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Effects of Rumen-protected Amino Acids and Levels and Sources of Energy on Milk Production and Nutrient Utilization in Dairy Cattle Fed Metabolizable Protein-deficient Diets

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ABSTRACT

Lactating dairy cows are characterized by poor N efficiency and dietary N not captured in milk protein is excreted in urine and feces, which then contribute to environmental N pollution. Nitrogen losses also shrink profit margins for dairy producers due to costly protein sources. Additionally, the dairy industry is an important anthropogenic source of greenhouse gases such as CO_2 and CH_4, with CH_4 representing a potent greenhouse gas and non-negligible energy losses in dairy cattle. Thus, my PhD program has focused on developing nutrition-based approaches to improve milk production efficiency (e.g., milk yield/dry matter intake) and reduce N excretion and greenhouse gas emissions from dairy cattle. Three experiments were conducted on lactating Holstein cows to investigate the effects of rumen protected AA and levels and sources of energy on milk production and nutrient utilization in dairy cows fed low protein diets. Dairy rations with high energy (≥ 1.60 Mcal of net energy of lactation/kg) and low protein (≤ 16% crude protein) concentrations have been shown to increase milk production and feed efficiency and decrease urinary N excretion and CH_4 emissions. We also discovered that feeding low protein dairy diets (≤ 16% crude protein) with fibrous byproducts and RP-fat as replacements for ground corn further enhanced milk production efficiency and milk fat yield without contributing more CO_2 and CH_4 to the environment. Furthermore, supplementation with rumen-protected Met, Lys, and His had limited effects on milk production and nutrient utilization in dairy cows fed low protein diets. These findings mean that farmers may feed cows diets high in fat and low in protein to achieve gains in profit margin and production efficiency. Further research is still needed to compare high protein (≥ 17% crude protein) diets and high fat, low protein diets on production performance and balance of N and energy in lactating dairy cows.

CHAPTER I: LITERATURE REVIEW

Utilization of Nitrogen in Dairy Cattle

Dietary protein (N) can be classified into RDP and RUP: RDP is utilized by ruminal microorganisms to synthesize microbial protein, whereas RUP pass through without ruminal degradation (NRC, 2001). Ruminal synthesized microbial protein, RUP and endogenous N contribute to duodenal N flow, and are then digested and absorbed by the small intestine (NRC, 2001). Free AA and ammonia not utilized by gut tissues enter the circulatory system and go to the liver via hepatic portal vein (Lapierre et al., 2005, 2006). In the liver, AA are either used for production of glucose and protein or pass through the liver without any modifications (Lapierre et al., 2005). After leaving the liver, AA are transported to peripheral tissues including the mammary gland for milk protein synthesis and muscle tissues for muscle replenishment (Wang et al., 2019).

Nitrogen Sources in Diets

Dietary protein is often expressed as CP, which includes true protein and NPN. Dietary CP concentration is determined by multiplying the N content of feeds by 6.25 because the average N content of protein is 16% (NRC, 2001). True protein based on solubility has been classified into globular proteins (e.g., albumins, globulins, glutelins, prolamines, and histones) and fibrous proteins (e.g., collagens, elastins, and keratins), and some examples of NPN are peptides, free AA, nucleic acids, nitrates, amines, and ammonia (NRC, 2001). According to NRC (2001), the main protein sources are plant-based products including soybean meal (~44 or 48% CP), canola meal (~38% CP), corn gluten meal (~40 or 60% CP), corn distillers grains with solubles (~30% CP), and brewers' grain (~29% CP). High quality forages contain high concentrations of CP: alfalfa silages provide more than 20% CP and grass silages supply approximately 17% CP on a DM basis (NRC, 2001; Schwab and Broderick, 2017), and the CP

content of pastures ranges from 12 to 18% CP on a DM basis depending on pasture forages, grazing time and rainfall (Pembleton et al., 2016). The NRC (2001) demonstrates that animal-based protein feeds contain high levels of CP (DM basis): fish meal (~71% CP), feather meal (~92% CP), and blood meal (~95% CP); however, only small amounts of them (< 1 kg per cow per day) can be included in dairy diets (Shaver, 2005).

Microbial Protein Synthesis in the Rumen

Rumen degradable protein such as peptides, free AA, and ammonia can be utilized by ruminal microorganisms to synthesize microbial protein, which represents 50 to 80% of the protein flowing to the small intestine (Storm and Ørskov, 1983). The remaining protein sources leaving the rumen are RUP and endogenous protein (NRC, 2001). Moreover, ruminally synthesized microbial protein is known as a good quality protein due to its high apparent digestibility and balanced AA profile, and the EAA composition of microbial protein is similar to that of milk and lean body tissue (Schwab and Broderick, 2017). As mentioned by Schwab and Broderick (2017), at least 200 species of bacteria, more than 20 species of protozoa, and over 12 species of fungi have been established in the rumen.

Bacteria are the most abundant microorganisms in the rumen ($10^{10\text{-}11}$/mL) and more than 40% of isolated species are capable of synthesizing proteases (Wallace, 1996; Schwab and Broderick, 2017). Approximately 10% of ruminal bacteria exhibiting proteolytic activity can release proteases into the rumen (Broderick, 1998), and the remainder are associated with cell-bound microbial proteases (Kopecny and Wallace, 1982). Thus, ruminal bacteria are first attached to feed particles and then degrade dietary protein into peptides and AA (Brock et al., 1982). Peptides and AA derived from bacterial-surface proteolysis activity are transported inside

bacteria where peptides can be further degraded by peptidases into AA. Next, AA inside bacteria are either used to synthesize microbial protein or deaminated to VFA, CO_2, and ammonia (Tamminga, 1979), whose fates are influenced by availability of ruminally fermentable energy. Compared to bacteria, protozoa are less abundant ruminal microorganisms (10^{5-6}/mL) and represent about 40% of ruminal microbial biomass by utilization of dietary fibrous and nonfibrous carbohydrates and protein (Russell and Rychlik, 2001; Schwab and Broderick, 2017). The substrates ingested by protozoa include bacteria, fungi, and small feed particles, and are degraded into peptides and AA, which are used for synthesis of protozoal protein (NRC, 2001). However, over 65% of protozoal protein remains in the rumen (Punia et al., 1992) so that protozoal protein only accounts for 10 to 30% of microbial protein in the small intestine (Shabi et al., 2000; Sylvester et al., 2005). Ruminal fungi (10^{3-4}/mL) play a limited role in microbial protein synthesis partly due to their low concentrations (Schwab and Broderick, 2017). According to Faichney et al. (1997), the anaerobic fungi only accounts for 1.1 to 3.5% of microbial protein in the rumen and 0.7 to 2.7% of microbial protein in the small intestine.

Microbial protein synthesis in the rumen can be impacted by types and amounts of carbohydrates (Sannes et al., 2002; Zhu et al., 2013). Carbohydrates are sources of carbon skeletons and energy, and nonfibrous carbohydrates such as starch and sugars are more effective in stimulating microbial protein synthesis than fibrous carbohydrates such as cellulose and hemicellulose (Voelker and Allen, 2003). It is widely recognized that ruminal fermentation rate of nonstructural carbohydrates is faster than that of structural carbohydrates (NRC, 2001). Amylolytic microbes degrading starch can utilize ammonia, peptides, and AA as N sources to synthesize microbial protein whereas cellulolytic bacteria that degrade structural carbohydrates mainly use ammonia as their N source (Russell et al., 1992). Additionally, compared with

ammonia, preformed AA such as free AA and peptides can promote microbial protein synthesis and fiber digestion in the rumen (Carro and Miller, 1999; Brito et al., 2007). Optimal microbial growth in pH-controlled culture fermenters was obtained when dietary NFC to RDP ratio was equal to 2:1 (Hoover and Stokes, 1991); however, the optimum ratio between NFC and ammonia N has not been identified in vivo. Moreover, synchronization of ruminal degradation between carbohydrates and protein is an important factor to determine efficiency of microbial protein synthesis (Cabrita et al., 2006). Conversely, Reynolds and Kristensen (2008) has concluded that urea recycling to the rumen alleviates the effect of asynchronous N and energy supply on microbial protein synthesis. Overall, ruminal microbial protein synthesis can be impacted by many factors including solubility and structure of protein, passage rate, ruminal pH and substrate, and nutrient interaction (Bach et al., 2015).

