.3
NE_L, Mcal/kg of DM	1.60
Ca, % of DM	0.84
P, % of DM	0.37
In vitro true DM digestibility, %	76.1

[1]Diet was fed for ad libitum (adjusted daily to yield 10% orts) or restricted intake (set to restrict feed consumption by 10% of baseline DMI).
[2]It provided as fed basis: 297 ppm monensin sodium (Rumensin; Elanco, Greenfield, IN), 11.3% Ca, 1.76% P, 5.98% Mg; 6% K, 3% S, 15 ppm Co, 650 ppm Cu; 50 ppm I; 1,200 ppm Mn, 8.97 ppm Se, 3,700 ppm Zn, and 87.1 KIU/kg vitamin A.
[3]ProvAAl ELITE (Purdue Agribusiness, Inc., Salisbury, MD) is a product containing blood meal and Smartamine-M [Rumen protected DL-Methionine (60% MP-Met); Adisseo, Antony, France].
[4]BergaFat F100 is a product containing palmitic acid (Berg+Schimidt GmbH & Co, Hamburg, Germany).
[5]Alfalfa pellets (guarantee analysis: 12% CP, 2% crude fat, 28% crude fiber, 0.9% Ca, and 0.3% P; Poulin Grain, Newport, VT) used as "bait" in the portable automated open circuit gas quantification system.

Table 2. Nutrient intake, and ADG and BCS changes in lactating dairy cows fed for ad libitum (AL) or restricted intake (RI)[1]

	Treatments			
	AL			Effect ($P > F$)[2]
Item		RI	SEM	
DMI-Measured, kg/d	23.8	22.4	0.68	0.001
N intake, g/d	674	625	18.72	<0.001
NDF intake, kg/d	7.98	7.49	0.21	<0.001
ADF intake, kg/d	5.25	4.91	0.14	<0.001
OM intake, kg/d	22.2	20.6	0.62	<0.001
TDN, kg/d	16.8	15.7	0.47	<0.001
GE[3] intake, MJ/d	456	428	13.1	0.001
ADG, kg/d	0.41	−0.36	0.16	0.002
BCS change, score/period	−0.13	−0.15	0.05	0.83

[1]Diet was fed for ad libitum (adjusted daily to yield 10% orts) or restricted intake (set to restrict feed consumption by 10% of baseline DMI).
[2]Probability of treatment effect (AL vs. RI); significance was declared at $P \leq 0.05$ and trends at $0.05 < P \leq 0.10$.
[3]GE = gross energy.

Table 3. Milk yield, milk composition, and feed efficiency in lactating dairy cows fed for ad libitum (AL) or restricted intake (RI)[1]

	Treatments			
Item	AL	RI	SEM	Effect ($P > F$)[2]
Milk yield, kg/d	39.7	39.1	1.58	0.29
Milk fat, %	3.83	3.81	0.10	0.76
Milk fat, kg/d	1.53	1.45	0.06	0.11
Milk protein, %	3.14	3.09	0.04	<0.001
Milk protein, kg/d	1.26	1.18	0.04	0.001
Milk lactose, %	4.83	4.82	0.02	0.21
Milk lactose, kg/d	1.94	1.86	0.07	0.006
Milk SNF, %	7.97	7.90	0.05	0.001
Milk SNF, kg/d	3.20	3.04	0.12	0.003
Milk total solids, %	11.8	11.7	0.13	0.32
Milk total solids, kg/d	4.73	4.49	0.17	0.01
MUN, mg/dL	17.1	15.9	0.42	0.002
ECM, kg/d[3]	42.1	39.9	1.50	0.03
4% FCM, kg/d[4]	39.1	37.2	1.41	0.05
Milk yield/DMI, kg/kg	1.68	1.72	0.04	0.10
ECM/DMI, kg/kg	1.76	1.79	0.04	0.31
4% FCM/DMI, kg/kg	1.63	1.67	0.04	0.28

[1]Diet was fed for ad libitum (adjusted daily to yield 10% orts) or restricted intake (set to restrict feed consumption by 10% of baseline DMI).
[2]Probability of treatment effect (AL vs. RI); significance was declared at $P \leq 0.05$ and trends at $0.05 < P \leq 0.10$.
[3]ECM (kg/d) = [0.0752 × milk yield (kg/d)] + [12.3 × fat yield (kg/d)] + [6.56 × SNF (kg/d)] (Tyrrell and Reid, 1965).
[4]4% FCM = [0.4 × milk yield (kg/d)] + [15 × milk fat yield (kg/d)] (NRC, 2001).

Table 4. Methane (QCH$_4$) and CO$_2$ (QCO$_2$) fluxes, estimated O$_2$ consumption, and dietary energy estimations in lactating dairy cows fed for ad libitum (AL) or restricted intake (RI)[1]

	Treatments			
Item	AL	RI	SEM	Effect (P > F)[2]
QCH$_4$, g/d	472	458	18.0	0.16
QCO$_2$, L/d	6,867	6,733	195	0.20
O$_2$ consumption[3], L/d	6,523	6,396	185	0.20
HP[4], MJ/d	150	147	3.34	0.08
Milk-energy[5], MJ/d	119	116	4.09	0.22
Tissue energy balance[6], MJ/d	13.3	−10.6	4.91	0.002
ME intake[7], MJ/d	282	253	8.21	0.01
CH$_4$-energy[8], MJ/d	26.2	25.5	1.00	0.15
Urinary-energy[9], MJ/d	18.3	16.4	0.53	0.01
DE intake[10], MJ/d	327	295	9.39	0.01
Fecal-energy[11], MJ/d	104	93.0	3.25	0.02
GE intake estimated[12], MJ/d	430	388	12.6	0.01
DMI-Measured, kg/d	23.8	22.4	0.68	0.001
DMI-Energy[13], kg/d	22.5	20.2	0.66	0.01
DMI-Predicted[14], kg/d	27.5	23.9	0.56	<0.001
DMI-CM[15], kg/d	22.9	22.4	0.83	0.20

[1]Diet was fed for ad libitum (adjusted daily to yield 10% orts) or restricted intake (set to restrict feed consumption by 10% of baseline DMI).
[2]Probability of treatment effect (AL vs. RI); significance was declared at $P \leq 0.05$ and trends at $0.05 < P \leq 0.10$.
[3]O$_2$ consumption was estimated assuming a respiratory quotient of 0.95 (QCO$_2$ ÷ O$_2$ consumption = 0.95 (Madsen et al., 2010).
[4]Heat production estimated (MJ/d) = (4.96 + 16.07 ÷ respiratory quotient) × QCO$_2$ (L/d) × 1,000 (Kaufmann et al., 2011).
[5]Milk-energy (MJ/d) = {[0.384 × fat (%)] + [0.223 × protein (%)] + [0.199 × lactose (%)]} × milk yield (kg/d) (NRC, 2001).
[6]Tissue energy balance = empty body weight energy needed to vary 1 kg was calculated according to the NRC (2001) with an efficiency of 0.85 for weight gain and 0.82 for weight loss.
[7]ME intake (MJ/d) = HP (MJ/d) + milk-energy (MJ/d) + tissue energy balance (MJ/d (NRC, 2001).
[8]CH$_4$-energy = QCH$_4$ (L/d) × 2.17 MJ/L.
[9]Urinary-energy = ME intake (MJ/d) × 0.065 (Ferris et al., 1999).
[10]Digestible energy intake (MJ/d) = ME intake (MJ/d) + CH$_4$-energy (MJ/d) + urinary-energy (MJ/d).
[11]Fecal-energy (MJ/d) = [digestible energy (MJ/d) ÷ in vitro true DM digestibility (%)] – DE (MJ/d).
[12]Gross energy intake estimated (MJ/d) = DE intake (MJ/d) + Fecal-energy (MJ/d).
[13]DMI-Energy (kg/d) = GE intake estimated (MJ/d) ÷ diet GE concentration (MJ/kg).

[14]Actual animal variables (i.e., milk yield and composition, DIM, and BW) were used in the NRC (2001) to predict DMI according to the equation DMI (kg/d) = {[0.372 × FCM (kg/d)] + [0.0968 × $BW^{0.75}$ (kg)]} × {1 − $e^{[-0.192 \times (\text{week of lactation} + 3.67)]}$}.

[15]DMI-CM: DMI (kg/d) = {821.3 + [(0.27 × QCO_2 (g/d)] + [(1.18 × milk yield (kg/d)]} ÷ 126 (Casper and Mertens, 2010).

Table 5. Regressions between actual DMI (DMI-Measured), DMI estimated by backward dietary energy partition calculations (DMI-Energy), DMI predicted by the NRC (2001) (DMI-NRC), and DMI predicted by the Cornell Net Carbohydrate and Protein System v 6.1 (DMI-CM) using a dataset from lactating dairy cows fed for ad libitum intake or restricted intake[1]

Regression variables	R^2	RMSPE[2]	Slope	Intercept	$P > F$[3]
DMI-Measured × DMI-Energy[4], kg/kg	0.28	3.19	0.54	8.97	0.002
DMI-NRC[5] × DMI-Energy, kg/kg	0.36	6.07	0.76	0.92	<0.001
DMI-Measured × DMI-NRC, kg/kg	0.50	4.36	0.57	13.9	<0.001
DMI-Measured × DMI-CM[6], kg/kg	0.30	2.81	0.60	8.74	<0.001
DMI-Energy × DMI-CM, kg/kg	0.49	2.60	0.76	6.34	<0.001
DMI-NRC × DMI-CM, kg/kg	0.28	5.09	0.73	2.97	0.003
Regression variables by treatment					
DMI-Measured × DMI-Energy, kg/kg for AL	0.39	2.88	0.62	7.53	0.01
DMI-Measured × DMI-Energy, kg/kg for RI	0.07	3.47	0.25	14.7	0.34
DMI-Measured × DMI-NRC, kg/kg for AL	0.66	3.88	0.69	10.9	<0.001
DMI-Measured × DMI-NRC, kg/kg for RI	0.26	4.79	0.41	17.5	0.05
DMI-Measured × DMI-CM, kg/kg for AL	0.29	2.91	0.58	9.17	0.03
DMI-Measured × DMI-CM, kg/kg for RI	0.31	2.72	0.69	7.04	0.03
DMI-Energy × DMI-CM, kg/kg for AL	0.56	2.08	0.79	5.19	0.001
DMI-Energy × DMI-CM, kg/kg for RI	0.51	3.03	0.96	2.86	0.003
DMI-NRC × DMI-Energy, kg/kg for AL	0.33	5.58	0.68	3.70	0.02
DMI-NRC × DMI-Energy, kg/kg for RI	0.34	6.53	0.69	1.97	0.02
DMI-NRC × DMI-CM, kg/kg for AL	0.34	5.21	0.73	2.86	0.02
DMI-NRC × DMI-CM, kg/kg for RI	0.22	4.97	0.75	2.38	0.08

[1]Diet was fed for ad libitum (adjusted daily to yield 10% orts) or restricted intake (set to restrict feed consumption by 10% of baseline DMI).

[2]RMSPE = Root mean square predictive error (Kohn et al., 1998).

[3]Probability of treatment effect (AL vs. RI); significance was declared at $P \leq 0.05$ and trends at $0.05 < P \leq 0.10$.

[4]DMI = gross energy intake estimated ÷ diet gross energy concentration.

[5]Actual animal variables (i.e., milk yield and composition, DIM, and BW) were used in the NRC (2001) to predict DMI according to the equation DMI (kg/d) = {[0.372 × FCM (kg/d)] + [0.0968 × $BW^{0.75}$ (kg)]} × {1 − $e^{[-0.192 \times (\text{week of lactation} + 3.67)]}$}.

[6] DMI-CM: DMI (kg/d) = {821.3 + [(0.27 × QCO_2 (g/d)] + [(1.18 × milk yield (kg/d)]} ÷ 126 (Casper and Mertens, 2010).

Table 6. Sensitivity analysis of DMI estimated by backward dietary energy partition calculations (DMI-Energy) by increasing the respiratory quotient (RQ = $QCO_2 \div O_2$ consumption) from 0.85 to 1.05 and reducing the in vitro true dry matter digestibility (IVTDMD) by 4% increments in lactating dairy cows fed for ad libitum intake (AL) or restricted intake (RI)[1].

Fixed variable					
IVTDMD		RQ[2]	Treatment	DMI-Energy	% Difference (DMI-Measured – DMI-Energy)
Measured	(76.1%)	1.05	AL	22.0	7.56
			RI	19.7	12.05
		1.00	AL	22.3	6.30
			RI	20.0	10.71
		0.95	AL	22.6	5.04
			RI	20.3	9.37
		0.90	AL	22.9	3.78
			RI	20.6	8.04
		0.85	AL	23.3	2.10
			RI	21.0	6.25
−4%	(72.1%)	1.05	AL	22.0	7.56
			RI	19.7	12.05
		1.00	AL	22.3	6.30
			RI	20.0	10.71
		0.95	AL	22.6	5.04
			RI	20.3	9.37
		0.90	AL	22.9	3.78
			RI	20.6	8.04
		0.85	AL	23.3	2.10
			RI	21.0	6.25
−8%	(68.1%)	1.05	AL	23.2	2.52
			RI	20.8	7.14
		1.00	AL	23.5	1.26
			RI	21.1	5.80
		0.95	AL	23.8	0.00
			RI	21.4	4.46
		0.90	AL	24.2	-1.68
			RI	21.7	3.13
		0.85	AL	24.5	-2.94
			RI	22.1	1.34
−12%	(64.1%)	1.05	AL	24.6	-3.36
			RI	22.1	1.34
		1.00	AL	24.9	-4.62
			RI	22.4	0.00

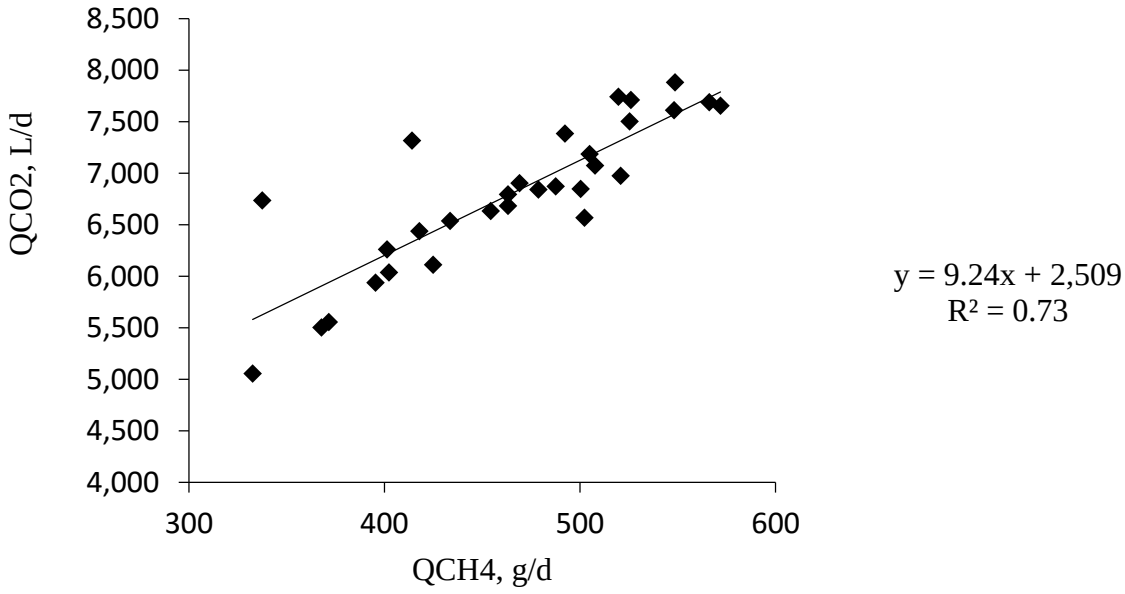
0.95	AL	25.2	-5.88
	RI	22.7	-1.34
0.90	AL	25.6	-7.56
	RI	23.0	-2.68
0.85	AL	25.9	-8.82
	RI	23.4	-4.46

[1]Diet was fed for ad libitum (adjusted daily to yield 10% orts) or restricted intake (set to restrict feed consumption by 10% of baseline DMI).
[2]Changes in the respiratory quotient according to Madsen et al. (2010) and Pereira et al. (2015).

Pereira et al. (2015).

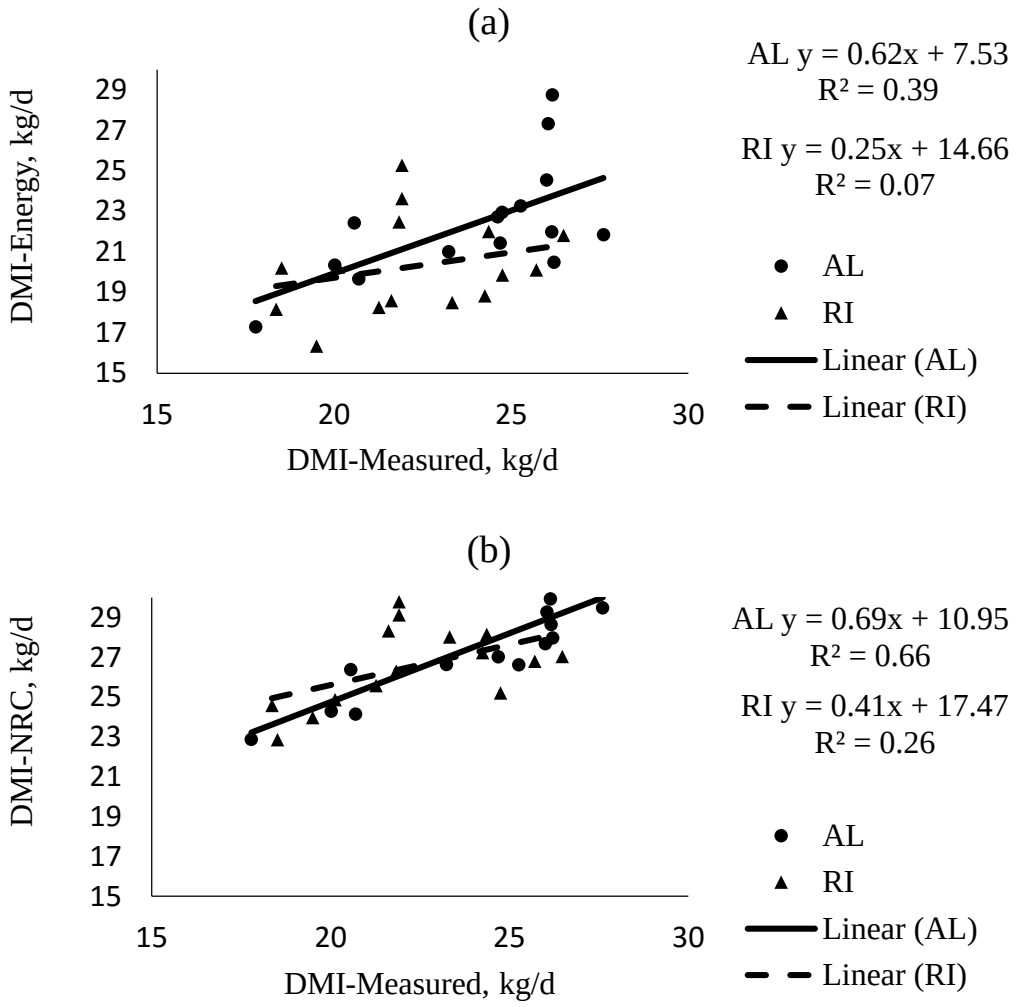
Figure 2. (a) Linear correlation between flux of CO_2 (QCO_2) and CH_4 (QCH_4) measured by the gas quantification system in lactating dairy cows fed for ad libitum or restricted intake.

(a)



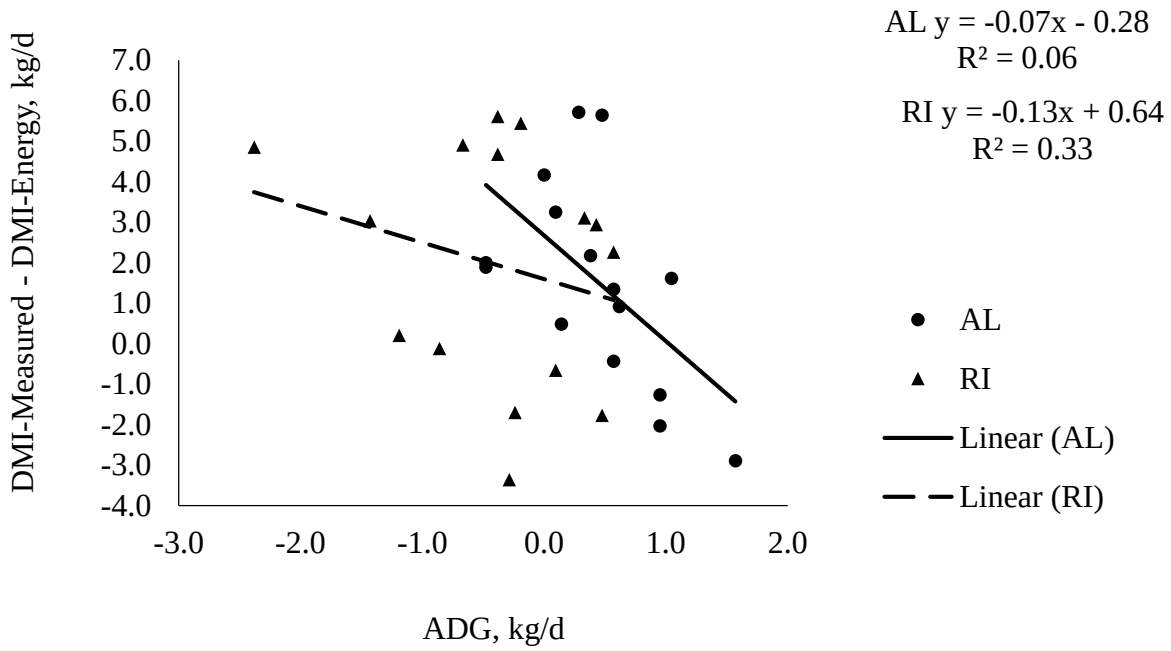
Pereira et al. (2015).