Protein Digestion and Absorption in the Gastrointestinal Tract

Rumen microbial protein, RUP and endogenous protein are the 3 protein sources flowing to the small intestine (NRC, 2001). Endogenous protein consists of saliva, sloughed epithelial cells and the remains of lysed ruminal microorganisms (NRC, 2001). Microbial protein accounts for from 50 to 80% of the protein entering the small intestine and is characterized by high intestinal digestibility and decent EAA profile that is similar to that of milk (Schwab and Broderick, 2017). The contribution of dietary RUP to protein in the small intestine can be impacted by the ingredient composition of the diet (NRC, 2001). Endogenous protein represents from 8 to 16% of the protein in the duodenum (Reynolds, 2005; Lapierre et al., 2006). Overall, the proportion of each fraction is dependent on the nutrition and ingredient composition of diets, ruminal environment and feed intake, and can be varied greatly.

According to NRC (2001), microbial protein yield can be calculated as 0.13 × TDN when RDP intake is more than 1.18 × microbial protein yield and as 0.85 × RDP when RDP intake is less than 1.18 × TDN-estimated microbial protein yield. Approximately 80% of microbial protein is true protein that is assumed to have 80% intestinal digestibility, with the remaining 20% of microbial protein composed of nucleic acid (NRC, 2001). Therefore, approximately 64% of microbial protein can be converted to MP in the small intestine. The intestinal digestibility of RUP has been measured using the mobile bag technique and can range from 50 to 100% depending on individual feeds (NRC, 2001). The proportion of RUP converted to MP is influenced by dietary ingredients. Moreover, 50% of endogenous N (g/d) calculated by multiplying DMI (kg/d) by 1.9 is considered as true protein and the intestinal digestibility of endogenous true protein is 80% (NRC, 2001). Thus, approximately 40% of endogenous protein is converted to MP in the duodenum.

Around 65% of AA derived from MP is transported to the blood mainly due to oxidation of AA in epithelial cells of the small intestine (Lapierre et al., 2005). Large variations in intestinal utilization exist among AA, with higher values for Leu, Thr, and some NEAA including Glu and Asp (Berthiaume et al., 2001). For instance, about 16-24% of intestinal Leu was oxidized in cells of gut tissues (Lapierre et al., 1999).

Metabolism of AA in the Liver

After being absorbed by the small intestine and transported to the portal vein, AA are extracted by the liver and then transported to peripheral tissues. The liver plays an central role in homeostasis of AA, and there are up to 2,000 L of blood passing through the liver each hour in dairy cows (Reynolds et al., 1988). Approximately 45% of portal absorbed AA were removed by

the liver, ranging from 16 to 69% among individual AA (Lapierre et al., 2005). There are 2 pathways for AA cleared by the liver: 1) being utilized to synthesize glucose (mainly from NEAA) and proteins (e.g., plasma export proteins), and 2) being deaminated to produce urea. The remaining AA pass through the liver without any modifications and are transported to peripheral tissues. Lapierre et al. (2005) demonstrated that the hepatic removal was low for Leu, Ile, Val and Lys, high for His (~36%), Met (~38%), and Phe (49%), and moderate for Thr (~25%). These variations in hepatic removal of EAA may be a result of differential hepatic affinities for EAA in dairy cows (Hanigan, 2005). Furthermore, hepatic removal was higher for total NEAA compared with total EAA, which may be explained by the theory that EAA would be prioritized to be used by the mammary gland (Doepel et al., 2009).

Hepatic AA removal has been shown to be associated with total liver input of AA determined by both blood AA concentrations and blood flow (Hanigan, 2005). Specifically, Hanigan et al. (2004) reported that the amounts of AA removed by hepatic tissue are largely impacted by blood supply, except for some NEAA including Ala, Asn, Asp, Gln, and Glu. Greater energy supply has been shown to improve hepatic blood flow (Reynolds, 1995); however, changes in MP supply did not modify blood flow in the liver (Blouin et al., 2002; Raggio et al., 2004). Nevertheless, hepatic removal of His, Met, Phe, and Thr and utilization of branched-chain AA, Lys, and Thr in peripheral tissues were decreased for reduced dietary protein content (12.7 vs. 16.6% CP; Raggio et al., 2004), suggesting that hepatic AA removal may be mainly regulated by blood AA concentrations in this study.

As mentioned above, one of the fates of AA removed by the liver is for urea synthesis and 40-80% of hepatic urea N is recycled back to the gastrointestinal tract, in particular the rumen (Harmeyer and Martens, 1980). Recycling of urea N entering the rumen could be

hydrolyzed into ammonia, which is then utilized for microbial protein synthesis and has a major effect on N metabolism of dairy cows (Lapierre and Lobley, 2001).

Metabolism of AA in the Mammary Gland

The mammary gland represents the primary user of the splanchnic flux of AA in lactating dairy cows. Approximately 90% of AA extracted by the mammary gland was utilized to synthesize milk protein (Cant et al., 1993) and the remaining AA is either used for tissue replenishment or for catabolism (Cant et al., 2018). According to Lapierre et al. (2005), the milk output to post-liver supply ratios for His, Met, and Phe were 98, 101, and 97%, respectively. Additionally, the ratios of milk output to splanchnic flux were 58, 61, 65, and 61% for Ile, Leu, Lys, and Val, respectively (Lapierre et al., 2005). Milk NEAA output is more than mammary NEAA uptake and thus de novo synthesis must occur, possibly through transamination of branched-chain AA and Lys (Guinard and Rulquin, 1994; Lapierre et al., 2003).

Mammary extraction for individual AA can be impacted by blood AA supply (Bequette et al., 2000), lactation stage (Schwab et al., 1992), and levels of hormones (Mackle et al., 2000). For instance, mammary clearance rate of His and other AA increased by 43 fold and decreased by 2-3 fold, respectively, and mammary blood flow improved by approximately 33% in lactating goats with His deficiency (Bequette et al., 2000). According to Mackle et al. (2000), there was a linear relationship between arterial concentration of EAA and arteriovenous difference of EAA. Infusion of insulin improved both mammary blood flow and mammary extraction rate of EAA (Mackle et al., 2000). Furthermore, short-term changes in mammary removal of AA may result from rearrangement of AA transporters between intracellular compartments and the plasma

membrane, and long-term modifications may be caused by differential gene expression of AA transport systems (Mackenzie and Erickson, 2004).

Regarding the pathways of individual AA in the mammary gland, branched-chain AA are often catabolized to provide carbon skeletons to synthesize NEAA (Bequette et al., 2003). Although Lys is considered the first or second limiting AA for milk protein synthesis, some Lys has been observed to be oxidized in lactating goats (Mabjeeshet et al., 2000). Methionine is another limiting AA, which is also involved in multiple metabolic functions such as synthesis of phospholipids, choline, and cysteine, and regulation of DNA (Bequette et al., 2003). Arginine also participates in several physiological functions such as nutrient perfusion and urea synthesis in mammary tissues (Bequette et al., 2003). Ultimately, EAA and NEAA extracted from bloodstream and NEAA synthesized by mammary epithelial cells are used to synthesize milk-specific proteins including caseins (α, β, κ, and γ) and whey proteins (e.g., α-lactalbumin and β-lactoglobulin), with caseins representing most milk proteins (~80%; NRC, 1988).

Environmental N Excretion

Lactating dairy cows are known to have poor N efficiency, and in North America, on average, only 24.7% of dietary N was utilized for milk protein synthesis (Huhtanen and Hristov, 2009). Dietary N not captured in milk protein is excreted in urine and feces. As indicated by Lapierre et al. (2005), approximately 34% (range, 17 to 46%) and 35% (range, 29 to 47%) of dietary N intake is excreted in urine and feces respectively. Urea is the major N metabolite in urine and hydrolyzed into ammonia rapidly after excretion. Aerobic microorganisms can use ammonia N as substrate to produce nitrite ($\mathbf{NO_2^-}$), nitrate ($\mathbf{NO_3^-}$), and nitrous oxide ($\mathbf{N_2O}$). Subsequently, Some NO_2^- and NO_3^- can then be converted by anaerobic microorganisms to N_2

and N_2O. Regarding fecal N excretion, ammonia N represents a small fraction of N in feces and the main fecal N is organically bound N. Taken together, the environmental N pollution from dairy cows includes ammonia, N_2O, and N oxides in the atmosphere and NO_3 in soil and ground water (Tamminga, 1992; Castillo et al., 2000). Approximately 23 tons of wet manure and 109 kg of N can be produced by an average dairy cow with 8,182 kg of milk per lactation (Van Horn et al., 1991). Unfortunately, only 30% of manure N produced by US dairy industry is recovered and applied to cropland (Kellogg et al., 2000). It has been widely recognized that dairy practices contribute to more N pollution than any other livestock operations (Rotz, 2004).

Strategies to Improve N Utilization

Poor N efficiency of dairy cows not only contributes to increased amounts of N excretion to the environment, but also reduces the profit margin for dairy producers. According to a recent survey done by Ishler (2017), feed costs account for approximately 58% of milk income with supplemental protein sources considered the most expensive feedstuffs. Thus, it is crucial to enhance milk N efficiency and reduce manure N excretion. There have been many management and nutritional strategies examined to improve efficiency of N utilization of dairy cows (Arriaga et al., 2009; Dijkstra et al., 2011). Animal grouping (St-Pierre and Thraen, 1999), increased frequency of ration balancing depending on forage availability and feedstuffs price (Arriaga et al., 2009), and feeding TMR instead of component feeding (Jonker et al., 2002) have all showed to reduce N waste and enhance efficiency of N utilization in dairy cows. Specifically, reducing dietary CP concentration and/or improving dietary energy level are often applied to improve milk N efficiency and reduce urinary N excretion (Broderick, 2003; Rius et al., 2010b). Feeding forages with higher RUP (birdsfoot trefoil vs. alfalfa) have been shown to improve milk

production and N utilization in lactating dairy cows (Hymes-Fecht et al., 2013). Condensed tannins of birdsfoot trefoil, natural polyphenolic compounds, can protect forage protein from being degraded by ruminal microorganisms into ammonia (Mueller‐Harvey, 2006).

Additionally, diets with forages harvested at the afternoon improve efficiency of N utilization of dairy cows, because afternoon-harvested forages contain higher levels of nonstructural carbohydrates, which promotes microbial protein synthesis in the rumen and milk protein synthesis in the mammary gland (Brito et al., 2008).

Maximization of Microbial Protein Synthesis

The synchronization of ruminal fermentable carbohydrates and RDP has been proposed to maximize microbial protein synthesis, improve efficiency of N utilization, and reduce environmental N excretion (Stokes et al., 1991; Aldrich et al., 1993). For instance, Hristov et al. (2005) compared different sources of carbohydrates (i.e., corn dextrose, corn starch, white oat fiber, and a combination of them) on the utilization of ruminal ammonia and demonstrated that corn starch improved uptake of ammonia for microbial protein synthesis compared with other carbohydrate sources. According to Castillo et al. (2001), the efficiency of N utilization increased, and urinary N excretion decreased with feeding the diet with low degradable starch (i.e., corn) compared with other carbohydrate sources including fiber, soluble sugars, and barley. As reported by Aguerre et al. (2011), the ratio of milk N to N intake and manure N to milk N increased linearly and tended to decrease linearly, respectively, as the forage-to-concentrate ratio decreased from 68:32 to 47:53. Furthermore, microbial N flow was elevated by 23 g/d with ruminal infusion of 1 kg of sucrose (Kim et al., 1999). Nevertheless, Cabrita et al. (2006) and Reynolds and Kristensen (2008) have pointed out that synchronizing dietary RUP and energy

supply has limited effects on microbial protein synthesis and production performance of lactating dairy cows.

Feeding Low Protein Diets to Dairy Cows

To maximize milk production, high CP diets have been widely fed to lactating dairy cows. For instance, dietary CP level averaged 17.6% for high-producing dairy cows in the western US (Hristov et al., 2006). However, feeding diets with high CP is often associated with decreased milk N efficiency and increased manure N excretion (Broderick, 2003; Olmos Colmenero and Broderick, 2006). Moreover, Yan et al. (2010) observed a strong positive relationship between manure N excretion and intake of dietary protein ($R^2 = 0.90$). High CP (18.4%) diets have also been observed to elevate ruminal degradation of protein and lower the efficiency of N utilization compared with low CP (15.1%) diets (Broderick, 2003). Thus, nutritional modifications are required to improve efficiency of N utilization and reduce N excretion to the environment.

Compared with non-ruminant animals, ruminant animals including dairy cows have better ability to recycle urea N synthesized in the liver (Lapierre and Lobley, 2001). In addition, greater proportions of urea N produced by the liver recycle back to the rumen when dietary CP level decreases (Reynolds and Kristensen, 2008). Chibisa and Mutsvangwa (2013) reported that higher proportion of hepatic urea N entered the rumen for microbial protein synthesis in dairy cows fed low protein versus high protein diets (15.2 vs. 17.3% CP). These results reveal that feeding low protein diets may have the potential to improve N utilization and reduce N excretion by promoting the transformation of hepatic urea N into microbial protein.

Consequently, low protein diets have been studied intensively by dairy researchers in recent years (Hristov, 2016). For instance, reducing dietary CP (16.1 vs. 18.8%) lowered urinary N and fecal N outputs without modifying milk and milk protein yields of mid-lactation cows (Leonardi et al., 2003). Urinary N excretion was reduced but milk production was not altered for 13.7 versus 15.5% CP of dairy diets (Lee et al., 2015). Likewise, milk production was not modified but urinary N excretion was decreased for 7.1% vs. 10.3% dietary RDP concentration (Agle et al., 2010). Reducing dietary CP concentration from 17.3% to 14.4% had no impact on milk and milk protein yields of dairy cows during mid to late lactation (151 to 305 DIM); however, reductions in milk and milk protein yields were observed during early to mid lactation (1 to 150 DIM; Lawrence, 2009). Moreover, when feeding diets with different CP levels ranging from 13.5 to 19.4%, obtained by replacing rolled high-moisture shelled corn with solvent extracted soybean meal, the highest milk N efficiency and lowest urinary N excretion were observed with feeding 13.5% CP, but maximal milk and milk protein yields were obtained for the treatment with 16.5% CP (Olmos Colmenero and Broderick, 2006). Taken together, relative to high CP diets, feeding low CP diets is an effective strategy to enhance efficiency of N use but may reduce production performance of lactating dairy cows.

The negative responses of milk production with feeding low protein diets may result from deficiencies of some limiting AA including Met, Lys, and His. In support, supplementation of MP-deficient diets with rumen protected (**RP**) -Met, Lys, and His elevated milk and milk true protein yields, while having similar milk N efficiency and manure N excretion (Lee et al., 2012). Similarly, RP-Met, Lys, and His improved milk true protein yield in lactating dairy cows fed MP-deficient diets without reduction in milk N efficiency (Giallongo et al., 2016). However, no response in milk yield was detected when adding RP-Met, Lys, and His to MP-deficient diets

(Giallongo et al., 2016). These inconsistent responses with RP-Met, Lys, and His may be related to amounts and bioavailability of RP-AA products, level of MP deficiency, dietary composition, and lactation stage. Thus, further research is required to better understand the interactions between level of dietary MP deficiency and RP-AA supplementation on milk production and efficiency of N utilization in lactating dairy cows.

Energy Supplementation

Increased supply of ruminally fermentable energy (e.g., corn starch) can capture more ammonia N to synthesize microbial protein in the rumen, which is then utilized for milk protein synthesis (Voelker and Allen, 2003; Hristov et al., 2005). Broderick (2003) demonstrated that milk yield, milk protein yield, and milk N efficiency were elevated, and urinary urea N and total N were reduced in response to increasing amounts of energy obtained by decreasing dietary forage to concentrate ratio (75:25, 63:37, and 50:50). Niu et al. (2016) reported that milk true protein N and milk N efficiency were increased, and total urine N and the proportion of N intake as urinary N decreased with feeding a low forage diet (37.4% forage of DM) compared with a high forage diet (53.3% forage of DM).

On the other hand, addition of ruminally fermentable energy can improve mammary uptake of AA and milk protein yield in dairy cows (Lemosquet et al., 2009; Omphalius et al., 2019). Mammary arterial fluxes of EAA and mammary uptake of all EAA except Arg and Val increased linearly with duodenal infusion of increasing amounts of glucose (0, 443, 963, and 2398 g/d; Rulquin et al., 2004). Moreover, the ratios of mammary uptake to milk output for His, Met, and Leu showed linear increases to duodenal infusions of glucose (Rulquin et al., 2004). Mammary net uptakes of most of EAA (i.e., Arg, Ile, Lys, Phe, and Trp) and milk and milk

protein yields were higher for abomasal infusion of starch (Rius et al., 2010a). Furthermore, milk protein output was elevated with ruminal infusions of propionate (Raggio et al., 2006) and postruminal infusions of starch (Reynolds et al., 2001). However, when supplemental energy was provided as RP-fat (e.g., palmitic acid), no change was observed for milk protein production (Lock et al., 2013; Mathews et al., 2016).