Figure 3. Correlations by treatment (AL = ad libitum intake and RI = restricted intake) between (a) actual DMI (DMI-Measured) and DMI estimated by backward dietary energy partition calculations (DMI-Energy) and (b) DMI-Measured and DMI predicted by the NRC (2001) (DMI-NRC). Diet was fed for ad libitum (adjusted daily to yield 10% orts) or restricted intake (set to restrict feed consumption by 10% of baseline DMI).



Pereira et al. (2015).

Figure 4. Correlations between (a) Average daily gain (ADG) and difference between actual DMI (DMI-Measured) and DMI estimated by backward dietary energy partition calculations (DMI-Energy).



CHAPTER III: FEEDING FIELD PEAS TO EARLY-LACTATION DAIRY COWS INCREASED HEAT PRODUCTION AND DECREASED METHANE EMISSIONS

INTRODUCTION

Ground field peas (**GFP**, *Pisum sativum*) are a pulse crop widely grown in the northern regions of United States and throughout Canada that can be used as a cover crop in the spring and can resist up to -6° C of frosts (McKay et al., 2003). This supplement is priced at US$ 200.00 / tonne (USDA, 2016), which is comparatively cheaper than a mix between corn meal and soybean meal (**SBM**), both priced at US$ 149.00 and US$ 440.00 / tonne (USDA, 2016). Ground field peas are a moderate source of protein for dairy cows, but the protein is highly degradable in the rumen, which could lead to high levels of N production and excretion to the environment (Vander Pol et al., 2009). The combination of both protein and starch in this supplement could replace a 60: 40 mix between corn meal and SBM and consequently decrease overall feeding costs.

Animal production was maintained when dairy cows were fed 16% GFP replacing SBM and corn meal, but milk and milk protein production was decreased when animals were fed either (DM basis) 24% and 36% of GFP in the total DMI (Albrecht, 2012). Microbial protein production should be maximized in order to maximize productivity and decrease N pollution to the environment (NRC, 2001). Most nutrition models predict microbial protein production is dependent on energy and an increase in microbial protein production indicates that dietary energy is highly digestible (Hristov et al., 2004). Although rolled peas or GFP caused a decrease in purine derivatives production (Vander Pol et al., 2009), the drop in production performance may be caused by a poor AA profile, more specifically a low proportion of Met in field peas that did result in a 7 : 1 ratio of Lys to Met (Vander Pol et al., 2008). Field peas had a high content of Lys, but this AA is not protected from ruminal degradation (Vander Pol et al., 2008; Vander Pol et al., 2009). Addition of RUP sources as rumen protected (**RP**) –Lys and RP – Met to the

metabolizable protein flow in the duodenum has the potential to improve milk and milk protein yield. Several studies were performed feeding GFP to dairy and beef cattle in several levels of DMI but none fed RP-Lys and RP-Met (Petit et al., 1997; Gilbery et al., 2007; Vander Pol et al., 2008).

Previous studies have shown that as cows produced more milk, the efficiency of energy utilization for milk production also increased, although animals have no change in net heat production (**HP**) (Aharoni et al., 2006). An increase in energy efficiency is expected when cows produce more milk and milk protein, as more proportion of energy is derived to milk production and a lower proportion is used for maintenance and HP. Heat production can be lower than expected if estimates are done using BW and milk yield as fixed variables (Aharoni et al., 2006). A higher proportion of energy may be directed to the mammary gland, with a reduced proportion directed to maintenance and HP, causing an increase in energy efficiency (Kebreab et al., 2003; Aharoni et al., 2006). Thus, cows can be separated in groups of high and low energy efficiency as cows with higher milk yield and no difference in HP can be considered more energy efficient (Phuong et al., 2013). In order to make this separation possible, new techniques to measure energy balance from several animals must be developed that can have the lowest effect on animal behavior and metabolism.

Calorimetric chambers have been used in the past for measurements of O_2 consumption (***QO*$_2$**) and estimations of energetic balance (Reynolds et al., 2001; Reynolds et al., 2010; Reynolds et al., 2014). This methodology is the most precise and has lower variability and higher repeatability when compared to spot short-term measurements (Hammond et al., 2015) but estimates are not practical for outdoor conditions and unrealistic, as they are made in controlled environments without complex environmental effects (Brosh, 2007). Recently, a portable gas

quantification system (**GQS**, GreenFeed system, C-Lock Inc., Rapid City, SD) was used to estimate HP from cattle according to spot short-term CO_2 flux (***Q*CO$_2$**) measurements (Pereira et al., 2015b, 2015c), but quantification of QO_2, to estimate HP (Aharoni et al., 2006; Brosh, 2007), and direct measurements of fecal energy were not performed. Estimates of HP using an O_2 sensor in the GQS, coupled with measurements of digestible energy (**DE**), ME and NE_L, will give estimates on the energy efficiency of dairy cows feeding GFP based diets.

Our hypotheses were: 1) compared with a positive control diet based on corn meal plus soybean meal and balanced for a 3:1 MP-Lys to MP-Met ratio (**CSAA** diet) according to the NRC (2001), feeding 25% of the diet DM as ground field peas (**FP** diet) would decrease milk yield and milk true protein synthesis due to a shortage of EAA especially MP-Met; 2) depression of both milk yield and milk true protein synthesis would be alleviated by balancing FP with RP-Lys and RP-Met to yield a 3:1 MP-Lys to MP-Met ratio (**FPAA** diet) similar to the positive control (i.e., the CSAA diet); 3) compared with feeding urea [i.e., the negative control diet = **U**], feeding FP diet would increase production performance, milk protein synthesis, and N use efficiency due to increased ruminal supply of true soluble protein from ground field peas versus NPN from urea. The objective of this study was to compare the effects of: 1) NPN from urea versus soluble true protein from ground field peas, and 2) starch and RDP from corn meal plus soybean meal balanced with RP-Lys and RP-Met versus starch and RDP from ground field peas balanced or not with RP-Lys and RP-Met on milk yield and composition, N use efficiency, nutrient digestibility, ruminal fermentation characteristics, and plasma concentrations of AA.

MATERIALS AND METHODS

The experiment was performed at the University of New Hampshire Fairchild Dairy Teaching and Research Center located in Durham, NH (43° 14'N, 70° 95'W) from June 29th to September 14th, 2014. Care and handling of animals were approved in accordance to the University of New Hampshire Institutional Animal Care and Use Committee guidelines (IACUC protocol no. 140402).

Animals, Experimental Design, and Diets

Twelve multiparous Holstein cows averaging (mean ± SD) 97 ± 36 DIM and 684 ± 61 kg of BW and 4 primiparous cows averaging 101 ± 23 DIM and 619 ± 35 kg of BW at the beginning of the study were selected. Cows were randomly assigned to 1 of 4 treatments in a 4 × 4 Latin Square design. The 4 squares were balanced for potential first-order carryover effects (Williams, 1949; Kim and Stein, 2009) in subsequent periods similar to Resende et al (2015). Animals were distributed in balanced squares resulting in 3 squares of multiparous cows (square 1 = 69 ± 13 DIM, 645 ± 47 kg of BW; square 2 = 83 ± 16 DIM, 662 ± 47 kg of BW; square 3 = 140 ± 22 DIM, 743 ± 46 kg of BW) and 1 square of primiparous cows (101 ± 23 DIM, 619 ± 35 kg of BW).

Animals were housed in a tie-stall barn. Individual feed intake and orts measurements were recorded using Super Data ranger (American Calan Inc., Northwood, NH) and animal's intakes were individualized using wooden feed tubs (90 × 90 × 90 cm) for each cow. The experimental diets (Table 7) contained 50% forage as corn silage (34.8%) and grass - legume mix silage (15.2%). The grass-legume silage and most of the corn silage were grown and harvested at the University of New Hampshire properties during the 2013 growing season.

Diets were formulated using the NRC (2001) model to be isonitrogenous and isoenergetic. Diets were formulated with a goal of reaching 16.5% CP and an average of -50 g/d or -300 g/d of MP balance, respectively for diets with corn and soybean meal and for diets with GFP as main sources of starch and protein. Low MP balance for diets containing GFP was due the high RDP of these diets. Addition of RP-Lys (Aji-Pro L, Ajinomoto Heartland Inc., Tokyo, Japan) and RP-Met (Smartamine-M, Adisseo, Antony, France) was calculated with the intention of replenishing AA requirements as estimated according to the NRC (2001) model. Average requirements of AA were calculated using BW, DMI, milk and milk protein variables as measured at the beginning of each experimental period after feedstuffs nutrient composition were added to the model.

Based on industry specifications for AA bioavailability (40% L-Lys monohydrochloride and 35% L-Lys bioavailability for AjiPro-L with 100% RUP digestibility; and 70% D-L-Met coated with 2-vinylpyridine-co-styrene with 75% D-L-Met bioavailability and 100% RUP digestibility for Smatamine-M), for every 100 g of AjiPro-L and Smartamine-M, 14 g and 53 g of Lys and Met, respectively, were expected to be delivered in the duodenum and be absorbed in the blood. The experimental diets (Table 7) contained (DM basis) 35.5% Corn silage, 15.5% grass-legume silage, 6% roasted soybeans, 2% alfalfa pellets and 1) corn meal (36%), SBM (2.4%) and urea (1.3%) [**U** diet], 2) corn meal (29.7%), SBM (9.8%) with RP-Lys (0.13%), and RP-Met (0.07%) [**CSAA** diet], 3) GFP (25%), corn meal (12.3%), SBM (2.4%) (**FP** diet) and 4) GFP (25%), corn meal (12.2%), SBM (2.3%) and addition of RP-Lys (0.15%) and RP-Met (0.05%) (**FPAA** diet).

Each experimental period lasted 21 d with 14 d for diet adaptation and 7 d for data and sample collection. Diets were fed as TMR and were prepared twice daily at 0630 h and 1630 h

using a Super Data Ranger mixer (American Calan Inc., Northwood, NH), placed on individual wood feed tubs (90 × 90 × 90 cm), and offered at 0700 h and 1700 h. Cows were fed 30 and 70% of the daily TMR allocation during the a.m. and p.m. feedings, respectively, to account for differences in feeding intervals between A.M. and P.M. The amount of TMR offered to the animals were recorded using the Super Data Ranger scale (American Calan Inc.). Refusals were collected daily before the p.m. feeding and weighed as done for the TMR. The TMR amounts offered to the cows were adjusted daily to yield approximately 5% refusals per animal.

Feed Sampling and Analysis

Two samples each of corn and grass-legume silages were obtained daily immediately before they were placed in the Super Data Ranger (American Calan Inc.). One sample was composited every 3 days and dried using a microwave (Model R-209KK 700 Watts, Sharp electronics, Osaka, Japan) for dietary DM adjustment. The second sample was pooled weekly and lyophilized for 48 h (Labconco freeze drier 5, Kansas City, MO) and used to determine nutrient composition. Feed amounts were adjusted every 3 days to leave approximately 5% refusals per cow. Feed TMR was weighed daily and samples were collected every 2 days after the a.m. and p.m. feeding. Orts samples were weighed daily and collected every 2 days the day after TMR samples were collected. Samples of orts and TMR were refrigerated immediately after collection and pooled weekly by treatment for DM and nutrient composition analysis. Corn meal, SBM, GFP, minerals, urea, RP-Lys, and RP-Met samples were collected weekly and lyophilized immediately. Orts and fecal samples were dried in an air forced oven (1380FMS; VWR Scientific, Radnor, PA) at 55ºC for 72 h. All samples (TMR, orts, feces, feeds, corn silage, grass-legume silage) were ground through a 1-mm screen in a Wiley mill (Arthur H. Thomas

Scientific, Philadelphia, PA), packed on labeled plastic bags, and shipped to a commercial laboratory for standardized wet chemistry analysis (Dairy One forage laboratory, Ithaca, NY). The following analysis were done in the 4 TMR and fecal samples: DM (method 930.15; AOAC International, 2006), total N (methods 990.03 and 992.23 967.07; AOAC International, 2006), NDF (method 6; Ankom Technology, Fairpoint, NY; solutions as in Van Soest et al., 1991), ADF (method 5; Ankom Technology, solutions as in method 973.18; AOAC International, 1998), crude fat (method 2003.05; AOAC International, 2006), starch (YSI 2700 Select Biochemistry Analyzer, application note no. 319, YSI Inc. Life Sciences, Yellow Springs, OH), ash (method 942.05; AOAC International, 2006), and minerals using a Thermo ICAP 6300 Inductively Coupled Plasma Radial Spectrometer after microwave digestion. Orts were analyzed for DM, ash, NDF, ADF, total N according to methods and procedures described above. Samples of TMR, feces, and individual feedstuffs were measured for gross energy at the University of New Hampshire Keener Dairy Research Building Laboratory (Durham, NH) using an adiabatic oxygen bomb calorimeter (Model 1241, Parr instruments, Moline, IL). After lyophilized (TMR and feedstuffs) or forced-air dried (feces), 1 g of each sample was pelleted using a manual press (1.27 cm diameter press) and placed in a metal jacket with a fuse wire (Platinum fuse wire, Parr instruments, 9.62 J/cm) that touched the pellet. The jacket was then flushed with 20 atm of 96.5% O_2 twice, and then compressed with 25 atm of O_2. The jacket was placed in a metal container with 2 L of water, and the sample was ignited after temperature stabilization. Temperature rise was measured every 1 min until measurements were stable for 3 consecutive min, when the final temperature was recorded. A 1 g pellet of benzoic acid (Parr instruments, 26,454 MJ/kg) was used as standard. Feedstuff samples were analyzed for AA composition at the University of Missouri Experimental Station Chemical Laboratories using a cation exchange

chromatograph (cIEC-HPLC) coupled with post-column ninhydrin derivatization and quantitation with norleucine as the internal standard (method 982.30; AOAC, 2006).

Apparent feed digestibility was estimated in 8 multiparous cows (squares 1 and 2) using the indigestible ADF (**iADF**) methodology (Huhtanen et al., 1994). Fecal grab samples were dried in an forced-air oven at 55ºC for 72 h and ground through a 1 mm screen using a Willey Mill. Individual feed samples and TMR samples were lyophilized and ground through a 1 mm screen. Half a gram of each feed and fecal sample was weighed inside 4 cm^2 Ankom F57 bags (Ankom technology, Macedon, NY). All F57 bags were place in 1 larger laundry bag (60 cm^2) and ultimately inserted into the rumen through the cannula of 1 ruminally-cannulated lactating cow for 288 h. The cow used for in situ incubation of feeds and feces was fed (DM basis) 42% corn silage, 9.6% grass-legume silage, and 48.4% concentrate mix with SBM, corn meal, steam flaked corn, beet pulp, blood meal, urea, Smartamine-M, calcium carbonate, mineral and vitamin mixes (diet was similar to that fed by Pereira et al., 2015b except for citrus pulp, which was replaced by beet pulp). After removed from the cow, bags were rinsed with water, added to an Ankom automated fiber analyzer (Ankom Fiber Analyzer A2000, Ankom technology, Macedon, NY), washed with acid detergent solution at 100°C for 1 h, rinsed with hot water (100°C water), soaked with acetone, and finally dried in an air forced oven at 105°C for 4 h. Dried samples were weighed in order to assess iADF. Estimated digestibility was calculated according to the increased proportion of indigestible ADF in the fecal sample compared to that from TMR.

Milk Sampling and Analyses

Cows were milked twice a day at 0500 h and 1600 h and milk yield was recorded throughout the experiment at each milking. A subsample of milk was collected on d 16, 17, and

18 of each experimental period during the morning and afternoon milking times in tubes containing 2-bromo-2-nitropropan-1,3 diol. Samples were pooled in duplicate by cow per day according to the proportion of milk yield in each of the milking events and kept at 4°C until sent for Dairy One Cooperative Inc. (Ithaca, NY) for determination of milk fat, true protein, lactose, and MUN by mid-infrared reflectance spectroscopy in a Milkoscan (Foss Inc., Hillerød, Denmark) and SCC by flow cytometry in a Fossomatic (Foss Inc.). Concentrations and yields of milk components were calculated as the average between the duplicate samples. Calculations of ECM and 4% FCM were performed according to (Tyrrell and Reid, 1965) and the NRC (2001), respectively. Energy contents of milk, in MJ/d, were calculated according to the NRC (2001). Efficiency was calculated using the ratios between DMI and milk yield, DMI and ECM, and DMI and 4% FCM.

Blood sampling and analyses

Blood samples were taken for 3 consecutive days, once daily 4 h after morning feeding at 1100 h on d 16, 17 and 18. Samples were taken from the coccygeal vein or artery of each cow into 2 vacutainer tubes containing EDTA (Monoject, Covidien, Mansfield, MA) and composited by cow. After collection, blood tubes were immediately transported to the laboratory, where they were centrifuged (2,155 × g for 20 min at 4°C) using an Eppendorf Centrifuge model 5810 (Eppendorf, Hamburg, Germany). Plasma from the first tube was sampled and stored at -20°C for further analyses of non-esterified fatty acids (NEFA) and plasma urea-N (PUN). Analysis of NEFA was performed colorimetrically using an UV/visible spectrophotometer (Beckman Coulter Inc., Brea, CA) set at a wavelength of 550 nm with a kit HR(2) Series (Wako Chemicals USA Inc., Richmond, VA). Analyses of PUN were performed colorimetrically [Blood Urea

Nitrogen Kit. Sigma Chemical Comp. Quantitative, Colorimetric Determination of Blood Urea Nitrogen in Serum or Plasma at 515-540 nm (Procedure No. 535)] using an UV/visible spectrophotometer (Beckman Coulter Inc., Brea, CA) set at a wavelength of 540 nm.

From the second tube, 4 mL of plasma was added to a glass 40 mL culture tube with 1 mL of 15% sulfosalicylic acid solution, added for protein precipitation and release of free AA. The solution was mixed using a Vortex (Mini Vortexer, VWR International, Bridgeport, NJ) and placed at 4° C for 10 min. In sequence, tubes were centrifuged for 20 min at 2,155 × g and 4°C and 0.6 µL of the supernatant was collected each day, pooled per cow per period into a cryovial, and stored at -80°C until sent to University of Missouri Experimental Station Chemical Laboratories. Samples were analyzed using cIEC-HPLC coupled with post-column ninhydrin derivatization and quantitation with norleucine as the internal standard (method 982.30; AOAC, 2006).

Rumen, Fecal and Urinary Sampling and Analyses

Ruminal samples were taken from 4 cows (square 1) on d 15 at the following times after feeding at 0700 h: 0, 1, 2, 4, 6, 8, 10, 12, 14, 16 and 18 h. Samples were taken using a 40 cm long PVC tube (2.5 cm diameter) hooked to a volumetric flask with an opening to an 85 mL vacuum bulb (VWR international, Radnor, PA). The PVC tube entered the rumen through a small opening on the cannula cap to prevent contamination from outside air into the gaseous phase. At each sampling, subsamples of approximately 400 mL were obtained from the cranial sac, ventral sac, and caudal sac of the rumen in random depths through the mat and liquid phase. The fluid was immediately transported to the laboratory, vortexed, and measured for pH using a portable pH meter (model SP20, VWR International, Bridgeport, NJ). After rumen pH

measurements, a subsample of 46.8 mL was acidified with 1.2 mL of 6 N hydrochloric acid into a centrifuge tube and frozen for later NH_3-N analysis. Samples were thawed at room temperature (23°C), mixed with a vortex, and centrifuged at 3,125 × g for 20 min. The supernatant was separated from the solid pellet and 10 mL was added to a beaker with 1 mL of ionic strength adjuster (ammonia pH adjusting ISA; Orion 951211; Thermo Fisher Scientific, Chelmsford, MA) that releases gaseous NH_3-N measured using a gaseous ISE meter (Orion Star A214 Benchtop pH/ISE Meter, Thermo Scientific, Waltham, MA). A subsample of 8 mL was added to 0.2 mL of 50% sulfuric acid into a cryovial and frozen at -80°C for later VFAs analysis. Samples were sent to West Virginia University Rumen Fermentation Profiling Laboratory (Morgantown, WV) for VFA analysis using a gas chromatograph with flame ionization detector (Varian model 3300, Varian Inc., Palo Alto, CA) and a 2-m × 2-mm glass column packed with 10% stationary phase 1200/1 H_3PO_4 on 80/100 Chromosorb W-AW media (Supelco Inc., Bellefonte, PA).

Urine was sampled by stimulation of the pudendal nerve massaging the area below the vulva using cows from squares 1 and 2 for 3 consecutive days (d 16, 17 and 18 of each period) every 6 h. On day 16, animals were sampled at 0000 h, 0600 h, 1200 h, 1800 h, and 0000 h. On days 17 and 18, sampling was advanced in 2 h increments in order to account for diurnal variation of urine concentration and component excretion (i.e., day 17, samples were taken at 0200 h, 0800 h, 1400 h, 2000 h; day 18, samples were taken at 0400 h, 1000 h, 1600 h, and 2200 h). If a cow refused to urinate, her sample for the respective time point was collected on d 19 of each period. Urine samples were immediately transported to the laboratory where they were mixed using a vortex. Subsamples of urine (800 µL each) were then added to 2 centrifuge tubes containing 38.4 mL of 0.072 N sulfuric acid, and then frozen at -20°C until analyses. One

subsample of urine (3.5 mL) from each cow was added to a centrifuge tube containing 1.2 mL of 6 N hydrochloric acid, frozen at -20°C, and later analyzed for NH_3-N concentration.

After thawing at room temperature, urinary samples were analyzed for concentration of creatinine (assay kit no. 500701; Cayman Chemical Co., Ann Arbor, MI) colorimetrically using a microplate reader set at a wavelength of 492 nm, allantoin (Chen et al., 1992), uric acid (assay kit no. 1045-225; Stanbio Laboratory, Boerne, TX), total N (micro-Kjeldahl analysis; AOAC, 1990; Dairy One Cooperative Inc.), urinary urea-N, and NH3-N using a gaseous ISE meter as described for ruminal samples. Urinary urea-N, allantoin, and uric acid were read at wavelengths of 540, 522, and 520 nm, respectively, on a UV/visible spectrophotometer (Beckman Coulter Inc., Brea, CA). Daily urinary volume and excretion of components were estimated from urinary creatinine concentration assuming a constant creatinine excretion of 29 mg/kg of BW (Valadares et al., 1999). Calculation of total purine derivative (PD = uric acid plus allantoin) excretion was performed according to the: PD/creatinine (mmol/L) ratio considering a constant creatinine excretion rate of 29 mg/kg of BW (Valadares et al., 1999; Chizzotti et al., 2008).

Fecal grab samples were collected at the same times as for urine samples directly from the rectum, pooled to one sample/cow/period, dried in an air-forced oven at 55ºC for 72 h and ground through a 1 mm screen using a Willey Mill.

Gaseous, Energy and Heart Rate Measurements and Analyses

Gaseous measurements were performed using the GQS mounted in a cart for use in a tie-stall barn. The system was previously described in Dorich et al. (2015), Huhtanen et al. (2015), and Pereira et al. (2015b; 2015c). Calculations of spot short-term measurements of QCO_2, CH_4 flux ($\boldsymbol{QCH_4}$) and QO_2 were performed as described in Pereira et al. (2015b). In order to decrease

bias for time of the day variation in spot short-term measurements, the GQS system was placed in front of the cows 3 times daily during 7 days. Sampling started at 0800h, 1600h and 0000h on day 14 of each experimental period, with sampling times advanced 2 hours daily (1000h, 1800h and 0200h for day 15 and so forth) in order to account for diurnal variation. Animals were not sampled between 0300h and 0600h and between 1500h and 1700h as they were being milked. Each sampling lasted 2 hours from the first cow to the last cow and had an average of 3 min and 41 sec per cow with 1 to 2 min interval between samples in order to assess background concentration of QO_2, QCO_2, and QCH_4. The RQ was calculated as the ratio between QCO_2 and QO_2 as described in Pereira et al. (2015b).

Heart rate monitors were used for 4 days in 4 multiparous cows (square 2) in order to calculate QO_2 per heartbeat similar to as described in (Aharoni et al., 2006). The transmitters (Polar H3; Polar Electro, Sweden) were placed into a girth strap (Polar equine belt; Polar Electro) to secure the transmitter in place. Two areas, the first below the right shoulder of the animal, behind the scapula/humeral joint on the 7th intercostal space, and the second right above where the heart rests, between the fourth and fifth intercostal space on the ventral chest left area, were clipped and covered in electrolyte gel (Lectron II; Pharmaceutical innovations Inc., Newark, New Jersey). Data were recorded every 5 sec for 96 h. A total of 9 min 12 sec could be correlated for O_2 consumption per heartbeat per cow daily to calculate the O_2 pulse (Aharoni et al., 2003).

Gross energy intake was calculated based on measurements of energy density of the TMR (MJ/kg) multiplied by DMI (kg/d). Fecal excretion of energy was estimated using the fecal energy concentration (MJ/kg) multiplied by estimated fecal DM output (kg/d). Tissue energy balance was calculated according to the empty BW energy needed to vary 1 kg of BW with an efficiency of

0.85 for weight gain (31.8 MJ/kg) and 0.82 for weight loss (29.2 MJ/kg, NRC 2001). Heat production (MJ/d) was calculated as: 20.47 (kJ/L) ÷ 1000 × QO_2 (L/d; Nicol and Young, 1990; Aharoni et al., 2006). Metabolizable energy was calculated as: HP + milk energy + tissue energy balance (MJ/d). Urinary energy was estimated as 6.5% of ME intake (Ferris et al., 1999; Ferrell and Oltjen, 2008). Digestible energy (**DE**) intake was calculated as: ME intake + QCH_4 energy + Urinary energy (MJ/d; Pereira et al., 2015b).

Statistical Analyses

Data were analyzed using the MIXED procedure of SAS (SAS version 9.4) according to a replicated 4 × 4 Latin square design. Pairwise orthogonal contrasts were constructed to compare the effects of 1) NPN vs. soluble true protein = U vs. FP; 2) GFP vs. corn meal plus SBM balanced for Lys and Met = FP vs. CSAA; and 3) GFP balanced for Lys and Met vs. corn meal plus SBM balanced for Lys and Met = FPAA vs. CSAA. The following model was fitted for DMI, milk yield, milk components, plasma analyses (squares 1, 2, 3 and 4), digestibility and urine (squares 1 and 2), QCH_4, QCO_2, QO_2, respiratory quotient, and minutes of spot short-term measurement (squares 2, 3 and 4):

$$Y_{ijklm} = \mu + S_i + P_j + C_{k(i)} + TRT_l + S \times TRT_{il} + E_{ijkl}$$

where Y_{ijkl} = dependent variable, μ = overall mean, S_i = fixed effect of i^{th} square, P_j = fixed effect of j^{th} period, C_k = random effect of k^{th} cow within i^{th} square, TRT_l = fixed effect of l^{th} treatment, $S \times TRT_{il}$ = interaction between i^{th} square and l^{th} treatment, and E_{ijkl} = error term ~ N (0, σ^2_e).

For analysis of ruminal VFA, pH, and NH_3-N, the following model was used:

$$Y_{ijklm} = \mu + P_i + C_{j(i)} + TRT_k + E_{ijk} + HOUR_l + TRT_k \times HOUR_l + E_{ijkl}$$

where Y_{ijkl} = dependent variable, μ = overall mean, P_j = fixed effect of i^{th} period, C_j = random effect of j^{th} cow within i^{th} period, TRT_k = fixed effect of k^{th} treatment, E_{ijk} = whole plot error, $HOUR_l$ = fixed effect

of l^{th} sampling hour and $TRT_k \times HOUR_l$ = interaction between i^{th} treatment and l^{th} sampling hour, and E_{ijkl} = subplot error term $\sim N (0, \sigma^2_e)$. The covariance structure of the R matrix used was "spatial exponential [SP(EXP)]" for estimating covariance with cow (period) as subject according to the lower akaike information criterion compared with unstructured, heterogeneous autoregressive, compound symmetry and spatial power.

For analysis of energy balance, QO_2, milk, fecal and urinary energy estimations, GE, DE, ME intakes, tissue energy retention, O_2 pulse, and heartbeat data, the following model was used with 1 square of cows (square 2):

$$Y_{ijklm} = \mu + P_i + C_{j(i)} + TRT_k + E_{ijk} + DAY_l (P_i) + E_{ijkl}$$

where Y_{ijkl} = dependent variable, μ = overall mean, P_j = fixed effect of i^{th} period, C_j = random effect of j^{th} cow within i^{th} period, TRT_k = fixed effect of k^{th} treatment, E_{ijk} = whole plot error, DAY_l = fixed effect of l^{th} sampling day within i^{th} period, and E_{ijkl} = subplot error term $\sim N (0, \sigma^2_e)$. The covariance structure of the R matrix used was "compound symmetry" for estimating covariance with cow as subject according to the lower akaike information criterion compared with unstructured, heterogeneous autoregressive, and spatial power. The interaction between hour and treatment was removed from the gas measurements model, as it was not significant ($P > 0.25$).

Individual 5-min measurements of gas flux from all animals were used to calculate repeatability, which was the variance of cow within period divided by the total variance (Huhtanen et al., 2015). The Kenward-Roger degrees of freedom correction was used for all variables. All results are expressed as least square means and were considered significant at $P \leq 0.05$. Trends were declared at $0.05 < P \leq 0.10$. Interaction terms were removed from the final model when $P \geq 0.25$.

RESULTS AND DISCUSSION

Dietary Nutrient Composition and Estimation of Duodenal AA Flow

Nutrient composition of dietary ingredients is shown in Table 7. Ground field peas had 21% CP, less than half of SBM (51.1% CP), but values were similar for NDF and ADF. Soybean meal had a numerically higher concentration of GE when compared to GFP (12.9 vs. 10.4 MJ/kg, respectively). Values of GE for GFP were lower than the 19.2 MJ/kg that was reported by (Petit et al., 1997), which may be caused by a poorer feed grade used in this experiment. Relative to AA composition, GFP had higher concentration of Lys as a percentage of CP when compared to SBM (7.36 vs. 6.26%, respectively), but lower concentration of Met (1.07 vs. 1.32%, respectively). Concentrations of branched chain AA and other essential AA (**EAA**) were similar between these 2 feeds except for Trp, which was almost half in GFP than in SBM (0.85 vs. 1.48%, respectively). The concentration of starch in GFP averaged 44.9%, which is comparable to results reported the literature (Vander Pol et al., 2008).

Ingredient and nutrient composition of diets are shown in Table 8. Dietary CP was similar across diets, but with a lower concentration of soluble CP in CSAA (+) compared to the other 3 diets. Numerically lower contents of NDF and ADF were observed in the U diet compared to CSAA, FP, and FPAA. Concentration of soluble protein was numerically lowest for the CSAA diet [34.8% vs. 49%, 46% and 47% for CSAA, U, FP and FPAA, respectively]. Starch content was numerically higher for U and CSAA diets compared to FP and FPAA [35.5% and 34% vs. 31.2% and 32.2% for CSAA, U, FP, and FPAA, respectively].

Estimations obtained from the NRC (2001) model are shown in Table 9. Dietary analysis showed diets had similar NE_L. Balance of MP was relatively similar among U (-397 g/d), FP (-422 g/d) and FPAA (-437 g/d), and less negative for CSAA (-163 g/d), which was consistent

with the low concentrations of dietary CP. Although there were numerical differences for MP balance among diets, MP-bacterial was similar (mean = 1,300 g/d). The main difference among diets was for MP-RUP, which was numerically higher for the CSAA diet [1,218 vs. 809, 826 and 854 g/d for CSAA, U, FP, and FPAA, respectively]. Diet U had the greatest MP-Lys to MP-Met ratio , which averaged 3.59:1, and a MP-Lys to MP-His ratio of 3.08:1. The 2 diets with RP-Lys and RP-Met had MP-Lys to MP-Met ratios of 3.07:1 (CSAA) and 3.13:1 (FPAA), respectively, which were slightly higher than the recommended range of 2.9 to 3.0:1.0 (NRC, 2001). The diets with RP Lys and RP Met were formulated to yield a 3.0:1 ratio, and the discrepancy relative to the target occurred because of differences in the concentrations of Lys and Met in SBM and roasted soybean, which had higher values in the NRC (2001) feed library compared to actual feed analysis.

Dry Matter Intake, Milk Yield and Milk Composition

Dry matter intake, milk and milk components results are shown in Table 10. As shown previously (Brito and Broderick, 2007), DMI was lower for cows fed U treatment compared to FP. Similarly, cows fed a diet with only urea as the supplemental protein source had lower DMI compared to other diets supplemented with true protein (Brito and Broderick, 2007). Increased DMI in the present study for FP compared to U was probably due to the presence in the rumen of preformed AA and peptides, which can stimulate microbial growth (Olmos Colmenero and Broderick, 2006; Brito et al., 2007), but we did not directly measured these values. No difference for DMI was observed between cows fed CSAA vs. FP and FPAA. Feeding cows RP-Lys and RP-Met should not have an effect in DMI according to previous research (Leonardi et al., 2003; Robinson, 2010). Albrecht (2012) when feeding lactating dairy cows increasing amounts (DM

basis) of GFP (0, 12, 24 and 36%) found that DMI decreased linearly from 24.2 kg/d to 21.8 kg/d as did Vander Pol et al. (2009) feeding rolled peas or GFP. On the other hand, other researchers reported that GFP did not decrease DMI, but proportions in the diet were at 20.2% for raw and extruded peas (Petit et al., 1997) or at 15% (Vander Pol et al., 2008).

Milk yield and 4% FCM were similar between U and FP and between CSAA vs. FP and FPAA (Table 10). Energy corrected milk had a trend ($P = 0.08$) to be lower for U when compared to FP, which was due to a lower ($P = 0.03$) milk protein yield from U compared to FP. No difference for the concentration and yield of milk fat was observed between the NPN-based diet (i.e., U) and the soluble true protein-based diet (i.e., FP). Milk fat % and yield were higher ($P = 0.01$ and 0.04, respectively) for cows fed FPAA vs. CSAA, which may have been caused by a difference in acetate to propionate ratio in the rumen. A decrease in milk yield was observed when cows were fed rolled or ground peas (Vander Pol et al., 2009) and a linear decrease was observed for milk and milk fat yield when increasing concentrations of GFP (0, 12, 24 and 36%) were added to the diet (Albrecht, 2012). Petit et al (1997), and Vander Pol et al. (2008) found no differences in production responses between diets with or without GFP.

Milk true protein yield for cows fed U was of 1.15 kg/d, lower than protein yield of cows fed FP, which may have been due to higher DMI, energy intake for cows fed FP, and higher concentration of preformed AA and peptides in the rumen for microbial protein production. Concentration of milk protein was higher for CSAA vs. FP ($P < 0.01$), and milk true protein concentration and yield were similar between CSAA and FPAA. Albrecht (2012) reported that cows had decreasing milk true protein yield when fed increasing amounts of GFP. Brito and Broderick (2007) observed lower milk protein concentration and yield in cows fed urea than true protein from SBM, canola meal, or cottonseed meal. Hristov et al. (2004) conducted a meta-

analysis with 846 diets examining the factors that contribute to higher milk protein yield and concluded that milk protein is affected by DMI and dietary concentration of soluble protein, carbohydrates and their interactions when using the Cornell – Penn – Miner model. When using the NRC (2001) model, factors that affected milk protein yield were BW and dietary components DMI and concentrations of CP, MP, NDF (Hristov et al., 2004). Cows fed FP had higher dietary NDF content compared to U, which was not enough to result in differences in milk protein yield. It is possible that no significant differences between NPN from urea and soluble protein from GFP exist that would improve milk protein yield. Improved milk protein yield in CSAA vs. FP was probably due to a better EAA profile reaching the duodenum as described in Allen (2000) and Lee et al. (2012), with a Lys to Met ratio of 2.99:1 (Table 9). When GFP-based diets were added with RP AA, milk protein yield responses were similar to a corn meal and SBM control diet (CSAA).

Values of MUN for U (9.85 mg/dL) were higher than FP (9.09 mg/dL, SEM = 0.27) Cows on FP produced more milk protein % and yield and, although no difference was found between diets for milk true N efficiency (milk N ÷ N intake), higher values of MUN can be explained by this difference in milk protein production. Milk urea nitrogen concentrations were lower for cows fed CSAA (7.93 mg/dL) vs. FPAA. A difference in soluble protein intake was pronounced between CSAA and FP or FPAA diets ($P < 0.001$) and the lower MUN concentrations can be due to the lower soluble protein intake for CSAA. Plasma urea nitrogen was higher for U (13.9 mg/dL) compared to FP (10.6 mg/dL) and were not different between CSAA and FP or FPAA. Cows fed CSAA had MUN values lower than the recommended range of 8.5 mg/dL to 11.5 mg/dL (Kohn et al., 2002). Although these values were low, milk yield was not affected.

Gaseous Measurements, Ruminal Metabolism and Apparent Digestibility

Results of spot short-term gaseous measurements are shown in Table 11. Lower QCH$_4$ production was found for cows fed U (341 g/d) compared to FP (390 g/d, P = 0.02). Cows fed CSAA had higher QCH$_4$ compared to FPAA (379 and 355 g/d, respectively, P = 0.04). Release of CH$_4$ from the rumen is directly related to DMI, and NDF digestibility (Hristov et al., 2013). Cows had low QCH$_4$ when fed U as a consequence of having lower DMI and NDF digestibility (Table 11). No difference was found between diets for the ratios between QCH$_4$ and DMI, milk yield, ECM and 4% FCM. Microbiome analysis due to feeding FP to dairy cows have not yet been assessed and should be the focus of further studies so relationships with QCH$_4$ can be made.

No difference was found between diets for QCO$_2$ and QO$_2$, showing that release of CO$_2$ from the rumen and metabolic energy consumption in the body were similar between all pairwise comparisons. In several occasions, cows did not keep their heads inside the GQS during all 5 minutes of each spot short-term measurement period (average of 3 min and 42 sec), which caused a loss in 62% of total measurements performed. When the GQS head position sensor identifies that the animal head is positioned at > 30 cm from the gas sampling area, the GQS automatically discards the data of that measurement. For 62% of all sampling times performed, cows did not maintain their nostril at ≤ 30 cm, and the data was not used in this analysis resulting in the lower than intended number of measurements per cow in each period, but higher quality of measurements (Huhtanen et al., 2015).

Repeatability of gas flux measurements (data not shown) was of 91.3% for QCH$_4$, 92.5% for QCO$_2$ and 74.1% for QO$_2$. The within cows' coefficient of variation for QCH$_4$, QCO$_2$ and

QO_2 was, respectively, 25.5, 15.2, and 22.2%. The between cow coefficient of variation was 7.04, 4.34, and 5.79 %, respectively for QCH_4, QCO_2 and QO_2. In a study using the GQS with 75 lactating cows in a free-stall barn in Sweden and 118 lactating dairy cows in Michigan, repeatability of QCH_4 and QCO_2 were of 75.0 and 86.0% on average (Huhtanen et al., 2015). In a previous experiment at the University of New Hampshire, 16 different cows using the same GQS had repeatability values of 88.0 and 87.0%, respectively for QCH_4 and QCO_2 (Pereira et al., 2015b). Besides of high number of measurements discarded, the GQS did capture a difference in QCH_4 between treatments and was able to estimate gaseous emissions with low error, such as previously described (Huhtanen et al., 2015; Velazco et al., 2015), although no difference was found between treatments for QCH_4 as a proportion of DMI, milk yield, ECM and 4% FCM.

Data shown herein had higher repeatability values for QCH_4 and QCO_2 than previously reported in literature (Huhtanen et al., 2015; Velazco et al., 2015), but values for QO_2 cannot be compared to other values using the GQS, as there are none reported in literature. Direct comparisons with respiratory chambers should not be done as the 2 systems are not comparable (Dorich et al., 2015). In our study, QO_2 measurements had high noise in the electrochemical sensor response when compared to the near infrared sensors of CH_4 and CO_2, which could have increased variability between measurements and caused a decreased repeatability. Noise was caused by the impact of the sensor's air pump piston on the sensor, which was further easily repaired using a longer air tube between the pump and the sensor variations in air humidity can affect the O_2 sensor, as it would measure the O_2 present in water if humidity reached the electrochemical plate, but this problem was mathematically accounted for before the results were calculated and O_2 from humidity was removed from the final QO_2 results according to the

following equation: {QO_2 (L/s) = [QO_2 measured from GF (L/s) × barometric pressure (kPa) ÷ (barometric pressure (kPa) – water vapor pressure (kPa)]} (Melanson et al., 2010).

The RQs were similar across treatments, but, on average (RQ = 0.88), lower than results from the literature of 0.9 to 1.1 using different gaseous measurement techniques (Ferris et al., 1999; Madsen et al., 2010). Respiratory quotient from the GQS was never assessed and direct comparisons are not possible. Although a high repeatability for QO_2 was found, the RQ used may not have been entirely representative of the daily QO_2 utilization because of the removal of 62% of the data due to poor head positioning. In addition, higher environmental concentration of either CH_4 or CO_2 can increase bias of QCH_4 and QCO_2 (Huhtanen et al., 2015) and RQ as a result. However, in the current experiment, the barn fans were turned on during all the time, so barn ventilation and background gas concentration likely had low interference with the results. Further studies are recommended focusing on how head proximity and other environmental factors can impact QO_2 as have been done for QCH_4 and QCO_2 (Huhtanen et al., 2015).