A combination of reducing dietary protein and increasing energy has been examined to improve efficiency of N use and reduce N excretion from dairy cows. Rius et al. (2010b) indicated that elevated dietary energy (forage-to-concentrate ratio: 39:61 vs. 50:50) or protein (RUP: 6.6 vs. 4.6% DM; constant RDP at 10.1% DM) elevated milk and milk protein yields independently; however, the maximal efficiency of N utilization was obtained when feeding the diet with high energy and low protein. Thus, further studies are needed to explore nutritional strategies to improve production performance, while improving great milk N efficiency in dairy cows fed low protein and high energy diets.

Energy Utilization in Dairy Cattle

Energy Sources in Diets

Energy mainly comes from carbohydrates, protein, and fat derived from grasses, legumes, crop residues, industrial byproducts, cereal grains, crop plants, animal protein meals, oilseeds, fat supplements, etc (NRC, 2001). Several approaches have been used to express energy values of feeds, including TDN, digestible energy (**DE**), ME, and net energy (**NE**). First, TDN is calculated by summing up truly digestible NFC, CP, 2.25 × fatty acid (**FA**), and NDF and then subtracting metabolic fecal TDN (assumed to be 7; NRC, 2001). Processing adjustment factors are applied to account for increased digestibility of NFC due to physical processing, and

heat and steam treatment (NRC, 2001). Digestible energy of a feed is calculated by multiplying the truly digestible nutrients by their heats of combustion (e.g., 4.2, 5.6, 9.4, and 4.3 Mcal/kg for carbohydrates, protein, long chain fatty acids (**FA**), and glycerol, respectively; NRC, 2001). Dietary DE at maintenance is calculated by adding DE of feeds in the diet (NRC, 2001). When calculating dietary DE at actual intake, a discount factor is applied to account for decline in digestibility resulted from increased feed intake (NRC, 2001). The ME and NE of the diet or individual feed are calculated using the equation: (1.01 × DE - 0.45) + 0.0046 × (EE - 3), and 0.703 × ME – 0.19 + (0.097 × ME +0.19)/97 × (ether extract - 3), as described by NRC (2001).

Energy Utilization in the Rumen

Carbohydrates including fibrous carbohydrates (e.g., cellulose and hemicellulose) and non-fibrous carbohydrates (e.g., sugars and starch) are the most important energy yielding nutrients for dairy cows (NRC, 2001). Non-structural carbohydrates have higher ruminal degradation rates than structural carbohydrates and thus can supply more energy per unit of DM basis (Sniffen et al., 1992). However, fibrous carbohydrates can stimulate rumination, ruminal contraction, and saliva production (NRC, 2001). Therefore, both types of carbohydrates are very important for milk production and health of dairy cows.

Ruminal bacterial species, particularly, *Ruminococcus flavefaciens, Rumincoccus albus,* and *Fibrobacter succinogenes* play an important role in hydrolyzing cellulose via cellulase enzyme complexes, as extracellular enzymes (Schwartz and Gilchrist, 1975). A few fungi and protozoa also showed the ability to have cellulolytic activities (NRC, 2001). Cellulose is first broken down to oligosaccharides, and then cellobiose, which is finally hydrolyzed to glucose (Lynd et al., 2002). Hemicellulose is degraded mainly by hemicellulolytic bacterial species (e.g.,

Prevotella ruminicola and *Butyrivibrio fibrisolvens*) and to lesser degree by certain protozoal species to xylose and other pentoses, which are then converted to fructose and trioses (Baldwin, 1965; Hungate, 1966). Starch is hydrolyzed into maltose and glucose via amylases and carbohydrases derived from amylolytic bacterial species such as *Succinomonas amylolytica and Streptococcus bovis* (Prins, 1977). Eventually, all carbohydrate monomers (mainly glucose and fructose) are catabolized into pyruvate, which is a central intermediate in ruminal carbohydrate metabolism. Pyruvate is converted to VFA mainly including acetate, propionate, and butyrate through a variety of pathways (Moss et al., 2000). Most of the energy requirement of a dairy cow is met by ruminal VFA production (NRC, 2001). During this process, CO_2, H_2, and CH_4 are also formed and released through eructation and respiration (Moss et al., 2000). Ruminal CH_4 production also represents 2-12% energetic losses (Johnson and Johnson, 1995).

As mentioned above, dietary N sources can be categorized into RDP and RUP with RDP including NPN, AA, peptides, and some true protein (NRC, 2001). When feeding adequate fermentable energy mainly provided by carbohydrates, RDP can be utilized by ruminal microorganisms to synthesize microbial protein, and microbial protein synthesis is an energy-requiring process. On the other hand, when energy supply is limiting in the rumen, microorganisms tend to deaminate AA or peptides into VFA, CO_2, and ammonia in order to provide energy (Bach et al., 2005).

Dairy diets generally contain 2-7% of lipids and 1-3% of supplemental fat can be added without adverse effects (Mosley et al., 2007; Piantoni et al., 2013). Various sources of fat are available to dairy cows, including oilseeds, dry-granular fat, animal and animal-vegetable blends, and RP-fat (NRC, 2001). These fat supplements mainly provide triglycerides, UFA, and SFA. Ruminal metabolism of these fat metabolites involves 2 major processes: lipolysis and

biohydrogenation. Hydrolysis of triglycerdies by lipolytic microorganisms (e.g., *Butyrivibrio fibrisolvens* and *Anaerovibrio lipolytica*) results in free FA, glycerol and small amounts of mono- and di-glycerides, and UFA can be hydrogenated to some degree during the biohydrogenation process (Jenkins, 1993; Buccioni et al., 2012). Glycerol is degraded rapidly, yielding propionate as a major end product (Garton et al., 1961). Small amounts of dietary FA are catabolized to CO_2 and VFA, which are then absorbed by the rumen wall (Jenkins, 1993). Taken together, free FA, mainly including C16 and C18, account for 85-90% of lipid leaving the rumen, and the remaining 10-15% of lipids are derived from ruminal de novo synthesis, mainly existing as phospholipids (NRC, 2001).

Digestion and Absorption of Energy Substrates in the Gastrointestinal Tract

In dairy cows, VFA (C2 to C6) produced by ruminal fermentation of carbohydrates are mainly absorbed by the ruminal epithelium and to a lesser degree, by the abomasal epithelial cells (Storm et al., 2012). According to Kristensen (2005), a slight amount of ruminal acetate, a small amount (5-10%) of propionate, and an extensive amount of butyrate are metabolized by the ruminal wall during absorption. Most of the absorbed butyrate by the ruminal wall is converted to ketones (i.e., β-hydroxybutyrate) that are later found in the portal vein. Notably, the liver releases more acetate than its uptake because the liver is able to synthesize acetate (Kristensen, 2005). The majority of the portal flux of VFA except acetate are extracted by the liver, and hepatic extraction for propionate is greater than that for butyrate (93 vs. 80%; Kristensen, 2005). Furthermore, propionate is the main precursor for gluconeogenesis in the liver.

Small amounts of FA, mainly medium chain FA can be absorbed or metabolized by ruminal epithelial cells (Doreau and Ferlay, 1994; Kristensen, 2005). Thus, most of the FA

attached to feeds and microbial particles flow out of the rumen and exist as salts of sodium, potassium, or calcium (Loften et al., 2014). These salts are dissociated and protonated in acidic abomasum and transformed into nonionized free FA in the duodenum (Loften et al., 2014). Within the duodenum, micelles are formed after dissociation of free FA by pancreatic secretions (lipases and bicarbonate) and bile, and then transported across intestinal epithelial cells. Average intestinal digestibility of total C16 and total C18 were 74.6 and 73.4%, respectively, and tended to increase with the chain length (Schmidely et al., 2008). Furthermore, FA absorbed are re-esterified into triglycerides and newly formed triglycerides and phospholipids are incorporated into chylomicrons and very-low density lipoproteins, which are then transported mainly via the lymphatic system to peripheral tissues including the mammary gland (Tso and Balint, 1986).