Results of ruminal samples analysis are presented in Table 11. No difference was found for ruminal pH across diets. The 24-h profile of ruminal pH is presented in Figure 5. Albrecht (2012) found that ruminal pH decreased quadratically in cows fed increasing amounts of field peas. Total NH_3-N concentration was higher ($P < 0.01$) for the U diet compared to FP and higher ($P = 0.05$) for FPAA compared to CSAA. High release of NH_3-N is expected for diets with high amounts of urea such as U (NRC, 2001). Higher concentration of NH_3-N was previously reported in cows fed 24% field peas (Albrecht, 2012). Addition of 0.9% of urea in a corn silage-based diet increased NH_3-N concentration in the rumen significantly more than a diet without urea added (Boucher et al., 2007). An increase in NH_3-N concentrations is desired to increase availability of substrate for rumen microbial protein synthesis, but excess NH_3-N not used by

microbes will be converted to urea and excreted, decreasing overall N efficiency (NRC, 2001; Colmenero and Broderick, 2006; Boucher et al., 2007). When comparing CSAA and FP or FPAA, the higher values of NH_3-N for the latter were probably due to increased intake of soluble protein when diets were fed with GFP when compared to corn meal and SBM.

Cows fed FPAA had higher concentration of total VFAs when compared to CSAA. Cows fed true protein FP had highest concentration of VFAs compared to cows fed U [112 mM per day vs. 103 mM, respectively for FP and U, $P < 0.01$]. Cows fed U had higher concentration of acetate than cows fed FP and less propionate. Cows fed FPAA had higher proportion of acetate than cows fed CSAA but less propionate, which can explain the highest concentration of milk fat with feeding FPAA (Table 10). Cows fed U fed higher acetate : propionate ratio than those fed FP, but no effect on milk concentrations of fat and lactose was found (Table 10). Beef steers fed 20.5% GFP, compared to a control fed corn meal and canola meal, had similar DMI, NDF, and ADF apparent total tract digestibility, similar concentration of propionate, but lower concentration of acetate (58.8 mM vs. 63.6 mM, respectively Gilbery et al., 2007).

No differences were found for DM, OM and starch digestibilities among diets (Table 11). Digestibility of NDF was lower for cows fed U compared to FP, which is expected as this diet had less concentration of true protein, necessary for cellulolytic microbes in the rumen in order to be able to digest fiber (Brito et al., 2007). No difference was found between CSAA and FP or FPAA diets for NDF digestibility.

Nitrogen and Energy Balance Results

Results for N balance are presented in Table 12. Total N intake, was not different among diets for this study. Total estimated urine production, total manure N excreted, urinary N-NH_3,

and consequent percentages of excretion relative to N intake were not different between diets. Cows fed U had similar values of urinary urea N excretion compared to cows fed FP (117 and 103 g/d, respectively), and cows fed CSAA had similar values of urinary urea N excretion compared to cows fed FPAA (respectively, 83.5 g/d and 80.5 g/d) but lower values compared to cows fed FP. Brito and Broderick (2007) found increased total N excretion and urinary urea N excretion when feeding a diet with urea as the only source of supplemental protein compared to diets with SBM or cottonseed meal or canola meal. In contrast with the present study, diets in Brito and Broderick (2007) did not contain RP AA. When fed a diet deficient in MP with addition of RP-Lys and RP-Met, cows had lower excretion of urinary urea N (48 g/d) compared to a diet with adequate levels of MP without RP AA (Lee et al., 2012). Cows fed a diet deficient in MP (Lee et al., 2012) or adequate in MP (Leonardi et al., 2003) with addition of RP-Met had total N excretion in urine of 97 and 201 g/d, respectively. Results presented herein for cows fed RP AA (185 and 165 g/d, respectively for CSAA and FPAA) showed similar excretion of total N in urine when feeding either SBM or GFP in a diet supplemented with RP AA.

No differences were found between treatments for estimations of manure total N excretion (Table 12). Cows fed a similar urea treatment without true protein had lower excretion of total feces (6.11 kg/d) and also lower total excreted N compared to our study (Brito and Broderick, 2007). Total N excretion was not different between treatments U vs FP and CSAA vs. FP or FPAA. In a study feeding late lactation cows diets deficient in MP with RP AA, a trend for higher efficiency in N retention and secretion in milk was found compared to a diet with RP AA added (Pereira et al., 2015a). Lee et al. (2012) also found less excretion of N from the body when feeding diets deficient in MP with addition of RP-Lys and RP-Met. Higher secretion of N in milk was only achieved when RP His was fed to low MP diets (Lee et al., 2012).

Purine derivatives results are shown in Table 12. Cows fed U had lower excretion of total allantoin when compared to cows fed FP ($P < 0.01$) but similar excretion of uric acid ($P = 0.52$). Cows fed FP had higher purine derivatives concentration, with no difference found between cows fed CSAA vs. FP or FPAA. As discussed before, higher concentration of purine derivatives may have been due to higher availability of preformed AA and peptides in the rumen, thus increasing ruminal microbial protein production (Brito et al., 2007).

Energy balance results are presented in Table 13 (n = 16 observations, square 2). Cows had higher GE intake when fed FPAA vs CSAA. No difference was found between other contrasts. Intake of DE was highest for cows fed FPAA compared to CSAA. This also led to higher estimated values of energy excretion in urine. As a consequence, the amount of energy retained in the body available for metabolic processes (i.e. ME intake) was significantly higher for FPAA (310.9 MJ/d) when compared to cows fed CSAA (252.8 MJ/d).

No difference was found among U and FP diets for release of energy in milk and ME efficiency (milk energy ÷ ME intake). Cows fed FPAA released more energy in milk and retained more tissue energy compared to CSAA. No difference was found for HP among FP or FPAA versus CSAA. Cows fed U had lower HP compared to cows fed FP. Increase in ADG resulted in a higher estimated value of ME intake for FPAA. One hypothesis for higher HP in FP vs. U is that an increase in nutrients supply to somatic cells results in higher metabolic rate and, consequently, increase in cellular HP metabolic processes, as DMI was higher for FP (Rolfe and Brown, 1997). Significant correlations have been found between DMI and QCH_4 (Hristov et al., 2013; Pereira et al., 2015b). A significant relationship between ME intake and HP was found in our study [Figure 1 (a)] and is consistent with previous findings (Rolfe and Brown, 1997; Dong et al., 2015), but work with dairy cows using spot short-term technology for HP measurements is

scarce (Hammond et al., 2015; Huhtanen et al., 2015, Dorich et al, 2015). In addition, higher levels of nutrient digestibility in the rumen can directly increase HP by increased microbial growth or by non-growth energy dissipation mechanisms (Russell and Cook, 1995), which was observed in our study (Table 12). Increased microbial metabolism can result in more QCH_4 being produced. A significant relationship between ME intake and QCH_4 was found in our study [$R^2 = 0.649$; Figure 6(b), n = 16 observations].

As HP increased, milk yield decreased in the present study [Figure 6 (c), n = 16 observations]. As milk yield increased, it was hypothesized that more energy was directed to the mammary gland and less was used for HP by the cow. The only energetic partition not directly assessed in the present study was maintenance requirements, which is not dependent on milk production. As described in the literature, energy required for maintenance is closely related to HP and studies have reported these values may not change (Aharoni et al., 2006) or slightly increase as DMI increases (Dong et al., 2015). Thus, our results suggest that animals producing more milk had lower HP and, consequently, less waste of energy as HP. Although cows fed FPAA had higher ME intake compared to CSAA, we could not conclude if this energy was directed or not to HP, as this variable was similar between the 2 diets. In fact, when observing the relationship between milk yield and HP, cows that produced more milk produced less heat [Figure 6 (c), n = 16]. The extra energy that did not go to HP may have been directed in the body for milk production. No relationship was found for milk components and HP fed any of the diets ($r^2 = 0.07$, data not shown).

Plasma Amino Acid Concentrations

Concentrations of plasma AA are shown in Table 14. Amino acid differences between primiparous and multiparous cows for plasma Arg concentration, were not found ($P > 0.05$). Contrary to our study, results of a recent meta-analysis showed that primiparous cows have a greater concentration of Arg in plasma when compared to multiparous cows (104.5 μ*M* and 81.2 μ*M* respectively; Patton et al., 2015).

Concentration of plasma Met was not different between U and FP, but was higher for animals on FPAA (31.9 μ*M*) compared to cows fed CSAA (27.7 μ*M*). According to the duodenal AA flow calculated by the NRC (2001) model, cows on CSAA and FPAA had a similar amount of available Met (67 g/d and 64 g/d, respectively), but when all production variables (BW, DMI, milk yield and milk composition) were added to the model, Met availability was only 2.15% of total MP flow for CSAA and 2.31 % of total MP for FPAA, which may be an explanation for plasma Met being higher for cows fed FPAA compared to cows fed CSAA. Cows fed U had lower plasma concentration of Lys than those fed FP, and cows fed CSAA had lower concentrations of plasma Lys than those fed FPAA. These findings may be due to the high concentration of Lys in FP and consequent high proportion of Lys as a % of total MP fed as modeled by the NRC (2001). As previously reported, Met and Lys are the first limiting AA in diets based on corn silage, alfalfa silage, corn meal and SBM (Leonardi et al., 2003; Broderick et al., 2008; Lee et al., 2012). Concentration of plasma Lys and Met increased linearly as a function of their duodenal availability independently if the requirements were met or not (Patton et al., 2015).

Milk protein yield response in our study suggests that cows were deficient in some EAA, independently of treatment fed. This suggestion is based on the results of a recent meta-analysis with 106 studies and 420 rations (Patton et al., 2015). All values of plasma AA, except for Thr

and Ile, presented herein (Table 14) were lower than values found in the developmental and validation databases of the meta-analysis (Patton et al., 2015). In addition, the mean concentration of milk protein in the meta-analysis of Patton et al. (2015) was higher than that of the current study (2.96 vs. 2.88%, respectively). Thus, it is possible that cows fed U were limited in both Lys and Met, cows fed CSAA were limited in Ile, cows fed FP were limited in Met, and cows fed FPAA were limited in either His or Leu.

Milk protein concentration was similar between U and FP, and between CSAA and FPAA, but overall lower than 3% for all dietary treatments, which corroborates with what would be expected for cows limiting in EAA. Looking at a whole body metabolism, cows that are deficient in EAA for milk production are also deficient in EAA for production of body proteins, which could result in a poor overall health. When EAA are insufficient from the diet, other metabolic pathways besides milk protein production may also be affected (Osorio et al., 2013). Besides milk protein, the body needs AA for protein synthesis in order to produce receptor proteins, messenger proteins, enzymes, immunoglobulins and overall regulatory proteins, which could lead to other metabolic problems in the long term such as ketosis (McCarthy et al., 1968), low immune function and limited liver lipid metabolism and release (Osorio et al., 2013).

Histidine may be unique compared to all EAA as it has labile pools [i.e., the intramuscular dipeptides carnosine (β-alanyl-l-histidine) and anserine (β-alanyl-N-methylhistidine), as well as circulating hemoglobin] that provide a source of endogenous His during short periods of deficiency (Lapierre et al., 2008, 2014; Ouellet et al., 2014). For instance, Lapierre et al. (2008) estimated that the total body concentration of carnosine in dairy cows would be approximately 420 g of His, which could substantially supply His while buffering its deficiency particularly in changeover studies with short experimental periods. In fact, short-term

studies (7- to 14-d periods) resulted in linear increases for plasma His and carnosine, a quadratic trend for increased muscle carnosine, and linear and quadratic trends for increased muscle anserine in lactating dairy cows abomasally infused with incremental amounts of His (Lapierre et al., 2014; Ouellet et al., 2014). Additional evidence for the role of His labile pools to buffer its deficiency was the lack of effect when feeding adequate- versus deficient-MP diets on the plasma concentration of His in a short-term, changeover design study using lactating dairy cows (Lee et al., 2015), but an opposite trend in long-term, continuous randomized complete block design studies (9- to 12-wk long; Lee et al., 2012a,b; Giallongo et al., 2016). In the current 21-d period Latin square study, the plasma concentrations of both His and carnosine were lower (P = 0.03; Table 14) in cows fed FPAA than in those fed CSAA, suggesting that mobilization of His from its labile pools may not have been enough to raise His to concentrations similar to those found in the remaining 3 diets. On the other hand, the plasma concentration of His did not change with feeding FP versus U, but plasma carnosine did decrease (21.8 versus 19.8 μM) indicating that cows fed U likely relied on carnosine and maybe hemoglobin or anserine to mitigate short-term deficiency in His. Brito et al. (2007) demonstrated that cows fed urea had lower omasal flow of His than those fed soybean meal, cottonseed meal, or canola meal. This reinforce the hypothesis that cows fed NPN-rich diets may rely on His labile endogenous pools to meet requirements. Because no anserine was detected in the plasma of cows used the present study, it can be hypothesized that this dipeptide was either not mobilized from muscles or was completely catabolized to provide His. Lee et al (2012a) also did not detect any anserine in plasma of cows fed adequate versus deficient-MP diets supplemented with RP-AA.

CONCLUSIONS

Feeding cows with FP and RP-AA (i.e., FPAA diet) had similar milk protein yield when compared to the positive control diet based on soybean meal and corn meal [i.e. CSAA]. Milk protein yield was lower for cows fed a negative control diet [i.e. U] based on urea vs. cows fed FP, which showed that true protein from FP improved milk protein yield in the current experiment, but no difference was found between diets supplemented with RP AA (i.e. CSAA vs. FAPP). Feeding FPAA decreased plasma concentration of His compared to CSAA, leading to the assumption that His could be the third limiting AA in diets based on GFP supplemented with RP AA, particular RP-Met. Overall, milk N efficiency was not different among all diets.

Overall, $Q\mathrm{CH_4}$ calculated using spot short-term measurements was low for all treatments. Feeding soluble non protein N [i.e. U] could have caused a decrease in bacterial population in the rumen when compared to feeding soluble true protein (i.e. FP), resulting in lower $Q\mathrm{CH_4}$. No differences were found among diets for $Q\mathrm{CO_2}$ and $Q\mathrm{O_2}$. The average RQ was 0.88, which was similar to the respiratory quotient found in literature. Although further studies are necessary for assessment of residual HP and energetic efficiency in dairy cows, in this study, higher ME intake resulted in higher HP. Cows fed U had lower HP than cows fed CSAA, mainly due to higher ME intake, which may have increased total tissue metabolism.

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Table 7. Nutrient composition of feed ingredients used in the experimental diets.

Item	Corn silage	Grass-legume silage	Corn meal	Soybean meal	Ground field peas	Roasted soybean	Urea	Alfalfa pellets[1]
DM, % of fresh matter	29.7	32.5	88.4	89.3	89.2	94.3	99	89.9
CP, % of DM	7.22	14.2	8.50	51.1	21.0	39.8	290	13.4
Soluble protein, % of CP	-	-	-	15.0	69.0	-	-	-
Gross energy, MJ/kg of DM	11.6	15.0	13.4	12.9	10.4	16.3	-	10.4
NDF, % of DM	40.6	62.3	7.90	10.8	11.9	21.7	-	58.3
ADF, % of DM	24.2	42.3	3.30	5.40	4.40	15.3	-	36.7
Starch, % of DM	43.3	-	71.0	-	44.9	-	-	-
EAA, % of CP								
Arg	1.78	1.83	4.64	6.99	7.58	7.46	-	
His	1.23	0.95	2.78	2.50	2.46	2.64	-	-
Ile	3.55	3.00	3.56	4.43	4.11	4.58	-	
Leu	8.47	4.98	10.8	7.40	7.26	7.89	-	
Lys	2.73	2.63	3.71	6.26	7.36	6.39	-	-
Met	1.64	1.10	2.16	1.32	1.07	1.39	-	-
Phe	3.41	3.07	4.64	4.88	4.80	5.28	-	
Thr	3.41	2.49	3.56	3.70	3.84	3.97	-	
Trp	0.55	0.73	0.77	1.48	0.85	1.15	-	
Val	4.37	3.37	4.48	4.31	4.59	4.72	-	
NEAA, % of CP								
Ala	8.06	5.12	6.96	4.10	4.38	4.32	-	-
Asp	6.15	5.42	6.96	10.7	11.1	11.6	-	-
Cys	1.23	0.66	2.16	1.26	1.39	1.33	-	-
Gly	11.2	5.34	16.9	16.8	4.48	4.29	-	-
Glu	3.82	3.22	4.17	4.04	16.0	17.3	-	-
Orn	0.68	0.44	0.00	0.08	0.05	0.08	-	-
Pro	5.60	2.78	8.04	4.51	4.22	5.17	-	-
Ser	3.00	2.05	4.64	4.21	4.16	4.45	-	-
Tyr	1.50	0.73	1.55	0.20	3.09	3.84	-	-

Tau	1.91	1.76	2.63	3.60	0.69	0.19	-	-

[1]Greenfeed pellets were available for cows 3 times a day when using the system and total a daily maximum of 750 g/cow.

Table 8. Ingredient and nutrient composition of the 4 experimental diets fed to 16 lactating dairy cows[1]

	Treatments			
Ingredients, % of diet DM	U	CSAA	FP	FPAA
Corn silage	34.8	34.8	34.8	34.8
Grass-legume silage	15.2	15.2	15.2	15.2
Corn meal	36.0	29.7	12.3	12.2
Ground field peas	0.00	0.00	25.0	25.0
Soybean meal	2.40	9.80	2.40	2.30
Roasted soybean	5.90	5.90	5.90	5.90
Urea	1.30	-	-	-
Minerals and vitamins[2]	2.40	2.40	2.40	2.40
AjiPro-L	-	0.13	-	0.15
Smartamine-M	-	0.07	-	0.05
Alfalfa pellets[3]	2.00	2.00	2.00	2.00
Penn State particle separator				
Top sieve, %	4.73	7.12	4.57	4.76
Medium top sieve, %	38.8	41.4	48.6	45.5
Medium bottom sieve, %	46.0	42.4	37.2	39.5
Bottom sieve, %	9.31	8.26	9.15	9.53
Nutrient composition, % of diet DM (unless otherwise noted)				
CP	15.4	15.1	14.9	15.0
Soluble protein, % of CP	49.0	34.8	46.0	47.0
NE_L, Mcal/kg	1.76	1.76	1.73	1.78
Gross energy, MJ/kg	14.3	13.1	15.3	13.8
Starch	35.5	34.0	31.2	32.2
Ethanol soluble carbohydrates	4.15	4.55	4.10	4.30
NDF	28.6	30.3	31.2	31.2
ADF	17.4	19.3	19.9	19.9
Lignin	3.30	2.60	2.78	2.63
Ether extract	4.45	4.28	3.98	4.43
NFC[3]	47.2	46.0	45.7	47.4
Soluble sugars	4.15	4.55	4.10	4.30
Ash	5.88	6.47	6.25	6.03
NDICP	1.08	1.58	1.48	1.43
ADICP	0.55	0.38	0.82	0.60
Ca	0.57	0.65	0.57	0.50
P	0.36	0.38	0.36	0.35
Mg	0.34	0.34	0.32	0.31
K	1.22	1.33	1.28	1.36
S	0.15	0.18	0.18	0.19
Na	0.44	0.47	0.42	0.38
Cl	0.49	0.51	0.47	0.46
Fe, mg/kg	233	241	254	248
Zn, mg/kg	98.3	109	102	96.5
Cu, mg/kg	17.0	17.5	16.3	17.5

Mn, mg/kg	49.3	52.0	48.0	50.5

[1]U = negative control diet based on urea; CSAA = positive control diet based on corn meal and soybean meal supplemented with rumen protected Lys and Met; FP = ground field peas-based diet (FP); FPAA = ground field peas-based diet supplemented with rumen protected Lys and Met.

[2] Mineral and vitamin mix provided on as fed basis: 297 mg/kg monensin sodium (Rumensin; Elanco, Greenfield, IN), 11.3% Ca, 1.76% P, 5.98% Mg, 6% K, 3% S, 15 mg/kg Co, 650 mg/kg Cu, 50 mg/kg I, 1,200 mg/kg Mn, 8.97 mg/kg Se, 3,700 mg/kg Zn, and 87.1 KIU/kg vitamin A.

[3]NFC = 100 – [CP + (NDF – NDICP) + fat + ash]

[3] Alfalfa pellets (guarantee analysis: 12% CP, 2% crude fat, 28% crude fiber, 0.9% Ca, and 0.3% P; Poulin Grain, Newport, VT) used as “bait” in the portable automated open circuit gas quantification system.