Utilization of Energy Substrates in the Mammary Gland

In addition to milk protein, milk fat is the other important component in dairy milk and determines its economic value (Jesse and Cropp, 2008). Milk fat is predominantly composed of triglycerides (~95%) containing more than 400 different FA (Jensen and Clark, 1988). Milk fat content and milk FA profile can be significantly impacted by dairy species and diets (Morales et al., 2000). There are two main sources for milk FA production: de novo synthesis within the mammary epithelial cells and preformed FA from the blood circulation (Dils, 1986).

Ruminal acetate and β-hydroxybutyrate are the major substrates for de novo FA synthesis in the mammary gland (Urrutia and Harvatine, 2017). Fatty acids derived from mammary de novo synthesis include all the C4 to C12 FA, around 95% of C14:0, and about 50% of C16:0 (Shingfield et al., 2010), and account for about 40% of milk FA (Chilliard et al., 2000). Acetate is converted to acetyl-CoA under the catalysis of acetyl CoA carboxylase, and β-hydroxybutyrate

is activated to butyryl-CoA (Bauman and Davis, 2013). Then, FA synthetase catalyzes the condensation cycles of malonyl-CoA derived from acetyl-CoA by using either acetyl-CoA or butyryl-CoA (Bauman and Davis, 2013).

The other 50% of C16:0 and long-chain FA are derived from circulating FA that are extracted by the mammary gland via very-low density lipoproteins, chylomicrons, and nonesterified FA (Shingfield et al., 2010) and represents approximately 60% of milk fat (Chilliard et al., 2000). Notably, nonesterified FA are originated from either intestinal absorption of lipids or from lipolysis in adipose tissues. Generally, the mobilization of body fat reserves contributes to less than 10% of milk FA except for postpartum dairy cows (Shingfield and Griinari, 2007). Preformed FA cannot be elongated but can be desaturated by adding a *cis*-9 double bond on the FA under the catalysis of Δ-9 desaturase (Shingfield et al., 2008). For instance, C18:0 is transformed to *cis*-9 C18:1, which accounts for 60-80% of total C18:1 in milk (Shingfield et al., 2013). Other FA such as C10:0, 12:0, 14:0, 15:0, 16:0, and 17:0 can also be catalyzed by Δ-9 desaturase to form *cis*-9 unsaturated FA (Shingfield et al., 2010).

Energy Efficiency in Dairy Cows

Gross energy of the diet is the sum of combustion energy of individual feeds. Digestible energy is calculated by subtracting fecal energy from gross energy. Urinary energy loss and gaseous energy losses (primarily CH_4) are subtracted from DE to calculate ME. Metabolizable energy is either lost as heat production or used for animal growth (NE_G) and milk production (NE_L). Fecal energy loss is the most variable portion, ranging from 10 to 60% of GE (Reid et al., 1980). Digestible energy contributes to the majority of the variation in NE (~86%), thus suggesting that feed digestibility plays an important role in improving the efficiency of energy

utilization in dairy cows (Moe et al., 1972). Digestibility of forages is mainly affected by maturity, followed by physical processing, feed intake, and dietary CP level (Buxton, 1996). There is less variation in the conversions of DE to ME or ME to NE compared with that from gross energy to DE (Reynolds et al., 2011). Approximately 50% of heat production is attributed to gastrointestinal tracts and the liver in ruminants (Reynolds et al., 2011).

For energy efficiency expressed as milk energy/DE, energy efficiency was reduced by 1.6% for each 10% increase in dietary ADF level and showed a quadratic response to proportion of dietary concentrate; whereas digestible CP, BW, and DIM did not alter energy efficiency (Phuong et al., 2013). It may be because that diets with high ADF level could fill up the rumen quickly and limit DMI, and also ADF could be less digestible and provide less energy to support the growth of microorganisms (Moore and Coleman, 2001). Increased dietary proportion of concentrates may supply more available fermentable energy to stimulate ruminal fermentation (Broderick, 2003); however feeding too much concentrate could result in ruminal acidosis, which in turn impairs production performance and energy efficiency (Beauchemin, 2007). In addition to dietary factors, energy efficiency was influenced by animal-related factors such as breeds, lactation stage, and parity (Britt et al., 2003; Prendiville et al., 2009). However, no significant effects of BW and DIM on energy efficiency were observed in the meta-analysis by Phuong et al. (2013).

Technologies to Measure Gas Fluxes

Global climate change has been a defining issue since the mid-20th century due to continued population growth and advancing economies. Global warming is attributed to greenhouse gas emissions from anthropogenic activities such as CO_2, CH_4, N_2O, and fluorinated

gases (FAO, 2019). The dairy industry contributes ~4% of global anthropogenic greenhouse gas emissions with CH_4, N_2O, and CO_2 representing 63, 25, and 12% of total emissions from dairy systems, respectively (FAO, 2019). Additionally, CH_4 is a potent greenhouse gas that has 28-36 times more global warming potential than CO_2 (USEPA, 2020). Several approaches have been developed to accurately quantify greenhouse gase missions from dairy cattle such as the open-circuit respiration chamber, the sulphur hexafluoride (**SF_6**) tracer gas technique, and the GreenFeed system (Hristov et al., 2015; Hammond et al., 2016).

Respiration Chambers

Whole animal open-circuit respiration chambers have been used for more than 100 years to measure CO_2 and CH_4 (Kellner, 1913). Inflowing air is constantly circulated through the chamber and mixes with incoming air and emitted gases from animals, and incoming air and exhaust air are sampling regularly for analyses of CH_4 and CO_2 (Hammond et al., 2016). Emissions of CO_2 and CH_4 are calculated by multiplying the airflow by the differences in the concentrations between inflowing and outflowing gases, respectively (Hammond et al., 2016). To avoid air loss, it is important for respiration chambers to be adequately air tight. It is also critical to calibrate the measured concentrations of gases and air flow to account for changes in temperature, air pressure and humidity (Hammond et al., 2016). Generally, experiment periods for measurements of CO_2 and CH_4 using respiration chambers ranged from 1 to 7 consecutive days (Van Zijderveld et al., 2010; Olijhoek et al., 2016).

The GreenFeed System

The GreenFeed system (C-Lock Inc., Rapid City, South Dakota, USA) has been recently used to measure short-term gas fluxes of CO_2 and CH_4 from individual animals (Pereira et al.,

2015; Harper et al., 2017). The animal is trained to voluntarily put its head in the chamber with regular delivery of pelleted feeds, and thus there is minimal disruption of daily routine and minimal stress to the cow (Hristov et al., 2015). Measurements of gases are typically completed over a 7-min period (5 min for sampling of emitted gases and 2 min for sampling of background gases) and multiple times over several days per period (Hristov et al., 2015). In contrast to respiration chambers, the GreenFeed system is able to measure gases from a large number of animals and uses radio frequency identification tags to identify individual animals (Hammond et al., 2015).

Sulphur Hexafluoride Tracer Technique

The SF_6 technique developed by Zimmerman (1993) relies on the installment of a permeation tube into the reticulorumen of the animal for the release of a known quantity of SF_6, the placement of a tube with in-line flow restrictors near the nose of the cow for collection of exhaled air, and a pre-evacuated collection vessel connected to tubing with in-line flow restrictors (Hammond et al., 2016). Samples are typically collected over at least 5 conseuctive days to account for diurnal variation in gaseous fluxes, with background gas samples collected simultaneously (Hammond et al., 2016).

Comparison of Techniques for Measurement of Gases

Studies with ruminants have been done to compare emissions of CH_4 and CO_2 measured using respiration chambers, SF_6 tracer techniques, and the GreenFeed system (Jonker et al., 2016; Doreau et al., 2018). Specifically, a study with 8 nonlactating Holstein cows in a 15 week experiment showed that CH_4 production was higher with using the respiration chamber (367 g/d)

relative to the SF_6 technique (310 g/d) and GreenFeed system (319 g/d), and CO_2 emission (g/d) was similar between the respiration chamber (9.89 kg/d) and GreenFeed system (10 kg/d) and lower for the SF_6 technique (7.72 kg/d) relative to the other two approaches (Doreau et al., 2018). For CH_4 production, the correlation coefficient was 0.78 between the respiration chamber and SF_6 technique and not significant between the respiration chamber and GreenFeed system or between the SF_6 technique and GreenFeed system and for CO_2 emission, the correlation coefficient was not significant between the respiration chamber and the GreenFeed system (Doreau et al., 2018). Furthermore, Huhtanen et al. (2013) has demonstrated that CH_4 emission was similar between the GreenFeed system and the respiration chamber in dairy cows and repeatability for both CH_4 and CO_2 measurements was high during 1-2 week experimental periods. Collectively, respiration chambers, SF_6 techniques, and the GreenFeed system all can provide reliable measurements of CH_4 and CO_2 and the differences among the 3 approaches are moderate.

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CHAPTER II: INCREMENTAL AMOUNTS OF RUMEN PROTECTED-HISTIDINE INCREASE PLASMA AND MUSCLE HISTIDINE CONCENTRATIONS AND MILK PROTEIN YIELD IN DAIRY COWS FED A METABOLIZABLE PROTEIN-DEFICIENT DIET

ABSTRACT

The dairy industry can benefit from low crude protein (CP) diets due to reduced N excretion, but shortages of Met, Lys, and His may limit milk protein synthesis. We studied the effect of incremental amounts of rumen protected (RP)-His on plasma and muscle AA profile, nutrient utilization, and yields of milk and milk true protein in dairy cows. Eight multiparous Holstein cows (130 ± 30 d in milk) were randomly assigned to treatment sequences in a replicated 4 × 4 Latin square design with 28-d experimental periods. Treatments included a basal diet composed (dry matter basis) of 50% corn silage, 15% haylage, and 35% concentrate supplemented with 0, 82, 164, and 246 g/d of RP-His and 11 g/d of RP-Met. Milk, plasma, and muscle samples were collected weekly or every other week during all 4 periods, whereas spot urine and fecal grab samples were taken only in wk 4 of each period. Data were analyzed individually by week using linear, quadratic, and cubic orthogonal polynomials and repeated measures. Plasma His increased linearly with RP-His during wk 1 (30.3 to 57.2 μ*M*) to wk 4 (33.2 to 63.1 μ*M*). Plasma carnosine increased linearly with supplemental RP-His except in wk 2. No treatment effect was observed for plasma 3-methylhistidine except a quadratic effect in wk 3. Inclusion of RP-His showed linear effects on muscle His in wk 2 (20.1 to 32.5 μ*M*) and 4 (20.3 to 35.5 μ*M*). Whereas muscle anserine and carnosine concentrations were not affected by treatments in wk 4, anserine responded quadratically and carnosine showed a trend for a quadratic response to RP-His in wk 2. During wk 4, treatments did not affect urinary excretion of total purine derivatives, as well as dry matter intake and milk concentrations of fat and true protein. In contrast, milk yield tended to increase linearly (31.2 to 32.7 kg/d) and milk true protein yield responded linearly (0.93 to 0.98 kg/d) to RP-His supplementation in wk 4. Also, milk urea-N (11.7 to 12.9 mg/dL) and urinary excretion of urea-N (23.7 to 27.0% of N intake)

increased linearly with feeding RP-His in wk 4. Overall, RP-His was effective to enhance plasma and muscle concentrations of His and milk protein synthesis. Elevated milk urea-N and urinary excretion of urea-N suggest that plasma His may have exceeded the requirement with excess N converted to urea in the liver. Future research is needed to determine the bioavailability of RP-His supplements to improve the accuracy of diet formulation for AA.

Key words: low crude protein diet, nitrogen efficiency, metabolizable protein, rumen-protected amino acid

INTRODUCTION

According to NRC (2001), maximum yields of milk and milk protein were obtained at 22 to 23% dietary CP based on regression approaches. However, such high-CP diets have been shown to reduce milk N efficiency (i.e., milk N/N intake), increase environmental N pollution, and shrink the profit margin of dairy producers due to the high costs of protein sources (Broderick, 2003; Olmos Colmenero and Broderick, 2006). Feeding low-CP diets improved milk N efficiency and reduced urinary N excretion, but also decreased yields of milk and milk true protein (Broderick et al., 2009; Lee et al., 2011), which may be attributed to deficient RDP supply or reduced intake of digestible Lys, Met, and His (**dLys**, **dMet**, and **dHis**) or both. Methionine and Lys are usually co-limiting AA (first or second) in typical US diets (Schwab et al., 1976; NRC, 2001), and His may become the third limiting AA in MP-deficient rations (Lee et al., 2012). One common way to mitigate dietary shortages of Lys, Met, and His is through supplementation of rumen protected (**RP**) AA (Whitehouse et al., 2017).

Rumen-protected AA supplements are characterized by low ruminal degradation and variable intestinal absorption of AA, thus resulting in different amounts of Lys, Met, and His transported into the blood (Lee et al., 2012; Whitehouse et al., 2017; Zang et al., 2017). Doepel and Lapierre (2010) suggested that increased plasma concentrations of EAA stimulated their uptake by the mammary gland, which in turn improved yields of milk and milk protein. While supplementation of MP-deficient diets with RP-Met and RP-Lys has steadily elevated the concentrations of these 2 EAA in plasma of lactating dairy cows, a similar consistent response has not been observed with feeding RP-His based on previous research (Lee et al., 2012; Giallongo et al., 2015, 2016). For instance, Lee et al. (2012) and Giallongo et al. (2016) observed that supplementing 120 g/d of RP-His to 10% MP-deficient diets increased plasma His

concentration by an average of 64% in two 10-wk randomized complete block design studies. However, plasma concentration of His did not change when adding 50 g/d of RP-His to a 5% MP-deficient diet in another 10-wk randomized complete block design experiment (Giallongo et al., 2015). These inconsistent results may be attributed to the use of labile pools of His including intramuscular carnosine (β-alanyl-L-His) and anserine (β-alanyl-*N*-methylHis) and blood hemoglobin during short-term His deficiency (Lapierre et al., 2008).

In recent years, a growing number of studies have been conducted to explore the metabolism of endogenous His reserves in lactating dairy cows. Lapierre et al. (2014) reported that muscle carnosine and anserine showed quadratic responses to abomasal infusions of His ranging from 0 to 22.8 g/d. In contrast, muscle carnosine and anserine were not different between a His-deficient and a His-adequate diet (Giallongo et al., 2015). Blood hemoglobin was not affected by infusing His abomasally (Lapierre et al., 2014) or feeding RP-His to lactating dairy cows (Giallongo et al., 2016). However, information is lacking regarding the metabolism of endogenous His pools in dairy cows fed MP-deficient diets supplemented with varying levels of RP-His. Our central hypothesis is that plasma and muscle concentrations of His would respond linearly to increasing amounts of RP-His fed to lactating dairy cows. The primary objective of our study was to investigate the effect of incremental amounts of RP-His on plasma and muscle AA profile, nutrient utilization, and yields of milk and milk true protein in mid-lactation Holstein cows. A secondary objective was to assess the temporal changes of His and His-containing metabolites by collecting blood samples weekly and conducting muscle biopsies every other week during a RP-His dose-response study in a Latin square design.

MATERIALS AND METHODS

All experimental procedures were approved by the Institutional Animal Care and Use Committee (protocol no. 170202) of the University of New Hampshire (Durham). The experiment was conducted at the University of New Hampshire Fairchild Dairy Teaching and Research Center (Durham) from March to July 2017.

Cows, Experimental Design, and Treatments

Eight multiparous Holstein cows averaging (mean ± SD) 130 ± 30 DIM, 42 ± 2 kg/d of milk, and 717 ± 53 kg of BW in the beginning of the study were used in a replicated 4 × 4 Latin square design with 28-d experimental periods. Cows were blocked by DIM and milk yield, and within each block, randomly assigned to treatment sequences. Squares were balanced for potential first-order carryover effects in subsequent periods as each treatment immediately preceded and followed every other exactly once in each square (Williams, 1949; Kim and Stein, 2009). Animals were housed in a tie-stall barn equipped with water bowels for free access to water and feed tubes for individual intake measurements. Dietary ingredients were mixed and offered as TMR twice daily at 0600 and 1700 h using a Super Data Ranger mixer (American Calan Inc., Northwood, NH). Orts were collected and weighed once daily before the afternoon feeding. Feed offered was adjusted daily to achieve 5 to 10% orts and cows were milked twice a day at 0530 and 1630 h. Feed intake and milk yield were recorded throughout the experiment. Cows were weighed (Northeast Scale Co., Hooksett, NH) immediately after the afternoon milking during 3 consecutive days before the beginning of the study and at the end of each period to compute BW change. Body condition score was determined by 3 trained individuals

before the beginning of the experiment and on the last day of each period following the procedures outlined by Wildman et al. (1982).