Table 9. Means for variables predicted by the NRC (2001) model in lactating dairy cows fed a negative control diet based on urea (U), a positive control diet based on corn meal and soybean meal supplemented with rumen protected Lys and Met (CSAA), a ground field peas-based diet (FP), or a ground field peas-based diet supplemented with rumen protected Lys and Met[1]

	Treatments			
Item	U	CSAA	FP	FPAA
NE_L allowable milk, kg/d	38.5	42.9	41.3	41.2
NE_L balance, MJ/d	-9.62	0.00	-4.18	-4.18
RDP, % of DMI	11.1	9.3	10.8	10.9
RUP, % of DMI	4.20	5.70	4.10	4.20
RDP balance, g/d	254	-146	235	248
RUP balance, g/d	-483	-192	-517	-534
MP allowable milk, kg/d	32.5	39.2	32.8	32.7
MP-bacterial, g/d	1,268	1,268	1,323	1,341
MP-RUP, g/d	809	1,218	826	890
MP-endogenous, g/d	110	118	116	118
MP balance, g/d	-397	-163	-422	-437
Duodenal flow Lys % of MP	6.75	6.58	7.06	7.31
Duodenal flow Met % of MP	1.96	2.20	1.85	2.35
Duodenal flow His % of MP	2.12	2.08	2.08	2.04
MP-Lys:MP-Met ratio	3.44:1	2.99:1	3.82:1	3.11:1

[1]Actual feed nutrient composition and animal variables were used (i.e., DMI, milk yield and composition, DIM, and BW) in the NRC (2001) evaluation software.

Table 10. Least square means for DMI, ADG, milk yield and composition, feed efficiency, and plasma concentration of urea-N in lactating dairy cows fed a negative control diet based on urea (U), a positive control diet based on corn meal and soybean meal supplemented with rumen protected Lys and Met (CSAA), a ground field peas-based diet (FP), or a ground field peas-based diet supplemented with rumen protected Lys and Met (n = 64 observations)

	Treatments					Contrasts (*P*-values)[1]		
Item	U	CSAA	FP	FPAA	SEM	U × FP	CSAA × FP	CSAA × FPAA
DMI, kg/d	23.3	25.0	24.6	25.0	0.39	<0.01	0.49	0.93
N intake, g/d	552	611	589	605	12.1	0.02	0.16	0.72
OM intake, kg/d	21.9	23.3	23.1	23.5	0.37	0.01	0.57	0.73
Soluble protein, % of DMI	11.4	8.67	11.3	11.8	0.19	0.84	<0.001	<0.001
Milk yield, kg/d	41.8	42.9	42.7	42.7	1.04	0.28	0.76	0.78
ECM, kg/d[2]	41.5	42.7	43.0	43.5	0.90	0.08	0.65	0.30
4% FCM, kg/d[3]	37.7	38.4	38.9	39.5	0.91	0.15	0.51	0.14
Milk yield/DMI, kg/kg	1.80	1.72	1.74	1.71	0.04	0.17	0.62	0.79
ECM/DMI, kg/kg	1.72	1.65	1.69	1.69	0.04	0.42	0.40	0.36
4% FCM/DMI, kg/kg	1.62	1.54	1.58	1.58	0.03	0.34	0.25	0.25
Milk N/N intake, %	33.4	32.7	33.1	32.8	0.91	0.71	0.70	0.88
Milk fat, %	3.40	3.35	3.43	3.57	0.09	0.70	0.33	0.01
Milk fat, kg/d	1.40	1.41	1.45	1.49	0.04	0.21	0.31	0.04
Milk true protein, %	2.78	2.94	2.86	2.94	0.04	0.02	<0.01	0.93
Milk true protein, kg/d	1.15	1.25	1.21	1.24	0.03	0.03	0.13	0.70
Milk lactose, %	4.98	4.93	5.01	4.93	0.03	0.16	<0.01	0.96
Milk lactose, kg/d	2.08	2.12	2.14	2.10	0.05	0.22	0.63	0.80
Milk SNF, %	7.76	7.87	7.87	7.87	0.05	<0.01	0.98	0.97
Milk SNF, kg/d	3.23	3.36	3.35	3.34	0.07	0.09	0.79	0.74
MUN, mg/dL	9.85	7.93	9.09	8.77	0.27	0.01	<0.01	<0.01
Plasma urea-N, mg/dL	13.9	10.6	11.4	10.3	0.63	<0.01	0.27	0.59
SCC, 1,000 cells/mL	306	150	72.0	165	72.0	0.61	0.96	0.78

[1]Orthogonal contrasts comparing the effects of: 1) NPN versus soluble true protein = U versus FP; 2) ground field peas versus corn meal plus soybean meal balanced for Lys and Met = FP versus CSAA; and 3) ground field peas balanced for Lys and Met versus corn meal plus soybean meal balanced for Lys and Met = FPAA versus CSAA. Significance was declared at $P \leq 0.05$ and trends at $0.05 < P \leq 0.10$.

[2]ECM (kg/d) = [0.0752 × milk yield (kg/d)] + [12.3 × fat yield (kg/d)] + [6.56 × SNF (kg/d)] (Tyrrell and Reid, 1965).
[3]4% FCM = [0.4 × milk yield (kg/d)] + [15 × milk fat yield (kg/d)] (NRC, 2001).

Table 11. Least square means for gaseous measurements, ruminal fermentation profile, nutrient intake, and apparent total tract digestibility of nutrients in lactating dairy cows fed a negative control diet based on urea (U), a positive control diet based on corn meal and soybean meal supplemented with rumen protected Lys and Met (CSAA), a ground field peas-based diet (FP), or a ground field peas-based diet supplemented with rumen protected Lys and Met (n = 48 observations for gaseous measurements. n = 32 observations for nutrient intake and digestibility variables. n = 16 observations for ruminal metabolism variables).

	Treatments					Contrasts (*P*- values)[1]		
Item	U	CSAA	FP	FPAA	SEM	U × FP	CSAA × FP	CSAA × FPAA
Gaseous measurements								
$Q CH_4$, g/d[2]	341	379	390	355	26.8	0.02	0.17	0.04
$Q CO_2$, L/d[3]	6,203	6,296	6,172	6,185	249	0.86	0.54	0.58
$Q O_2$, L/d[4]	7,485	7,213	7,458	7,249	329	0.93	0.46	0.91
$Q CH_4$ / DMI	14.8	15.5	16.1	15.6	1.04	0.21	0.57	0.91
$Q CH_4$ / milk yield	8.67	9.65	9.70	9.21	0.76	0.12	0.48	0.37
$Q CH_4$ /ECM	8.39	8.93	9.28	9.04	0.68	0.15	0.59	0.85
$Q CH_4$ / 4% FCM	9.26	9.96	10.3	9.90	0.75	0.14	0.66	0.92
$Q O_2$ / heartbeat	0.04	0.04	0.05	0.05	0.01	0.76	0.55	0.76
Respiratory quotient[5]	0.87	0.90	0.87	0.89	0.02	0.95	0.50	0.72
Minutes per measurement	3.76	3.51	3.72	3.81	0.23	0.77	0.20	0.06
Measurements/cow/period	9.37	7.27	8.80	7.25	1.44	0.76	0.43	0.99
Ruminal metabolism								
pH, -log10 [H+]	6.19	6.06	6.13	5.96	0.11	0.66	0.64	0.44
NH_3-N, mg/dL	5.53	3.16	4.13	4.46	0.54	0.05	0.15	0.05
Total VFA, m*M*	103	113	112	121	3.52	<0.01	0.65	<0.01
Acetate, mol/100 mol	61.2	57.8	57.9	59.3	1.66	<0.001	0.79	<0.01
Propionate, mol/100 mol	24.8	28.0	27.5	26.5	1.60	<0.001	0.20	<0.001
Butyrate, mol/100 mol	11.0	11.3	11.6	11.2	0.26	<0.001	0.14	0.57
Isobutyrate, mol/100 mol	0.82	0.74	0.87	0.83	0.03	0.09	<0.001	<0.01
Valerate, mol/100 mol	1.62	1.57	1.58	1.42	0.09	0.57	0.82	0.03
Isovalerate, mol/100 mol	0.57	0.60	0.63	0.67	0.03	0.17	0.53	0.11
Acetate:Propionate ratio	2.50	2.10	2.20	2.29	0.20	<0.001	0.05	<0.001
Intake, kg/d								

DM	23.1	25.2	24.8	25.8	0.69	0.06	0.63	0.48
OM	21.7	23.6	23.2	24.2	0.63	0.08	0.66	0.39
Starch	8.17	8.57	7.69	8.31	0.25	0.12	<0.01	0.36
NDF	4.58	3.98	4.09	4.07	0.28	0.23	0.77	0.81
ADF	2.65	2.36	2.49	2.36	0.17	0.52	0.59	0.98
CP	3.41	3.86	3.69	3.93	0.13	0.13	0.33	0.70
Digestibility, % of intake								
DM	64.3	70.5	68.5	70.8	2.15	0.18	0.51	0.91
OM	65.5	71.3	69.5	71.5	2.04	0.18	0.50	0.94
Starch	98.0	97.9	97.4	97.7	0.47	0.28	0.35	0.71
NDF	30.2	46.6	46.0	44.8	4.05	0.01	0.92	0.75
ADF	37.6	50.8	50.4	52.9	3.67	0.02	0.95	0.65
CP	59.6	67.3	61.2	67.3	2.74	0.68	0.12	0.99

[1]Orthogonal contrasts comparing the effects of: 1) NPN versus soluble true protein = U versus FP; 2) ground field peas versus corn meal plus soybean meal balanced for Lys and Met = FP versus CSAA; and 3) ground field peas balanced for Lys and Met versus corn meal plus soybean meal balanced for Lys and Met = FPAA versus CSAA. Significance was declared at $P \leq 0.05$ and trends at $0.05 < P \leq 0.10$.

[2] *Q*CH4 = flux of CH_4 measured using spot short-term measurements with a gas quantification system

[3] *Q*CO2 = flux of CO_2 measured using spot short-term measurements with a gas quantification system

[4] *Q*O2 = consumption of O_2 measured using spot short-term measurements with a gas quantification system

[5] *Q*CO2 ÷ *Q*O_2

Table 12. Least square means for N intake, balance, and efficiency and urinary concentration of creatinine and excretions of allantoin, uric acid, and purine derivatives (PD = allantoin plus uric acid) in lactating dairy cows fed a negative control diet based on urea (U), a positive control diet based on corn meal and soybean meal supplemented with rumen protected Lys and Met (CSAA), a ground field peas-based diet (FP), or a ground field peas-based diet supplemented with rumen protected Lys and Met (n = 32 observations)

	Treatments					Contrasts (*P*-values)[1]		
Item	U	CSAA	FP	FPAA	SEM	U × FP	CSAA × FP	CSAA ×FPAA
N intake, g/d	545	617	590	628	20.9	0.13	0.33	0.70
Urinary creatinine, m*M*	4.14	3.57	3.88	4.03	0.29	0.52	0.43	0.22
Urinary excretion, L/d	40.3	48.9	47.5	42.0	3.10	0.12	0.75	0.10
N balance, g/d								
Milk	186	208	196	209	6.30	0.18	0.09	0.93
Urine	192	185	203	165	14.7	0.58	0.36	0.30
Feces	210	197	216	197	16.0	0.78	0.38	0.99
Manure (feces plus urine)	402	381	419	361	23.0	0.59	0.24	0.50
Retained	-43.7	27.4	-24.0	56.9	29.7	0.64	0.22	0.45
N balance, % of N intake								
Milk	34.4	33.8	33.2	33.3	1.19	0.46	0.73	0.78
Urine	35.9	30.4	34.7	26.4	3.12	0.78	0.32	0.34
Feces	38.9	32.0	36.8	31.4	2.65	0.58	0.20	0.86
Manure (feces plus urine)	74.8	62.3	71.5	57.7	4.62	0.61	0.16	0.45
Retained	-9.27	3.89	-4.62	8.92	50.5	0.52	0.23	0.45
Urinary metabolites								
NH_3-N, g/d	3.27	3.10	3.40	2.50	0.30	0.75	0.47	0.14
NH_3-N, % of total urinary N	1.72	1.69	1.82	1.62	0.19	0.71	0.71	0.65
NH_3-N, % of N intake	0.63	0.51	0.62	0.42	0.05	0.87	0.24	0.26
Urea-N, g/d	117	80.5	103	83.5	5.57	0.05	<0.01	0.62
Urea-N, % of total urinary N	62.0	44.4	55.3	54.5	4.69	0.29	0.08	0.09
Urea-N, % of N intake	22.3	13.4	18.5	13.8	1.22	0.02	<0.01	0.79
Allantoin, mmol/d	321	370	390	373	17.6	<0.01	0.40	0.90
Uric acid, mmol/d	21.3	23.7	23.0	21.3	2.45	0.52	0.77	0.32
Total PD, mmol/d[5]	343	394	414	394	18.9	<0.01	0.37	0.99

[1]Orthogonal contrasts comparing the effects of: 1) NPN versus soluble true protein = U versus FP; 2) ground field peas versus corn meal plus soybean meal balanced for Lys and Met = FP versus CSAA; and 3) ground field peas balanced for Lys and Met versus corn meal plus soybean meal balanced for Lys and Met = FPAA versus CSAA. Significance was declared at $P \leq 0.05$ and trends at $0.05 < P \leq 0.10$.

[3] Estimated based on creatinine excretion assumed as 29 mg/kg of BW (Valadares et al., 1999).

[7] Feces were collected from 8 multiparous cows (2 squares) for 2 days every 6 hours to account for diurnal variation.

Table 13. Leas square means of gross energy (GE) intake, excretion, balance and efficiency of 4 multiparous lactating Holstein cows fed a negative control diet based on urea (U), a positive control diet based on corn meal and soybean meal supplemented with rumen protected Lys and Met (CSAA), a ground field peas-based diet (FP), or a ground field peas-based diet supplemented with rumen protected Lys and Met (n = 16 observations)

	Treatments					Contrasts (*P*-values)[1]		
Item	U	CSAA	FP	FPAA	SEM	U × FP	CSAA × FP	CSAA ×FPAA
GE intake, MJ/d[2]	447	422	424	475	18.7	0.36	0.91	0.05
Fecal energy, MJ/d[3]	157	135	139	124	16.2	0.44	0.88	0.63
DE intake, MJ/d[4]	289	287	286	352	18.2	0.84	0.95	0.01
CH_4 energy, MJ/d[5]	16.0	17.6	18.2	20.4	2.39	0.20	0.71	0.13
Urine energy, MJ/d[6]	16.7	16.4	16.3	20.2	1.02	0.74	0.93	0.01
ME intake, MJ/d[7]	257	253	251	311	15.7	0.74	0.93	0.01
Milk energy, MJ/d[8]	131	123	128	134	2.88	0.51	0.23	0.03
ADG, kg/d	-0.11	-0.33	-0.59	1.01	0.238	0.19	0.46	<0.01
Tissue energy balance, MJ/d[9]	-2.97	-9.85	-17.9	32.0	7.38	0.19	0.46	<0.01
Heat production, MJ/d[10]	129	140	141	145	10.8	0.04	0.78	0.28
Milk energy / GE intake, %	40.6	38.1	32.9	38.9	2.45	0.03	0.10	0.78
Milk energy / DE intake, %	46.5	43.0	45.3	38.3	2.77	0.68	0.42	0.12
Milk energy / ME intake, %	52.3	48.8	51.5	43.3	3.05	0.82	0.43	0.14
Heat production / GE intake, %	39.9	43.1	35.8	42.6	4.01	0.21	0.05	0.87

[1] Orthogonal contrasts comparing the effects of: 1) NPN versus soluble true protein = U versus FP; 2) ground field peas versus corn meal plus soybean meal balanced for Lys and Met = FP versus CSAA; and 3) ground field peas balanced for Lys and Met versus corn meal plus soybean meal balanced for Lys and Met = FPAA versus CSAA. Significance was declared at $P \leq 0.05$ and trends at $0.05 < P \leq 0.10$.

[2]Calculated as: GE (MJ/d) = DE + Fecal energy.

[3] Calculated based on fecal DM (indigestible ADF analysis) and measurement of energy density of fecal samples.

[4] Digestible energy intake (MJ/d) = metabolizable energy intake (MJ/d) + CH_4 energy (MJ/d) + urinary energy (MJ/d). (Pereira et al., 2015b).

[5] Calculated based on 2.17 MJ/L.

[6] Calculated based on 6.5% of metabolizable energy intake (Ferris et al., 1999; Ferrell and Oltjen, 2008).

[7] Metabolizable energy intake (MJ/d) = Heat production (MJ/d) + milk energy (MJ/d) + tissue energy balance (MJ/d)

[8]Milk-energy (MJ/d) = {[0.384 × fat (%)] + [0.223 × protein (%)] + [0.199 × lactose (%)]} × milk yield (kg/d) (NRC, 2001).

[9] Calculated according to the empty BW energy needed to vary 1 kg with an efficiency of 0.85 for weight gain (31.8 MJ/kg) and 0.82 for weight loss (29.2 MJ/kg; NRC 2001).
[10] Heat production (MJ/d) = 20.47 kJ/L × oxygen consumption L/d ÷ 1000 (Nicol and Young, 1990; Aharoni et al., 2006).

Table 14. Least square means for the plasma concentrations (µ*M*) of EAA and NEAA in lactating dairy cows fed a negative control diet based on urea (U), a positive control diet based on corn meal and soybean meal supplemented with rumen-protected Lys and Met (CSAA), a ground field peas-based diet (FP), or a ground field peas-based diet supplemented with rumen-protected Lys and Met (n = 64 observations).

	Treatments					Contrasts (*P*-values)[1]		
Item (µ*M*)	U	CSAA	FP	FPAA	SEM	U × FP	CSAA × FP	CSAA × FPAA
EAA								
Arg	69.6	65.4	73.7	70.2	3.68	0.26	0.03	0.19
His	47.1	46.7	44.5	37.9	2.26	0.21	0.28	<0.001
Ile	96.6	106	122	114	6.09	<0.001	<0.01	0.12
Leu	130	124	123	109	6.66	0.22	0.88	0.01
Lys	67.2	69.9	77.2	77.0	3.96	0.02	0.07	0.08
Met	19.9	27.7	19.6	31.9	1.06	0.83	<0.001	<0.01
Phe	42.1	40.1	41.5	37.4	1.33	0.72	0.42	0.14
Thr	101	108	111	103	4.85	0.09	0.64	0.41
Trp	40.6	40.4	41.7	39.2	1.40	0.55	0.52	0.49
Val	180	201	224	212	9.24	<0.001	<0.01	0.15
NEAA								
Ala	255	275	277	284	11.6	0.02	0.81	0.36
Asn	50.2	50.9	54.4	49.9	2.10	0.07	0.13	0.64
Asp	2.91	3.19	3.02	2.86	0.24	0.70	0.53	0.23
Cit	95.1	81.7	83.0	80.4	4.05	0.02	0.79	0.79
Cystathionine	1.55	2.02	1.43	2.17	0.08	0.15	<0.001	0.07
Cys	21.1	21.9	19.4	21.7	0.60	<0.01	<0.001	0.69
Glu	38.0	37.5	37.9	40.3	1.93	0.92	0.79	0.06
Gln	258	225	230	222	7.37	<0.01	0.55	0.71
Gly	377	320	334	304	13.7	<0.01	0.32	0.28
Homocysteine	2.53	2.79	2.78	2.76	0.20	0.11	0.96	0.85
Orn	38.9	39.4	40.4	41.7	2.28	0.79	0.58	0.21
Pro	93.3	87.6	80.8	80.6	3.98	<0.001	0.05	0.05
Ser	98.4	88.8	96.7	85.8	2.63	0.55	<0.01	0.30

Tau	41.0	50.4	42.9	47.4	1.93	0.48	<0.01	0.28
Tyr	40.3	40.7	42.1	35.7	1.91	0.39	0.52	0.02
3-Methylhistidine	4.09	3.60	3.73	3.52	0.18	0.11	0.55	0.72
Total BCAA[2]	407	431	469	435	21.4	<0.01	0.04	0.79
Total EAA	724	764	804	762	30.8	<0.01	0.16	0.96
Total urea cycle AA[3]	204.6	186.5	197.0	192.3	8.89	0.41	0.24	0.52
Total sulfur AA[4]	86.0	105	86.2	106	3.08	0.97	<0.001	0.79
Total NEAA	1,484	1,392	1,420	1,371	33.71	0.11	0.47	0.59
Total AA[5]	2,208	2,156	2,225	2,133	59.15	0.80	0.29	0.72

[1]Orthogonal contrasts were used to compare the effects of: 1) NPN from urea versus soluble true protein from ground field peas [i.e., U versus FP]; 2) starch and true protein from corn meal plus soybean meal balanced with rumen-protected Lys and Met versus starch and soluble true protein from ground field peas [i.e., CSAA versus FP]; and 3) starch and true protein from corn meal plus soybean meal balanced with rumen-protected Lys and Met versus starch and soluble true protein from ground field peas balanced with rumen-protected Lys and Met [i.e., CSAA versus FPAA]. Significance was declared at $P \leq 0.05$ and trends at $0.05 < P \leq 0.10$.

[2]Dipeptide (β-alanyl-l-His).

[3]Total branched-chain amino acids (BCAA) = Ile + Leu + Val.