During the 2-wk covariate period, all cows received the same diet fed as TMR (17.2% CP, 28.1% NDF, and 4.2% ether extract) consisting of (DM basis): 44.7% corn silage, 12.6% mixed-mostly grass-legume haylage, and 42.7% concentrate. The covariate period was used to reduce animal variation in Latin square designs as reported by Whitehouse et al. (2017). Milk samples were collected on d 13 and 14 of the covariate period, and blood (coccygeal vessels) and muscle (longissimus dorsi) samples were taken on d 14.

Treatments included a basal diet supplemented with 0, 82, 164, and 246 g/d of RP-His (0, 5, 10, and 15 g/d of dHis, respectively) in addition to 11 g/d of RP-Met (6.6 g/d of dMet); the RP-AA supplements were top-dressed on top of the TMR. The basal diet consisted of (DM basis): 50% corn silage, 15% mixed mostly grass-legume haylage, and 35% concentrate. It was formulated using the NRC (2001) to meet 100% NE_L and 82.5% MP requirements for a dairy cow averaging 680 kg of BW, 130 DIM, 42 kg/d of milk, 3.70% of milk fat, and 2.75% of milk true protein. According to NRC (2001), dietary His represented 2.06, 2.28, 2.52, and 2.75% of MP supply, respectively. The RP-His supplement used is a prototype product (Ajinomoto Co. Inc., Kawasaki-shi, Japan) containing 44% of His with 14% bioavailability (A. Haruno, senior researcher at Ajinomoto Co. Inc.; personal communication). Smartamine® M (Adisseo USA Inc., Alpharetta, GA), which contains 75% DL-Met and 80% bioavailability (Graulet et al., 2005), was used as the RP-Met supplement.

Feed Sampling and Analyses

Corn silage, mixed mostly grass-legume haylage, TMR, and ort samples were collected twice weekly and composited by week. Samples of concentrates (i.e., ground corn, beet pulp, soybean meal, steam-flaked corn, canola meal, liquid molasses, and corn dried distillers grains with solubles) were collected by Poulin Grain Inc. (Newport, VT) every time a new batch of grain mix was shipped. All feed samples were equally divided into 2 subsamples, with the first set dried (55°C, 48 h) in a forced-air oven (VWR Scientific, Radnor, PA) for determination of DM to adjust the TMR on an as-fed basis and to calculate DMI. The second set was lyophilized for 48 h (Labconco Inc., Kansas City, MO), composited by period, ground with a Wiley mill (A. H. Thomas Co., Swedesboro, NJ) to pass through a 1-mm screen, and stored in air-tight glass jars until nutritional analysis.

Lyophilized and ground (1 mm) samples of dietary ingredients were shipped to Dairy One Cooperative Inc. (Ithaca, NY) and analyzed for DM, CP, soluble protein, aNDFom, ADF, NDIN, ADIN, ADL, starch, ethanol soluble carbohydrates, ether extract, and ash using the procedures reported by Pereira et al. (2017) and Ghedini et al. (2018). Individual minerals (Ca, P, Mg, K, Na, S, Fe, Zn, Cu, Mn, and Mo) were also analyzed with the procedures mentioned by Ghedini et al. (2018), while Cl ion was determined by a Brinkmann Metrohm 716 Titrino Titration Unit with a silver electrode (Metrohm application bulletin no. 130, Metrohm Ltd., Herisau, Switzerland).

Lyophilized and ground (1 mm) samples of dietary ingredients were further ground (Wiley mill, A. H. Thomas Co.) to pass through a 0.5-mm screen and used for determination of AA by cation exchange chromatography-HPLC coupled with postcolumn ninhydrin derivatization with norleucine as the internal standard (method 982.30; AOAC International,

2016; University of Missouri Agricultural Experiment Station Chemical Laboratory, Columbia). Tryptophan was determined after alkaline hydrolysis and sulfur AA were analyzed after performic acid oxidation (method 988.15; AOAC International, 2016). Additionally, TMR and orts were analyzed for CP, NDF, ADF, and ash at Dairy One Cooperative Inc. laboratory.

Blood and Muscle Sampling and Analyses

Blood samples were collected into vacutainer EDTA tubes (Monoject, Mansfield, MA) via the coccygeal vein or artery approximately 4 h after the morning feeding on d 7 of wk 1 to 4 of each experimental period. For plasma collection, tubes were immediately placed in a chill bucket with beads (Chemglass Life Sciences, Vineland, NJ) and transported to the laboratory for centrifugation (2,155 × *g*, 20 min, 4ºC) using an Eppendorf centrifuge (model 5810, Eppendorf, Hamburg, Germany). Plasma samples were used to determine the profile of AA, His-containing metabolites, and urea-N (**PUN**) at Ajinomoto Co. Inc. using a High-Speed AA analyzer L-8900 (Hitachi High-Technologies Co., Tokyo, Japan) following the procedures stated by the manufacturer (http://www.hitachi-hta.com/sites/default/files/literature/L-8900%20Brochure.pdf). Codified plasma samples were sent to Ajinomoto Co. Inc. to preserve the identity of treatments.

Biopsies were performed by sampling the longissimus dorsi muscle on d 7 of wk 2 and 4 of each experimental period. Cows were moved out of their stalls, brought to a surgical room, and immobilized in a hoof trimming chute. The surgical area (between the 12th and 13th transverse processes) was clipped and sanitized by scrubbing with povidone surgical scrub and 91% isopropyl alcohol (vol/vol). Next, cows were anesthetized by injecting 4 mL of 2% lidocaine hydrochloride (wt/vol) subcutaneously down to the musculature on either side of the biopsy site (8 mL total). A 2.5-cm incision was made through the skin with a sterile scalpel blade

to expose the muscle and obtain a sample of tissue using an 8-mm Baker's dermal punch (Patterson Veterinary Supply, Devens, MA). Samples (~1.0 g/cow) were immediately placed in a container with dry ice, transported to the laboratory, and stored at −80°C until analysis. The biopsy sites were closed with non-absorbable sutures (Braunamid, Patterson Veterinary Supply) and monitored closely to avoid infection until sutures were removed within 2 wk. After thawing at room temperature, muscle tissues were homogenized with a tissue homogenizer (Omni International Inc., Kennesaw, GA), deproteinized with 0.61 *N* trichloroacetic acid, and finally treated with n-hexane to extract AA and dipeptides. Sample extracts were shipped codified to Ajinomoto Co. Inc. and analyzed for carnosine, anserine, and AA profile using a High-Speed AA analyzer L-8900 as reported previously.

Fecal and Urinary Sampling and Analyses

Fecal grab samples were taken directly from the rectum or during voluntary defecation at 8 timepoints (1000, 1600, and 2200 h on d 25; 0400, 1300, and 1900 h on d 26; and 0100 and 0800 h on d 27) in wk 4 of each experimental period. Fecal samples (~200 g/sampling) were collected into 100-mL specimen containers and transferred into 4-L storage bags to obtain composited samples by cow per period. Next, samples were dried in a forced-air oven (VWR Scientific) at 55°C for approximately 72 h and ground (Wiley mill, A. H. Thomas Co.) to pass through a 1-mm screen. Fecal samples were analyzed for DM, CP, NDF, ADF, and ash at Dairy One Cooperative Inc. laboratory. Moreover, duplicate samples (~0.5 g) of feces and TMR were weighed into Ankom F57 bags (25 µm pore size; Ankom Technology, Macedon, NY), placed in larger laundry nylon bags, and inserted in the rumen of 2 ruminally cannulated lactating Holstein cow for 12 d. After removal from the rumen, bags were rinsed with tap water and analyzed in-

house for ADF using an Ankom^{2000} fiber analyzer (Ankom Technology). Indigestible ADF (**iADF**) obtained from TMR and feces was used as the internal marker to estimate fecal output of DM and apparent total-tract digestibility of nutrients (Cochran et al., 1986; Huhtanen et al., 1994).