[4]Total urea cycle AA = Arg, Cit and Orn

[5]Total sulfur AA = Cystathionine + Cys + Homocysteine + Met + Tau

[6]Total AA = total EAA + total NEAA.

Figure 5. Measurement of ruminal pH of 4 lactating ruminally canullated dairy Holstein cows fed a negative control diet based on urea (U), a positive control diet based on corn meal and soybean meal supplemented with rumen protected Lys and Met (CSAA), a ground field peas-based diet (FP), or a ground field peas-based diet supplemented with rumen protected Lys and Met (n = 176 observations). Measurements were done before feeding (0h) and 1, 2, 4, 6, 8, 10, 12, 14, 16 and 18 h after morning feeding.

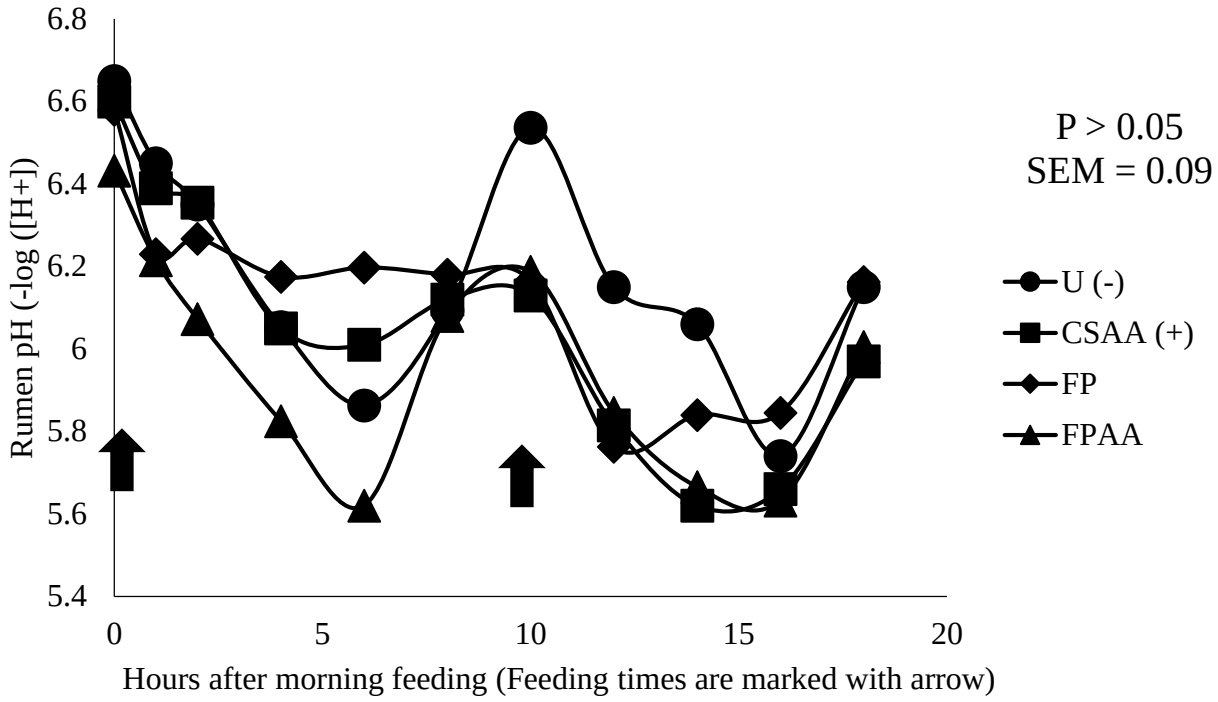
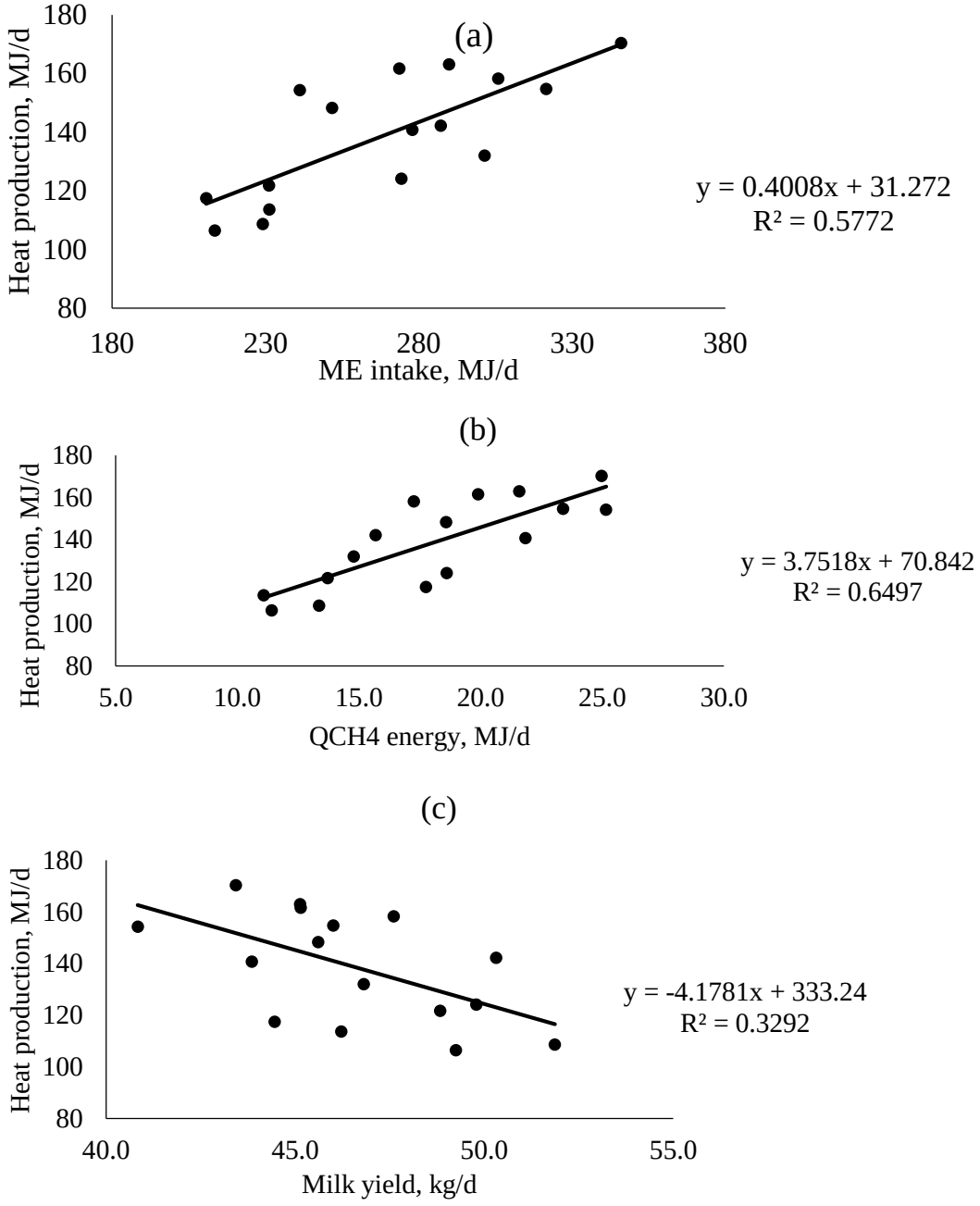


Figure 6. Relationship between heat production (MJ/d) and metabolizable energy (ME) intake (a), CH_4 flux energy (QCH_4-E, MJ/d), and milk yield (kg/d) of 4 lactating dairy cows fed a negative control diet based on urea (U), a positive control diet based on corn meal and soybean meal supplemented with rumen protected Lys and Met (CSAA), a ground field peas-based diet (FP), or a ground field peas-based diet supplemented with rumen protected Lys and Met (n = 16 observations) Higher intake of DE resulted in higher heat production by the cows. On the other hand, higher milk production meant less heat produced by the cows.



CHAPTER IV: EFFECTS OF REPLACING SOYBEAN MEAL FOR CANOLA MEAL IN DIETS WITH FIELD PEAS WITH OR WITHOUT RUMEN PROTECTED METHIONINE

INTRODUCTION

As shown in Chapter III, feeding ground field peas (**GFP**) with rumen protected (**RP**) Lys and RP Met increased yields of milk and milk protein in the same proportion compared to a diet based on soybean meal (**SBM**) and corn meal also supplemented with RP-Lys and RP-Met. When feeding GFP supplemented with both RP-Lys and RP-Met, significant increases in plasma concentrations of Lys and Met occurred, concomitant with a decrease in plasma concentration of His. The main hypothesis is the observed decrease in His was caused by addition of RP-Met to the diet. According to Patton et al. (2015), addition of RP AA could decrease AA absorption of other essential AA (**EAA**) in the duodenal mucosa. Patton et al. (2015) performed a meta-analysis in order to correlate diet and plasma concentrations of AA and used 420 treatment means from 104 studies. According to the authors, addition of Met to the diet caused a decrease in all EAA except Lys and Arg, while addition of Lys caused a decrease in His and Phe. When fed with a source of RP Met, diets based on GFP can result in a decrease of all plasma EAA concentrations.

When feeding GFP with RP Lys and RP Met in the last chapter, we hypothesize that His became the first limiting AA, preventing higher yields of milk protein. Limitation of His could have been caused by 2 main reasons: 1) decreased uptake of His in the intestine caused by addition of RP Met and 2) decreased duodenal availability of His due to the high content of RDP and negative MP balance of diets based on GFP.

Canola meal (**CAM**) has been previously shown as a feed that increased plasma EAA concentration (Martineau et al., 2014) when compared to other protein sources despite high ruminal degradability (NRC, 2001). Meta-analysis studies have shown that cows fed CAM had increased DMI and milk yield (Martineau et al., 2013) and also had increased delivery of all

EAA in the duodenum (except for His, Leu and Phe, which had a trend to increase, $P < 0.10$) as a direct effect of increased DMI, consequently increasing concentration of EAAs in plasma (Martineau et al., 2014). Studies are necessary to assess if feeding GFP and CAM can improve DMI and milk yield of lactating dairy cows when compared to a diet based on GFP and SBM as it has been observed in the literature (Martineau et al., 2014). In addition, feeding GFP with CAM can result in increased DMI and plasma EAA concentration, mitigating the detrimental effects of addition of RP Met for other plasma EAA as described by Patton et al. (2015). According to a study done in vitro (Maxin et al., 2013), canola meal has a high RUP value and can yield protein residues that have high concentration of Met, Lys and His when compared to SBM. It is possible that there is no need to supplement a diet with CAM and GFP with RP Met if the conjunction of the 2 feeds are complementary for replenishing AA requirements of lactating dairy cows.

The objectives of this study were to analyze if addition of CAM to a diet with 25% GFP can increase DMI and AA availability in the duodenum, causing an increase in plasma AA concentration of His and other EAA and hence improving milk and milk protein yield. Our hypothesis is that CAM will improve milk and milk protein yield when fed in conjunction with FP as microbial protein production and consequently MP balance may be higher than predicted by the NRC (2001) model, allowing for cows to have higher DMI and produce more milk than what is estimated. As diets with 25% FP can be deficient in Met, it is hypothesized that addition of RP Met will cause an increase in milk protein concentration and possibly an increase in milk protein concentration when fed to diets with 25% GFP and either SBM or CAM.

MATERIALS AND METHODS

The experiment was performed at the University of New Hampshire Fairchild Dairy Teaching and Research Center located in Durham, NH (43° 14'N, 70° 95'W) from February 17th to May 11th, 2016. Care and handling of animals was approved in accordance to the University of New Hampshire Institutional Animal Care and Use Committee guidelines (IACUC protocol no. 151206).

Animals, Experimental Design, and Diets

Twelve multiparous Holstein cows averaging (mean ± SD) 153 ± 31 DIM and 769 ± 64 kg of BW and 4 primiparous cows averaging 122 ± 16 DIM and 651 ± 41 kg of BW at the beginning of the study were used. Cows were randomly assigned to 1 of 4 treatments in replicated 4 × 4 Latin square design balanced for carryover effects, in which all combinations of treatments are present in each square as described in Chapter IV. Animals were distributed in squares balanced for DIM and parity resulting in 3 squares of multiparous cows (square 1 = 117 ± 18 DIM, 736 ± 62 kg of BW; square 2 = 162 ± 13 DIM, 776 ± 67 kg of BW; square 3 = 181 ± 12 DIM, 796 ± 65 kg of BW) and 1 square of primiparous cows (square 4 = 122 ± 16 DIM, 651 ± 41 kg of BW).

Animals were housed in a tie-stall barn. Individual feed intake and orts measurements were recorded using Super Data ranger (American Calan Inc., Northwood, NH) and animal's intakes were individualized using wooden feed tubs (90 × 90 × 90 cm) for each cow. The experimental diets (Table 7) contained 49% forage as corn silage (35.0%) and grass - legume mix silage (14.0%) both grown and harvested at the University of New Hampshire properties during the 2015 growing season.

The amount of RP-Met (Smartamine-M, Adisseo, Antony, France) used was calculated with the intention of replenishing AA requirements as estimated according to the NRC (2001) model. Average requirements of AA were calculated using BW, DMI, milk and milk protein variables as measured at the beginning of each experimental period after feedstuffs were added to the model. Based on industry specifications for AA bioavailability (70% D-L-Met coated with 2-vinylpyridine-co-styrene with 75% D-L-Met bioavailability and 100% RUP digestibility for Smatamine-M), for every 100 g of Smartamine-M, 53 g of Met was expected to be delivered in the duodenum and be absorbed in the blood.

The experimental diets contained (DM basis) 35.0% Corn silage, 14.0% grass-legume silage, 25% GFP, 1.5% citrus pulp, and corn meal, flaked corn and dry distillers' grains in variable amounts with 1) SBM (11%) as the major source of supplemental protein, (**FPSB diet**), 2) Canola meal (13.5%) as the major source of supplemental protein (**FPCM diet**). For each experimental diet, RP Met was top dressed to cows in the respective treatments in a 4 ×4 Latin Square factorial design (27 g/d) resulting in a total of 4 treatments: 1) FPSB diet with no RP Met supplemented, 2) FPSB diet with supplementation of RP Met, 3) FPCM diet with no RP Met supplemented and 4) FPCM diet with supplementation of RP Met. Diets had negative balance of MP according to the NRC (2001) model due to addition of 25% GFP, which is predicted with low MP.

Each experimental period lasted 21 days, with 16 days for diet adaptation and 5 days for data and sample collection. Diets were fed as TMR and were prepared twice daily at 0515h and 1615h using a Super Data Ranger mixer (American Calan Inc., Northwood, NH). Cows were feed 35% and 65% of the daily TMR allocation during the a.m. and p.m. feedings, respectively.

This feeding schedule was chosen in order to reduce the effect of uneven cycles of TMR intake for better estimates of plasma AA, urine, and feces chemical profile.

Feed Sampling and Analysis

Two samples each of corn and grass-legume silages were obtained daily immediately before they were placed in the Super Data Ranger (American Calan Inc.). One sample was composited every 3 days and dried using a microwave (Model R-209KK 700 Watts, Sharp electronics, Osaka, Japan), for dietary DM adjustment. The second sample was pooled weekly and lyophilized for 48 h (Labconco freeze drier 5, Kansas City, MO) for nutrient composition analysis.

Feed amounts were adjusted every 3 days to leave 5% to 10% refusals per cow. Feed TMR was weighed daily and samples were collected between days 14 and 21 in each experimental period after the a.m. and p.m. feeding. Orts samples were weighed daily and collected on the same days as TMR was collected. Samples of orts and TMR were refrigerated immediately after collection and pooled weekly by treatment for DM and nutrient composition analysis. The energy mix meal, SBM, GFP, and minerals samples were collected weekly and lyophilized immediately. Orts and fecal samples were dried in an air forced oven (1380FMS; VWR Scientific, Radnor, PA) at 55ºC for 72 h. All samples (TMR, orts, feces, silages and concentrates) were ground through a 1-mm screen in a Wiley mill (Arthur H. Thomas Scientific, Philadelphia, PA), packed on labeled plastic bags and shipped to a commercial laboratory for standardized wet chemistry analyses (Dairy One forage laboratory, Ithaca, NY). The following analyses were done in the 2 TMR and all fecal samples: DM (method 930.15; AOAC International, 2006), total N (methods 990.03 and 992.23 967.07; AOAC International, 2006),

NDF (method 6; Ankom Technology, Fairpoint, NY; solutions as in Van Soest et al., 1991), ADF (method 5; Ankom Technology, solutions as in method 973.18; AOAC International, 1998), crude fat (method 2003.05; AOAC International, 2006), starch (YSI 2700 Select Biochemistry Analyzer, application note no. 319, YSI Inc. Life Sciences, Yellow Springs, OH), ash (method 942.05; AOAC International, 2006), minerals using a Thermo ICAP 6300 Inductively Coupled Plasma Radial Spectrometer after microwave digestion. Orts were analyzed for DM, ash, NDF, ADF, total N according to methods and procedures described above. Feed samples were analyzed for AA composition at the University of Missouri Experimental Station Chemical Laboratories using a cation exchange chromatograph (cIEC-HPLC) coupled with post-column ninhydrin derivatization and quantitation with norleucine as the internal standard (method 982.30; AOAC, 2006).

Milk sampling and Analyses

Cows were milked twice a day at 0500 h and 1600 h and milk yield was recorded throughout the experiment at each milking. A subsample of milk was collected on d 19, 20, and 21 of each experimental period during the morning and afternoon milking times in tubes containing 2-bromo-2-nitropropan-1,3 diol. Samples were pooled in duplicate by cow per day according to the proportion of milk yield in each milking events and kept at 4°C until sent for analysis at Dairy One Cooperative Inc. (Ithaca, NY) for determination of milk fat, protein, lactose, SNF, total solids, and MUN by mid-infrared reflectance spectroscopy in a Milkoscan (Foss Inc., Hillerød, Denmark) and somatic cells count (SCC) by flow cytometry in a Fossomatic (Foss Inc.). Concentrations and yields of milk components were calculated as the average between the duplicate samples. Calculations of ECM and 4% FCM were performed according to

(Tyrrell and Reid, 1965) and the NRC (2001), respectively. Efficiency was calculated using the ratios between DMI and milk yield, DMI and ECM, and DMI and 4% FCM.

Blood sampling and analyses

Blood samples were taken for 2 consecutive days, once daily between 3.5 h and 4 h after the morning feeding, at 0945h on days 19 and 20. Samples were taken from the coccygeal artery or vein of each cow into 1 Vacutainer tube containing EDTA (Monoject, Covidien, Mansfield, MA) and composited by cow. After collection, blood tubes were immediately transported to the laboratory, where they were centrifuged (2,155 × g for 20 min at 4°C) using an Eppendorf Centrifuge model 5810 (Eppendorf, Hamburg, Germany). Plasma was sampled and stored at -20°C for further analyses of plasma urea nitrogen (**PUN)**. Analysis of PUN were performed colorimetrically using an UV/visible spectrophotometer (Beckman Coulter Inc., Brea, CA) set at a wavelength of 540 nm. From the same tube, 4 mL of plasma was added to a glass 40 mL culture tube with 1 mL of 15% sulfo-salicylic acid solution, added for protein precipitation and release of free amino acids (as described in Mondino et al., 1972). The solution was mixed using a Vortex (Mini Vortexer, VWR International, Bridgeport, NJ) and placed at 4° C for 10 min. In sequence, tubes were centrifuged for 20 min at 2,155 × g and 4°C and 0.6 µL of the supernatant was collected each day, pooled per cow per period into a cryovial, and stored at -80°C until sent to University of Missouri Experimental Station Chemical Laboratories. Samples were analyzed for AA using a cation exchange chromatograph (cIEC-HPLC) coupled with post-column ninhydrin derivatization and quantitation with norleucine as the internal standard (method 982.30; AOAC, 2006).

Statistical Analyses

Data were analyzed using the MIXED procedure of SAS (SAS version 9.4) according to a replicated 4 × 4 Latin square design with a 2 × 2 factorial arrangement of treatments. The following model was fitted for DMI, milk yield, concentrations and yields of milk components, and plasma concentrations of AA:

$$Y_{ijklm} = \mu + S_i + P_j + C_{k(i)} + PROT_l + RPMET_m + PROT \times RPMET_{lm} + S \times PROT_{il} + S \times RPMET_{im} + E_{ijklm}$$

where Y_{ijkl} = dependent variable, μ = overall mean, S_i = fixed effect of i^{th} square, P_j = fixed effect of j^{th} period, C_k = random effect of k^{th} cow within i^{th} square, $PROT_l$ = fixed effect of l^{th} protein source (SBM or CAM), $RPMET_m$ = fixed effect of m RP Met (supplementation or no supplementation with RP Met), $S \times PROT_{il}$ = interaction between i^{th} square and l^{th} protein source, $S \times RPMET_{im}$ = interaction between i^{th} square and m^{th} RP Met supplementation, and E_{ijklm} = error term ~ N (0, σ^2_e). All reported values are LSM with significance declared at $P < 0.05$ and trends at $0.05 < P < 0.10$. Interaction terms were removed from the model when $P \geq 0.25$.

RESULTS AND DISCUSSION

Dietary Nutrient Composition and model outputs

Chemical composition of feedstuffs used in this experiment are shown in Table 15. Ground field peas had 21.3% CP and 69% soluble protein as a percentage of total CP. Starch concentration averaged 45.6%, which was similar to the GFP used in Chapter IV. Soybean meal had 52.1% of CP with 27% soluble protein, and CAM had 41.8% with 24% soluble protein.

Ingredient profile of feedstuffs in this study were added to the NRC (2001) model and output results are reported in Table 17.

The ingredient and nutrient composition of the 2 diets are shown in Table 16. Dietary CP was slightly lower for FPCM compared to FPSB. Concentration of NDF and ADF were numerically higher for FPCM when compared to FPSB due to a higher numerical concentration of ADF and NDF in CAM when compared to SBM and lower proportion of corn meal, steam flaked corn and dry distillers' grains (which have low concentration of ADF and NDF) in the FPCM diet compared to FPSB. Both diets had a concentration of NDF higher than the minimum recommended by the NRC (2001) of 25% NDF with 19% from forage for a diet based on corn silage, alfalfa silage and ground corn grain.

Diets were formulated using the NRC (2001) model meeting all nutrient requirements, except for MP, of a lactating Holstein cow producing 42 kg/d of milk with 3.6% milk fat and 3.2% milk protein at 25 kg/d of DMI and weighing 650 kg of BW. Values from the NRC model (2001) are shown in Table 17 and were estimated using actual average cow DMI, milk yield, milk composition, BW and average temperature throughout the study (10° C). Cows fed FPSB had an estimated DMI of 26.7 kg/d, which was numerically lower than the estimated DMI of 27.2 kg/d for cows fed FPCM. In this study, as milk production was lower than predicted NEL allowable milk 45.3 kg/d and 46.4 kg/d, respectively for FPSB and FPCM), energy was likely not limiting for milk production. The RDP content of the diets was slightly higher for FPCM (12.1%) compared to FPSB (11.9%).

According to the NRC (2001), CAM has high degradability in the rumen, with 23.2% of the CP in the A fraction and 70.4% in the B fraction with a degradation rate of 10.4%/h. Harstad and Prestløkken (2001) conducted a study comparing in situ ruminal CP digestibility and total

intestinal CP digestibility of CAM compared to corn gluten meal and 2 types of fish meal. The main objective of Harstad and Prestløkken (2001) was to observe if prediction of total AA from the diet could be inferred from ruminal degradability of CP in diets with CAM. The authors reported a CP ruminal degradation of CAM of 70.6% after 16 h of in situ incubation and 83.5% after 24 h, which was higher than corn gluten meal. The degradability of all AA followed the same trend observed for CP. (Harstad and Prestløkken, 2001). In our study, the high degradability of CAM predicted by the model caused a lower RUP balance FPCM compared to FPSB (-445 g/d vs. -91 g/d, respectivelly. The RUP balance for CM was of -445, lower than -91 from SB treatment, resulting in an MP balance of -77 g/d for SB and -341 g/d for CM. As a result, the MP allowable milk was lower for CM (33.7 kg/d) when compared to SB (37.4 kg/d) (Table 17).

A production study (Brito and Broderick, 2007) showed that the greater RDP value predicted by the NRC (2001) model for CAM in diets was over-predicted. According to Huhtanen et al. (2011) it is possible that over-prediction of RDP by the NRC (2001) model occurred because the model used only studies with a positive balance of MP (Huhtanen et al., 2011). As a result of high ruminal degradability, the NRC (2001) model predicts that CAM-based diets need higher concentration of CP when compared to SBM-based diets (NRC, 2001; Huhtanen et al., 2011). In the present study, after dietary treatments were chemically evaluated, the FPCM diet contained lower concentration of CP and consequently numerically lower concentrations of MP when compared to FPSB.

Duodenal flows, estimated by the NRC (2001) model, of Lys, Met and His, as a % of MP were numerically higher for FPCM diets compared to FPSB (Table 20). The Lys to Met ratio

was of 3.8 : 1 for FPSB and 3.6 : 1 for FPCM. When Met was supplemented, ratios decreased to 3.01 : 1 for FPSB and 2.94 : 1 for FPCM.

Dry Matter Intake, Milk Yield and Milk Composition

Dry matter intake, milk yield and concentrations and yields of milk components are shown in Table 18. Cows fed FPCM had higher DMI when compared to cows fed FPSB ($P < 0.01$). No difference was found for DMI related to RP Met supplementation, following what has been reported in literature (Leonardi et al., 2003; Broderick et al., 2008). Differences in DMI between diets based on SBM or CAM have been reported in literature. For instance, Brito and Broderick (2007) observed that cows fed a CAM-based diet had higher DMI than those fed SBM-based diets (24.9 vs 24.2 kg/d, respectively). Martineau et al. (2013) conducted a meta-analysis examining the effects of feeding CAM at expense of several protein supplements in an average replacement rate of 11.7% of the diet DM. Responses in DMI were mostly positive with an increase of 0.24 kg/day per cow per 10% inclusion of CAM in the diet (Martineau et al., 2013). In the current study, the increase in DMI was of 0.59 kg/d per 10% of CAM added to the diet.

Following DMI, cows fed FPCM had higher milk yield ($P < 0.001$) compared to cows fed FPSB, but no difference was observed between cows supplemented or not with RP-Met (Table 18). The same responses were observed for ECM and 4% FCM. No effect of protein source or RP-Met supplementation was found for feed efficiency. Cows fed FPCM gained more weight compared to those fed FPSB (Table 18). Martineau et al. (2013) reported that when cows were fed diets based on CAM, milk yield, ECM, and 4% FCM increased with an increase of milk efficiency (i.e. ECM ÷ DMI) of 0.035 kg/kg of CAM supplemented. In the present study, no

difference in feed efficiency was observed between FPSB and FPCM treatments, due to proportionate increases in ECM and DMI. Brito and Broderick (2007) observed similar yield of milk when cows were fed either SBM- or CAM-based diets. No difference was found in Leonardi et al. (2003) for milk yield in cows supplemented or not with RP-Met. Broderick et al. (2008) feeding 4 increasing concentrations of CP (14.8%, 16.1%, 17.3% and 18.6%) to diets with decreasing levels of RP Met (15 g/d, 10 g/d, 5 g/d and 0 g/d, respectively for increasing concentrations of CP) and found that for diets with 17.3% CP and 5 g/d of RP-Met and 16.1% CP and 10g/d of RP Met, had highest levels of milk yield when compared to the other diets. Cows fed 15% CAM and supplemented or not with RP-Lys and RP-Met had an increase in DMI, but no response in milk yield was observed in cows supplemented with RP AA (Broderick et al., 2015). With this information, it is safe to conclude that the increase in milk yield observed in the present study was due to an increase in DMI between for FPCM compared to FPSB and not by supplementation of RP Met.

No difference was observed for milk fat concentration across treatments (Table 18). Milk fat yield was higher ($P < 0.001$) for cows fed FPCM compared to FPSB with no difference found for addition of RP-Met. Concentration of milk protein was highest for cows fed RP-Met while milk protein yield was highest for cows fed FPCM with no effect of RP Met on this trait. Martineau (2013) found higher milk protein concentration when CAM was added to diets replacing other protein supplements, which disagrees with the data from the present study. It is possible that for the studies used in Martineau (2013), replacement of another protein supplement improved concentration of EAA in plasma and consequently replenished mammary gland limiting AA requirements; and that this improvement in EAA did not occur in the present study.

Responses in milk fat yield and milk protein yield were positive when cows were fed CAM in the meta-analysis of Martineau et al. (2013) due to a combined effect of positive increase in milk yield and milk component concentrations. Canola meal had high RDP concentrations, similar to urea, as shown in (Brito et al., 2007) but had higher levels of microbial protein flow from the rumen when compared to a diet based on urea and similar values compared to a SBM based diet. Broderick et al. (2015) found no differences in milk fat and milk protein percentages and yields when cows were supplemented with fed RP Lys and RP Met compared to no supplementation. In the present study, the increase in milk protein and fat yields was probably due to an increase in total milk yield when cows were fed FPCM, and not due to addition of RP Met.

Cows fed CM had lower values of MUN when compared to cows fed SB (11.9 vs. 12.8 mg/dL, respectively, $P < 0.001$). No difference was found between diets supplemented or not with RP Met. Brito and Broderick (2007) found similar values for MUN between diets based on SBM and CAM. Higher levels of MUN for FPSB may have been caused by a combination of factors, such as total CP concentration of FPSB (17.1%) being higher than FPCM (16.7%), even though total N intake was not different between diets (Table 18), and higher milk protein yield for cows fed FPCM. These cows used more protein for milk, consequently secreting less N as MUN.

Cows fed FPCM had higher ($P = 0.04$) values of apparent milk true N efficiency (milk true N ÷ total N intake) when compared to cows fed FPSB (1.75 and 1.70 kg/kg, respectively). Higher milk N efficiency may have caused a decrease in total MUN, as more N was used by the mammary gland as true protein and less is excreted as MUN in milk. Brito and Broderick (2007) found no differences between SBM- and CAM-based diets for milk true N efficiency. Martineau

et al. (2013) reported that the response for apparent milk true N efficiency was positive for cows fed CAM based diets due to higher milk true protein yield. Huhtanen et al. (2011) compared SBM- with CAM-based diets either raw or heat treated. A total of 122 studies were used in the dataset with the prerequisite that at least 1 of the following protein supplements were used (CAM, SBM, heat treated CAM, and sunflower meal) in 2 different levels. The authors reported that cows fed CAM had higher DMI, N intake, and milk and milk protein yields when compared to cows fed SBM. In addition, cows fed CAM had a higher slope for organic matter and CP digestibility. In conclusion, the authors stated that dietary models overestimate the MP value of SBM compared to CAM. In addition, treating CAM with heat reduced the feeding value of the supplement because of Maillard reactions and reduced bioavailability of CP (Huhtanen et al., 2011). Huhtanen et al. (2011) and Martineau et al. (2013) reports on increased milk true protein yield in CAM-based diets, compared to other protein sources, agree with the present study's results for lower MUN values due to higher milk protein yields and consequent higher milk true N efficiency.

Predictions with the NRC (2001) model were done with observed values added to the model. Predictions of milk yield were under estimated for cows all diets, as shown in Table 18. Reduction in MP values were due to addition of 25% GFP to the diets, as discussed in Chapter IV. The NRC (2001) model predicted MP allowable milk as 33.7 kg/d (34 kg/d when RP Met was added) while actual milk yield was 40.5 kg/d for FPCM. Cows gained weight during the study, which suggests that body tissue was not depleted due to lack of MP or by limitations in NE_L. Previous research reported that the NRC (2001) model MP allowable milk values under-predicted milk yield in cows fed CAM (Brito and Broderick, 2007; Huhtanen et al., 2011; Martineau et al., 2013).

Plasma Amino Acid Concentrations

Results for plasma concentration of AA are shown in Table 19. Plasma concentrations of Ile, Leu, Met, Phe, Thr, Trp and Val were higher for cows fed FPCM compared to FPSB ($P < 0.05$). Cows fed FPCM had higher total DMI compared to FPSB, which can explain why animals fed CAM had higher plasma concentration of most EAA, which is similar to what has been found in Martineau et al. (2014).

Cows fed FPCM and fed RP Met had higher plasma concentration of Met. These results clearly show that balancing GFP through supplementation with CAM and RP-Met was an effective strategy to raise plasma Met to a concentration greater than the FPSB diet. Patton et al. (2015) demonstrated, using a meta-analysis, that His or Met plus Lys added alone was associated with increases in the plasma concentrations of these EAA without affecting others. However, addition of either Lys or Met alone caused significant changes in the plasma concentrations of several EAA, and Met appeared to be much more potent than Lys, causing a decrease in the concentrations of all EAA except Arg and Lys, whereas addition of Lys only decreased His and Phe (Patton et al., 2015). Our study did not find any differences in plasma EAA concentrations when RP Met was added.

An interaction was observed for His concentration, with the highest values (61.8 μM) found for cows fed FPCM and not supplemented with RP Met ($P = 0.05$). Results supports the hypothesis that RP Met supplementation may cause a decrease in plasma concentrations of His, although direct comparison between no addition and supplementation of RP Met had no significant difference for plasma concentration of His. Lapierre et al. (2014) fed cows a diet deficient in MP (72% MP requirements) and infused in the abomasum with His at 0, 7.6, 15.2 or

22.8 g/d in addition to an AA mixture, representing 1.60, 1.95, 2.30 and 2.65% of MP supply, respectively. The researchers found that milk yield was high for cows infused with either 2.30% or 2.65% of His on total dietary MP, and milk protein yield reached a plateau at 1.95% of infused His in total supplied MP. In the present study, diets contained 2.10 and 2.20% (FPSB and FPSM supplemented with RP Met), which according to Lapierre et al. (2014) would be enough to replenish His requirements. Using the AA oxidation indicator technique, Ouellet et al. (2014), evaluated His requirements using 6 lactating Holstein cows in a 6 × 6 Latin square fed 75% of total MP requirements. Cows were infused into the abomasum with a mixture of all AA and His. Histidine was infused in rates of 0, 7.6, 15.2, 22.8, 30.4, and 38.0 g/d, which is relative to 1.5, 1.83, 2.15, 2.46, 2.78 and 3.09% of total MP fed. From the results, authors identified that infusion of 7.6 g/d was sufficient to meet the requirements. At 2.46% of MP, plasma concentration of His and milk protein yield reached a plateau. (Ouellet et al., 2014).

In the study presented herein, model estimations of His as a % of MP was of 2.13% and 2.23%, respectively for FPSB and FPCM, showing that feeding CAM in diets based on GFP can improve duodenal supply of His and have the capacity to increase plasma concentrations of AA. Diets with high soluble protein and consequently high concentration of RDP may have limitations in His in the diet, due to low availability of His from the rumen, and diets with CAM hold potential to improve plasma His concentrations and consequently the efficiency for milk protein yield.

CONCLUSIONS

Cows fed FPCM in this study had higher DMI, higher milk yield and milk protein yield when compared to cows fed FPSB. As hypothesized, cows fed RP Met in diets with 25% GFP

had an increase in milk protein concentration independent of protein supplement fed (CAM or SBM). The present study shows that diets with 25% GFP and 14% CAM are under-predicted in the amount of MP available for milk production, with an MP allowable milk of 33.7 kg/d and actual milk production of 40.5 kg/d. It is possible that CAM have higher value of MP when compared to what the NRC (2001) predicts and this number that should be re-evaluated in future models as shown in the current study, in meta-analysis and other recent publications.

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Table 15. Nutrient composition of feed ingredients used in the experimental diets.

Item	Corn silage	Grass-legume silage	Canola meal	Soybean meal	Ground field peas	Corn meal	Steam flaked corn	DDGS[1]	Citrus pulp
DM, % of fresh matter	44.0	36.1	93.8	94.6	89.4	94.0	96.6	92.6	91.7
CP, % of DM	7.50	15.8	41.8	52.1	21.3	8.30	7.50	29.3	6.80
Soluble protein, % of CP	60.0	62.0	24.0	27.0	69.0	26.0	16.0	15.0	-
NDF, % of DM	39.3	57.0	28.8	7.9	12.1	9.00	6.90	36.5	2.60
ADF, % of DM	23.8	39.7	21.2	7.2	7.6	3.00	3.00	12.5	0.70
Starch, % of DM	37.6	0.8	1.2	1.2	45.6	73.9	72.8	2.00	6.30
Lignin, % of DM	2.80	5.9	9.2	0.3	-	1.10	1.00	2.80	1.70
ADICP, % of DM	0.40	1.0	2.9	4.2	-	0.20	0.40	2.60	0.70
NDICP, % of DM	1.00	2.5	6.6	5.9	-	0.90	1.10	6.90	2.60
Ether Extract, % of DM	3.10	4.6	4.7	1.5	-	3.7	3.30	17.5	3.80
Ash, % of DM	3.30	8.7	7.3	6.7	-	1.30	1.20	6.60	7.60
Ca, % of DM	0.20	0.65	0.72	0.32	-	0.02	0.02	0.03	1.59
P, % of DM	0.25	0.35	1.09	0.83	-	0.33	0.25	0.94	0.12
Mg, % of DM	0.14	0.25	0.58	0.32	-	0.11	0.08	0.34	0.15
K, % of DM	0.96	3.06	1.24	2.49	-	0.42	0.32	1.26	0.80
Ir, mg/kg	121	199	213	112	-	30.0	22.0	94.0	77.0
Zn, mg/kg	24.0	32.0	60.0	53.0	-	23.0	19.0	68.0	20.0
Cu, mg/kg	4.00	8.00	6.00	16.0	-	2.00	1.00	9.00	8.00
Mn, mg/kg	9.00	59.0	66.0	37.0	-	5.00	4.00	13.0	17.0
EAA, % of CP									
Arg	1.67	2.50	6.32	6.99	7.58	4.02	3.85	4.45	3.98
His	1.52	2.05	3.17	2.50	2.46	3.01	3.39	3.11	2.23
Ile	3.64	4.36	4.35	4.43	4.11	3.14	3.70	4.12	3.51
Leu	9.24	7.50	7.58	7.40	7.26	10.1	12.9	12.2	6.85
Lys	3.03	5.26	6.37	6.26	7.36	3.64	3.54	3.47	3.51
Met	1.82	1.60	2.11	1.32	1.07	1.63	2.00	1.95	1.27
Phe	3.79	4.68	4.21	4.88	4.80	4.14	5.08	4.85	4.14

Thr	3.18	4.04	4.48	3.70	3.84	3.14	3.54	3.94	3.51
Trp	0.45	0.58	1.25	1.48	0.85	0.75	0.92	0.72	1.12
Val	5.15	5.71	5.79	4.31	4.59	4.52	5.24	5.25	4.78
NEAA, % of CP									
Ala	8.79	6.93	4.72	4.10	4.38	6.28	7.70	7.05	4.94
Asp	5.76	7.89	7.50	10.7	11.1	6.53	7.08	6.48	10.4
Cys	1.52	0.96	2.51	1.26	1.39	1.88	2.16	1.99	1.43
Gly	11.1	7.31	17.6	16.8	4.48	15.3	18.9	13.5	11.2
Glu	4.09	5.07	5.39	4.04	16.0	3.64	3.85	3.94	4.78
Orn	1.06	1.22	0.08	0.08	0.05	0.13	0.15	0.11	0.16
Pro	6.36	4.68	6.45	4.51	4.22	7.03	9.09	7.71	7.65
Ser	3.03	3.08	3.81	4.21	4.16	3.77	4.62	4.52	3.66
Tyr	0.30	0.32	0.16	0.20	3.09	0.13	0.15	0.04	1.91
Tau	1.67	2.12	2.69	3.60	0.69	1.76	1.85	3.76	2.23

[1] DDGS: Dry distiller's grains with solubles.

Table 16. Ingredient and nutrient composition of the 2 experimental diets fed to 16 lactating dairy cows[1]. Rumen protected Met was top dressed to 4 cows in each diet in the amount of 27 grams per day (10.8 g in the a.m. and 16.2 g in the p.m.).

	Treatments	
Ingredients, % of diet DM	FPSB	FPCM
Corn silage	35.0	35.0
Grass-legume silage	14.0	14.0
Corn meal	6.5	5.0
Steam-flaked corn	3.2	2.5
Dry distiller's grains	1.3	1.0
Ground field peas	25.0	25.0
Soybean meal	11.0	-
Canola meal	-	13.5
Citrus pulp	1.5	1.5
Minerals and vitamins[2]	2.50	2.50
Penn State particle separator, %		
> 19.0 mm	7.20	9.40
8.0 – 19.0 mm	33.9	30.8
1.18 – 8.0 mm	42.6	37.9
< 1.18 mm	16.3	21.9
Nutrient composition		
CP, %	17.1	16.7
NE_L, Mcal/kg	1.57	1.55
NDF, %	26.9	29.6
ADF, %	16.3	18.2
Ether extract, %	2.8	3.1
NFC[3], %	52.1	49.4
Ca, %	0.6	0.6
P, %	0.4	0.5

[1]FPSB = ground field peas plus soybean meal. FPCM: ground field peas plus canola meal. Two diets were top dressed with 27 g/d of RP D-L-Met (Smartamine-M, Adisseo, France).
[2]Mineral and vitamin mix provided on as fed basis: 297 mg/kg monensin sodium (Rumensin; Elanco, Greenfield, IN), 13.6% Ca, 1.31% P, 4.77% Mg, 0.18% K, 0.72% S, 32 mg/kg Co, 422 mg/kg Cu, 1,290 mg/kg Mn, 8.97 mg/kg Se, 2,260 mg/kg Zn, and 87.1 KIU/kg vitamin A.
[3]NFC = 100 – [CP + (NDF – NDICP) + fat + ash]

Table 17. Means for variables predicted by the NRC (2001) model[1] in lactating dairy cows fed a diets containing 25% field peas and soybean meal or canola meal as main protein sources with or without supplementation of rumen protected (RP) Met (Smartamine-M, Adisseo).

	Treatment			
Item	FPSB	FPCM	FPSB + RP Met[2]	FPCM + RP Met[2]
Estimated DMI, kg/d	26.7	27.2	26.7	27.2
NE_L allowable milk, kg/d	45.3	46.4	45.3	46.5
NE_L balance, Mcal/d	4.60	4.20	4.60	4.30
RDP, % of DMI	11.9	12.1	11.9	12.1
RUP, % of DMI	5.10	4.60	5.20	4.70
RDP balance, g/d	578	679	580	682
RUP balance, g/d	-91.0	-445	-71	-421
MP allowable milk, kg/d	37.4	33.7	37.7	34.0
MP-bacterial, g/d	1,504	1,528	1,505	1,529
MP-RUP, g/d	1,225	1,023	1,242	1,039
MP-endogenous, g/d	132	136	132	136
MP balance, g/d	-77.0	-341	-60.0	-324
Duodenal flow Lys % of MP	6.84	7.05	6.80	7.00
Duodenal flow Met % of MP	1.78	1.96	2.16	2.38
Duodenal flow His % of MP	2.13	2.23	2.11	2.21
MP-Lys:MP-Met ratio	3.8 : 1	3.6 : 1	3.15 : 1	2.94 : 1

[1]Actual feed nutrient composition and animal variables were used (i.e., DMI, milk yield and composition, DIM, and BW) in the NRC (2001) evaluation software.

[2] Rumen protected Met was added at 27 g/d and increased duodenal flow of Met from 1.78% to 2.31% for SB diet and from 1.96% to 2.55% for CM diet.

Table 18. Least square means for DMI, ADG, milk yield and composition, and feed efficiency N in lactating dairy cows fed diets with 25% ground field peas and soybean meal (FPSB) or canola meal (FPCM) without rumen protected (RP) Met (None) and with supplementation of RP Met.

	Protein source		RP-Met			*P*-value[1]		
Item	FPSB	FPCM	None	Added	SEM	Protein	RP-Met	Interaction
DMI, kg/d	28.0	28.8	28.4	28.4	0.74	<0.01	0.80	0.26
N intake, g/d	765	771	767	769	19.9	0.50	0.81	0.27
ADG, kg/d	0.52	1.00	0.60	0.93	0.10	<0.001	<0.01	0.31
Milk yield, kg/d	38.9	40.5	39.9	39.5	1.96	<0.001	0.44	0.25
ECM, kg/d[2]	43.0	44.7	44.0	43.7	1.94	<0.01	0.46	0.17
4% FCM, kg/d[3]	36.1	37.5	37.0	36.5	1.61	<0.01	0.24	0.09
Milk yield/DMI, kg/kg	1.39	1.41	1.41	1.40	0.06	0.47	0.51	0.96
ECM/DMI, kg/kg	1.54	1.55	1.55	1.54	0.05	0.61	0.47	0.74
4% FCM/DMI, kg/kg	1.29	1.30	1.31	1.29	0.04	0.50	0.25	0.50
Milk N/N intake, %	26.6	27.4	26.8	27.4	0.94	0.04	0.18	0.61
Milk fat, %	3.56	3.54	3.56	3.53	0.11	0.64	0.80	0.25
Milk fat, kg/d	1.37	1.42	1.41	1.38	0.06	0.01	0.22	0.07
Milk true protein, %	3.38	3.35	3.32	3.41	0.07	0.17	<0.001	0.12
Milk true protein, kg/d	1.30	1.35	1.31	1.34	0.06	<0.01	0.14	0.74
Milk lactose, %	4.88	4.85	4.89	4.85	0.04	0.06	0.05	0.99
Milk lactose, kg/d	1.90	1.97	1.95	1.92	0.10	<0.01	0.22	0.27
Milk SNF, %	9.15	9.10	9.09	9.16	0.06	0.02	<0.01	0.12
Milk SNF, kg/d	3.55	3.69	3.62	3.61	0.17	<0.01	0.88	0.43
Milk total solids, %	12.71	12.63	12.66	12.69	0.15	0.10	0.50	0.80
Milk total solids, kg/d	4.92	5.11	5.03	5.00	0.23	<0.01	0.59	0.24
MUN, mg/dL	12.8	11.9	12.3	12.3	0.51	<0.001	0.93	0.22
SCC, 1,000 cells/mL[4]	74.6	78.9	78.7	74.8	0.25	0.92	0.91	0.42

[1] Probability of treatment effect (SB vs. CM or None vs. Added RP-Met and interaction between all treatments); significance was declared at $P \leq 0.05$ and trends at $0.05 < P \leq 0.10$.

[2]ECM (kg/d) = [0.0752 × milk yield (kg/d)] + [12.3 × fat yield (kg/d)] + [6.56 × SNF (kg/d)] (Tyrrell and Reid, 1965).

[3]4% FCM = [0.4 × milk yield (kg/d)] + [15 × milk fat yield (kg/d)] (NRC, 2001).

[4]Somatic cell count results were transformed using a natural log and back-transformed for least square means report. SEM and *P* values are from natural log transformed data. 1 cow was removed from 4 periods from SCC (n = 4) analysis due to being an outlier using a Shapiro-Wilk test, $P < 0.05$.

Table 19. Least square means for the coccygeal artery or vein plasma concentration of EAA and NEAA amino acids in lactating dairy fed a diets containing 25% field peas and soybean meal or canola meal as main protein sources with or without supplementation of rumen protected (RP) Met (Smartamine-M, Adisseo). (Preliminary data from 3 of 4 experimental periods).

	Protein source		RP-Met			*P*-value[1]		
Item, (μ*M*)	FPSB	FPCM	None	Added	SEM	Protein	RP-Met	Interaction
EAA								
Arg	83.7	84.9	84.0	84.6	2.64	0.57	0.77	0.48
His	60.1	63.0	62.2	61.0	2.66	0.09	0.46	0.05
Ile	135	142	139	138	4.78	0.04	0.98	0.19
Leu	151	161	157	154	6.21	0.02	0.38	0.86
Lys	88.1	93.2	90.0	91.3	3.87	0.06	0.62	0.71
Met	29.4	32.6	23.6	38.4	0.57	<0.001	<0.001	0.65
Phe	43.8	46.2	44.9	45.1	1.06	0.02	0.82	0.41
Thr	105	117	111	110	4.17	<0.001	0.79	0.77
Trp	37.7	41.0	39.2	39.5	0.93	<0.001	0.74	0.63
Val	273	299	288	284	9.96	<0.001	0.52	0.77
NEAA								
Ala	274	286	277	283	6.62	0.04	0.37	0.81
Asn	54.6	53.6	53.5	54.8	1.76	0.30	0.17	0.28
Asp	3.20	3.51	3.55	3.16	0.42	0.53	0.43	0.61
Cit	98.5	95.9	96.6	97.7	4.23	0.30	0.67	0.45
Cystathionine	2.43	2.62	2.02	3.02	0.09	0.07	<0.001	0.61
Cys	21.7	21.2	20.8	22.1	0.45	0.33	0.01	0.32
Glu	40.4	41.1	41.5	39.9	1.26	0.53	0.17	0.25
Gln	267	260	264	263	6.68	0.33	0.88	0.48
Gly	309	293	305	296	9.97	<0.01	0.07	0.59
Homocysteine	2.98	3.06	2.94	3.09	0.25	0.44	0.15	0.06
Orn	51.8	53.1	51.9	53.0	2.00	0.33	0.39	0.71
Pro	89.7	86.9	88.9	87.7	2.86	0.08	0.42	0.83
Ser	88.5	83.9	87.4	85.0	2.54	<0.01	0.14	0.27
Tau	54.5	58.4	53.2	59.7	2.29	0.44	<0.01	0.02

Tyr	44.3	49.3	46.0	47.6	1.43	0.001	0.25	0.88
3-Methylhistidine[2]	3.72	3.75	3.81	3.66	0.18	0.78	0.21	0.08
Total BCAA[3]	558	602	584	577	19.9	<0.001	0.55	0.66
Total EAA	1006	1079	1039	1047	29.4	<0.001	0.68	0.95
Total urea cycle AA[4]	234	234	232	235	6.81	0.99	0.55	0.42
Total sulfur AA[5]	106	112	98	120	2.25	<0.01	<0.001	0.06
Total NEAA	1406	1396	1399	1402	21.4	0.55	0.86	0.82
Total AA[6]	2138	2189	2161	2167	39.6	0.09	0.83	0.82

[1] Probability of treatment effect (SB vs. CM or None vs. Added RP-Met and interaction between all treatments); significance was declared at $P \leq 0.05$ and trends at $0.05 < P \leq 0.10$.

[2]Dipeptide (β-alanyl-l-His).

[3]Total branched-chain amino acids (BCAA) = Ile + Leu + Val.

[4]Total urea cycle AA = Arg, Cit and Orn

[5]Total sulfur AA = Cystathionine + Cys + Homocysteine + Met + Tau

[6]Total AA = total EAA + total NEAA.

GENERAL CONCLUSIONS

As shown in Chapter III, feeding GFP with RP-Lys and RP-Met increased milk and milk protein yield in the same proportion compared to a diet based on SBM and corn meal supplemented with RP Lys and Met. But when feeding FP with both RP-Lys and RP-Met, a significant increase in plasma Lys and Met occurred, with a decrease in plasma concentrations of His. One reason might be that after Lys and Met requirements were met, His became the most limiting AA and was used in the mammary gland, causing a decrease in its plasma concentrations. Another reason for a decrease in His could be from influence of RP-Met addition to the diet. Patton et al. (2015) performed a meta-analysis in order to correlate diet and plasma concentrations of AA and used 420 treatment means from 104 studies. According to the authors, addition of Met to the diet caused a decrease in all EAA except Lys and Arg, whereas addition of Lys caused a decrease in His and Phe (Patton et al., 2015). Ground field peas has a high concentration of Lys and diets with FP had high estimated duodenal flow and plasma concentration of Lys (see Chapter IV). Further studies should be performed using either a natural source of His or RP-His fed with FP supplemented with RP-AA in order to better understand the observed reduction in the plasma concentration of His.

The last chapter of this dissertation (Chapter IV) aimed to understand the effects of feeding CAM to diets with 25% GFP and addition of rumen protected Met. The objective of this study was to evaluate if feeding GFP and CAM with a source of rumen protected Met to balance duodenal availability of Met will increase plasma AA concentrations and possibly increase milk and milk protein yield. We hypothesize that feeding CAM instead of SBM will cause an increase in plasma AA that can be due either to higher DMI or to higher absorption of AA, thus increasing the availability of essential AA at the mammary gland for milk protein production.

Cows fed ground field peas and canola meal had higher DMI, higher milk yield and milk protein yield when compared to cows fed ground field peas with soybean meal. Cows fed a diet with canola meal replacing soybean meal in diets with 25% GFP had significant higher values of Met, Thr and Trp and Val and a trend for higher concentration of Phe and Leu. Cows fed RP Met in diets with 25% GFP had an increase in milk protein concentration independent of protein supplement fed (canola meal or soybean meal).

Overall, despite high concentrations of starch in the diet, feeding ground field peas to lactating dairy cows increased productivity when compared to a diet with urea as main source of N in the diet. True soluble protein from ground field peas increased microbial fermentation and improved digestibility parameters. Diets with ground field peas with rumen protected amino acid yielded similar animal performance when compared to a positive control composed of corn meal and soybean meal as main starch and protein sources.

Feeding canola meal in diets with 25% ground field peas was done with the null hypothesis that canola meal would maintain DMI, intake of AA and consequently, concentration of plasma amino acids. With the results of Chapter IV, the null hypothesis was rejected showing that canola meal improved all variables described, consequently causing an increase in milk yield and milk protein yield. Milk protein concentration was increased when cows were fed RP Met, independently of which protein supplement was fed, soybean meal or canola meal.

In conclusion, dairy farmers can feed ground field peas and canola meal in their diets with RP Met supplementation for increase in dry matter intake and consequently milk and milk protein yield.

APPENDIX I: COPYRIGHT PERMISSION FROM THE JOURNAL OF DAIRY SCIENCE FOR CHAPTER II:

JOURNAL OF DAIRY SCIENCE

1800 South Oak St. STE 100, Champaign, IL 61820 FAX: 217.378.4083 journals@assochq.org

April 5, 2016

Andre B. D. Pereira
University of New Hampshire

Dear Mr. Pereira:

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Pereira, A.B.D., A.F. Brito, L.L. Townson, and D.H. Townson. 2013. Assessing the research and education needs of the organic dairy industry in the northeastern United States. J. Dairy Sci. 96:7340–7348. doi:10.3168/jds.2013-6690.

Pereira, A.B.D., S.A. Utsumi, C.D. Dorich, and A.F. Brito. 2015. Integrating spot short-term measurements of carbon emissions and backward dietary energy partition calculations to estimate intake in lactating dairy cows fed ad libitum or restricted. J. Dairy Sci. 98:8913–8925. doi:10.3168/jds.2015-9659.

Sincerely,

Susan Pollock
Susan Pollock
Managing Editor
Journal of Dairy Science

APPENDIX II: REQUIRED DOCUMENTS FROM IACUC

IACUC no. 121203 from chapter III:

University of New Hampshire

Research Integrity Services, Service Building
51 College Road, Durham, NH 03824-3585
Fax: 603-862-3564

13-Dec-2012

Brito, Andre Fonseca De
UNH Farms, Dairy T & R Ctr
Durham, NH 03824

IACUC #: 121203
Category: C
Project: Comparison of Two Techniques to Measure Methane Emissions in Lactating Dairy Cows

The Institutional Animal Care and Use Committee (IACUC) has reviewed and recommended approval of the protocol submitted for this study contingent upon your response to the following:

1. In Section IV, A of the application, the researcher needs to add use of a log book to record monitoring of animals wearing the harnesses.
2. In Section VI, D, table v, the researcher needs to revise the animal numbers to reflect those in Section V, Table 1.

As soon as the IACUC receives an appropriate response to its concerns, above, it will issue you an approval letter for this protocol. **You may not commence activities in this protocol involving vertebrate animals until you have received IACUC approval.** Please respond to the IACUC within sixty days of this letter. If the IACUC does not receive a response within sixty days, your protocol will be withdrawn from further consideration by the IACUC.

If you have any questions, please contact either Dean Elder at 862-4629 or Julie Simpson at 862-2003.

For the IACUC,

Jill A. McGaughy, Ph.D.
Chair

cc: File

University *of* New Hampshire

Research Integrity Services, Service Building
51 College Road, Durham, NH 03824-3585
Fax: 603-862-3564

29-Apr-2016

Brito, Andre Fonseca De
Biological Sciences
Keener Dairy Research
Durham, NH 03824

IACUC #: 121203
Project: Comparison of Two Techniques to Measure Methane Emissions in Lactating Dairy Cows
Approval Date: 13-Dec-2012

The Institutional Animal Care and Use Committee (IACUC) reviewed and approved the protocol submitted for this study under Category C on Page 5 of the Application for Review of Vertebrate Animal Use in Research or Instruction - *the research potentially involves minor short-term pain, discomfort or distress which will be treated with appropriate anesthetics/analgesics or other assessments.*

Approval is granted for a period of three years from the approval date above. Continued approval throughout the three year period is contingent upon completion of annual reports on the use of animals. At the end of the three year approval period you may submit a new application and request for extension to continue this project. Requests for extension must be filed prior to the expiration of the original approval.

Please Note:
1. All cage, pen, or other animal identification records must include your IACUC # listed above.
2. Use of animals in research and instruction is approved contingent upon participation in the UNH Occupational Health Program for persons handling animals. Participation is mandatory for all principal investigators and their affiliated personnel, employees of the University and students alike. Information about the program, including forms, is available at http://unh.edu/research/occupational-health-program-animal-handlers.

If you have any questions, please contact either Dean Elder at 862-4629 or Julie Simpson at 862-2003.

For the IACUC,

Jill A. McGaughy, Ph.D.
Chair

cc: File
,
,

IACUC no. 140402 from Chapter IV:

University of New Hampshire

Research Integrity Services, Service Building
51 College Road, Durham, NH 03824-3585
Fax: 603-862-3564

21-Apr-2014

Brito, Andre Fonseca De
UNH Farms, Dairy T & R Ctr
Durham, NH 03824

IACUC #: 140402
Category: D
Project: Effects of Feeding Two Levels of Field Peas with or without Addition of Rumen Protected Amino Lysine and Methionine in Lactating Holstein Dairy Cows

The Institutional Animal Care and Use Committee (IACUC) has reviewed and recommended approval of the protocol submitted for this study contingent upon your response to the following:

1. Kate Homan needs to receive occupational health program approval to handle vertebrate animals prior to doing so.
2. In Section II, F of the application, the investigator needs to remove the check from "field study."
3. In the last sentence of III, B of the application, the investigator needs to change "nutrient" to "nitrogen."
4. The investigator needs to make the number of animals (16) consistent through the application.
5. The investigator needs to add to Section VII, A, #4 of the application information about the catheter.
6. The investigator needs to add to Section VII, A, #3, part e of the application information for the second type of blood sampling.

As soon as the IACUC receives an appropriate response to its concerns, above, it will issue you an approval letter for this protocol. **You may not commence activities in this protocol involving vertebrate animals until you have received IACUC approval**. Please respond to the IACUC within sixty days of this letter. If the IACUC does not receive a response within sixty days, your protocol will be withdrawn from further consideration by the IACUC.

If you have any questions, please contact either Dean Elder at 862-4629 or Julie Simpson at 862-2003.

For the IACUC,

Jill A. McGaughy, Ph.D.
Chair

cc: File

University *of* New Hampshire

Research Integrity Services, Service Building
51 College Road, Durham, NH 03824-3585
Fax: 603-862-3564

16-May-2014

Brito, Andre Fonseca De
UNH Farms, Dairy T & R Ctr
Durham, NH 03824

IACUC #: 140402
Project: Effects of Feeding Two Levels of Field Peas with or without Addition of Rumen Protected Amino Lysine and Methionine in Lactating Holstein Dairy Cows
Category: D
Modification Approval Date: 16-May-2014
Annual Approval Expiration Date: 18 Apr 2015
Protocol Three-Year Approval Expiration Date: 18-Apr-2017

The Institutional Animal Care and Use Committee (IACUC) has reviewed and approved the requested modification to the protocol for this study:

Changes per 4/30/2014 request

If you have any questions, please contact either Dean Elder at 862-4629 or Julie Simpson at 862-2003.

For the IACUC,

Jill A. McGaughy, Ph.D
Chair

cc: File

IACUC no. 151206 from chapter V:

University *of* New Hampshire

Research Integrity Services, Service Building
51 College Road, Durham, NH 03824-3585
Fax: 603-862-3564

22-Dec-2015

Brito, Andre Fonseca De
Biological Sciences
Keener Dairy Research
Durham, NH 03824

IACUC #: 151206
Project: Effects of Feeding Lactating Holstein Cows Canola Meal as a Source of Histidine in Diets with Field Peas as a Main Source of Protein With or Without Rumen Protected Methionine
Approval Date: 17-Dec-2015

The Institutional Animal Care and Use Committee (IACUC) reviewed and approved the protocol submitted for this study under Category C on Page 5 of the Application for Review of Vertebrate Animal Use in Research or Instruction - *the research potentially involves minor short-term pain, discomfort or distress which will be treated with appropriate anesthetics/analgesics or other assessments.*

Approval is granted for a period of three years from the approval date above. Continued approval throughout the three year period is contingent upon completion of annual reports on the use of animals. At the end of the three year approval period you may submit a new application and request for extension to continue this project. Requests for extension must be filed prior to the expiration of the original approval.

Please Note:
1. All cage, pen, or other animal identification records must include your IACUC # listed above.
2. Use of animals in research and instruction is approved contingent upon participation in the UNH Occupational Health Program for persons handling animals. Participation is mandatory for all principal investigators and their affiliated personnel, employees of the University and students alike. Information about the program, including forms, is available at http://unh.edu/research/occupational-health-program-animal-handlers.

If you have any questions, please contact me at 862-4629 or Julie Simpson at 862-2003.

For the IACUC,

Dean Elder, D.V.M.
Vice Chair

cc: File

University *of* New Hampshire

Research Integrity Services, Service Building
51 College Road, Durham, NH 03824-3585
Fax: 603-862-3564

24-Feb-2016

Brito, Andre Fonseca De
Biological Sciences
Keener Dairy Research
Durham, NH 03824

IACUC #: 151206
Project: Effects of Feeding Lactating Holstein Cows Canola Meal as a Source of Histidine in Diets with Field Peas as a Main Source of Protein With or Without Rumen Protected Methionine
Modification Approval Date: 23-Feb-2016
Annual Approval Expiration Date: 17-Dec-2016
Protocol Three-Year Approval Expiration Date: 17-Dec-2018

The Institutional Animal Care and Use Committee (IACUC) has reviewed and approved the requested modification to the protocol for this study:

Addition of blood sample from mammary vein

If you have any questions, please contact either Dean Elder at 862-4629 or Julie Simpson at 862-2003.

For the IACUC,

Jill A. McGaughy, Ph.D.
Chair

cc: File