Spot urine samples were collected concurrently with fecal samples into 100-mL specimen containers through stimulation of the pudendal nerve by massaging the area below the vulva or during voluntary urination in wk 4 of each experimental period. After each sampling, 1 mL of urine was pipetted into 50-mL centrifuge tubes containing 32 mL of 0.072 *N* H_2SO_4 to obtain composited urine samples by cow per period. Urine samples were stored at −20°C before analyses of nitrogenous compounds. After thawing at room temperature, samples were analyzed for concentrations of creatinine (assay kit no. 500701, Cayman Chemical Co., Ann Arbor, MI) using a chromate microplate reader set at a wavelength of 492 nm (Awareness Technology Inc., Palm City, FL), allantoin (Chen et al., 1992), uric acid (assay kit no. 1045–225; Stanbio Laboratory, Boerne, TX), urea-N (diacetyl-monoxime method of Rosenthal, 1955), and total-N (micro-Kjeldahl analysis, AOAC, 1990; Dairy One Cooperative Inc.). Allantoin, uric acid, and urea-N were read at wavelengths of 540, 522, and 520 nm, respectively, on a UV/visible spectrophotometer (Beckman Coulter Inc., Pasadena, CA). Daily urine volume was estimated from urinary creatinine concentration assuming a constant creatinine excretion rate of 29 mg/kg of BW (Valadares et al., 1999). Urinary excretion of urea-N, total-N, allantoin, uric acid, and purine derivatives (allantoin + uric acid) were calculated by multiplying the concentration of each of these metabolites by the urinary volume.

Milk Sampling and Analyses

Milk samples were collected using automatic samplers during 4 consecutive milkings starting in the afternoon milking of d 6 in wk 1 to 4 of each experimental period. Milk samples were transferred into tubes preserved with 2-bromo-2-nitropropan-1,3 diol (Broad Spectrum Microtabs II; Advanced Instruments Inc., Norwood, MA), pooled by cow proportionally to the morning and afternoon milk weights, and stored at 4°C until analysis. Milk samples were shipped to Dairy One Cooperative Inc. and analyzed for concentrations of fat, true protein, lactose, and MUN by Fourier transform infrared spectroscopy using a MilkoScan FT+ (Foss Inc., Hillerød, Denmark).

Statistical Analyses

The present study was conducted as a replicated 4 × 4 Latin square design with milk, plasma, and muscle samples collected weekly or every other week. This sampling regime allowed the statistical analyses to be conducted using data from individual week of each period to test linear, quadratic, and cubic effects in response to incremental amounts of RP-His supplementation as follows: wk 1 (d 1 to 7 of periods 1 to 4), wk 2 (d 8 to 14 of periods 1 to 4), wk 3 (d 15 to 21 of periods 1 to 4), and wk 4 (d 22 to 28 of periods 1 to 4). In addition, repeated measures were used to evaluate the temporal changes of plasma and muscle AA concentrations in response to RP-His supplementation. Data on apparent total-tract digestibility of nutrients and urinary N excretion are from wk 4 of each experimental period because no collections of spot urine and fecal grab samples were conducted during wk 1 to 3.

Data (i.e., plasma and muscle AA concentrations, DMI, yields of milk and milk components) were analyzed as a replicated 4 × 4 Latin square design using the MIXED

procedure of SAS (SAS version 9.4, SAS Institute Inc., Cary, NC) according to the following model:

$$Y_{ijkl} = \mu + S_i + C_{j(i)} + P_k + T_l + S_i \times T_l + \beta Cov_{ijkl} + e_{ijkl}$$

where, Y_{ijkl} = dependent variable, μ = overall mean, S_i = fixed effect of square (i = 1 to 2), $C_{j(i)}$ = random effect of cow nested within square, P_k = fixed effect of period (k = 1 to 4), T_l = fixed effect of treatment (l = 1 to 4), $S_i \times T_k$ = interaction between ith square and lth treatment, β = regression coefficient of the covariate term Cov_{ijkl}, Cov_{ijkl} = covariate variable for the jth cow within the ith square of the lth treatment in the kth period, and e_{ijkl} = residual error. The covariate term was removed from the statistical model when $P > 0.25$. Apparent total-tract digestibility of nutrients and urinary excretion of nitrogenous metabolites were analyzed with the same model presented above without the covariate term. Orthogonal polynomials were used to test linear, quadratic, and cubic effects in response to incremental amounts of RP-His supplementation. All data except digestibility of nutrients and urinary N excretion were reported as covariate-adjusted LSM ± SEM. Significance was declared at $P \leq 0.05$ and trends at $0.05 < P \leq 0.10$.

Plasma and muscle concentrations of His and His-containing metabolites were further analyzed as repeated measures using the MIXED procedure of SAS (SAS version 9.4) according to the following model:

$$Y_{ijklm} = \mu + S_i + C_{j(i)} + P_k + T_l + S_i \times T_l + e1_{ijkl} + W_m + T_l \times W_m + \beta Cov_{ijklm} + e2_{ijklm}$$

where, Y_{ijklm} = dependent variable, μ = overall mean, S_i = fixed effect of square (i = 1 to 2), $C_{j(i)}$ = random effect of cow nested within square, P_k = fixed effect of period (k = 1 to 4), T_l = fixed effect of treatment (l = 1 to 4), $S_i \times T_k$ = interaction between ith square and lth treatment, $e1_{ijkl}$ = whole plot error, W_m = fixed effect of week (m = 1 to 4) analyzed as repeated measure, $T_l \times W_m$ = interaction between lth treatment and mth week, β = regression coefficient of the covariate

term Cov_{ijklm}, Cov_{ijklm} = covariate variable for the jth cow within the ith square of the lth treatment in the mth wk of the kth period, and $e2_{ijklm}$ = subplot error. The SAS command REPEATED was used to model distinct residual variances. The covariance structures (compound symmetry, autoregressive, and heterogeneous first-order autoregressive) were tested and the one with the smallest Akaike's information criterion coefficient was retained in the final model. The covariate term was removed from the statistical model when $P > 0.25$. Significance was declared at $P \leq 0.05$ and trends at $0.05 < P \leq 0.10$.

RESULTS

The nutritional composition of the dietary ingredients is presented in Table 2.1. Table 2.2 shows the AA profile (% of CP) of different feeds used in the basal diet. The ingredient and nutritional composition of the basal diet are presented in Table 2.3, and the NRC (2001) evaluation of the experimental diets is shown in Table 2.4.

Plasma AA and His-Containing Metabolites

Treatment effects on plasma concentrations of AA and His-containing metabolites from wk 1 to 3 of each experimental period are shown in Supplemental Tables S2.1 to S2.3, and Table 2.5 shows plasma AA values during wk 4. The plasma concentrations of His increased linearly in all 4 wk with feeding incremental amounts of RP-His. Plasma His also showed a quadratic effect in wk 1 (Supplemental Table S2.1) and a quadratic trend ($P = 0.08$) in wk 3 (Supplemental Table S2.3). Except for His, treatments did not affect the plasma concentration of the remaining EAA during wk 4 (Table 2.5); however, RP-His supplementation modified the plasma concentrations of other EAA during wk 1 and 3. For instance, the plasma concentrations of Lys, Met, Phe, Thr,

and Trp all responded quadratically to supplemental RP-His during wk 1 ($P \leq 0.05$). Although no changes were observed in wk 2, the plasma concentrations of Arg, Leu, Ile Lys, Phe, and Val decreased linearly ($P \leq 0.06$) in dairy cows fed various levels of RP-His during wk 3. Treatments did not change the plasma concentration of 3-methylhistidine (**3-MHis**) apart from a quadratic effect ($P = 0.03$) in wk 3. Incremental amounts of RP-His increased the plasma concentration of carnosine linearly in all but wk 2.

Muscle AA Profile and His-containing Dipeptides

The effects of various dietary levels of RP-His on concentrations of muscle AA, carnosine, and anserine during wk 2 and 4 of each experimental period are presented in Supplemental Table S2.4 and Table 2.6, respectively. The concentrations of muscle His increased linearly in wk 2 and 4 in response to incremental amounts of RP-His. While muscle Lys concentration increased linearly during wk 2, no change was observed in wk 4. Muscle Met concentrations were not altered by RP-His throughout the study. The muscle concentrations of other EAA and all NEAA were not affected by treatments during wk 2 or 4. Likewise, no treatment effects were observed for muscle carnosine and anserine concentrations during wk 4. However, the concentration of muscle carnosine tended ($P = 0.08$) to respond quadratically and that of muscle anserine changed quadratically during wk 2, with the lowest values observed in cows offered 82 to 164 g/d of RP-His.

Nutrient Digestibility and Urinary N Excretion

Apparent total-tract digestibility of nutrients and urinary excretion of nitrogenous metabolites during wk 4 of each experimental period are shown in Table 2.7. Treatments did not

affect the apparent total-tract digestibilities of DM (mean = 75.3%), OM (mean = 76.8%), NDF (mean = 65.6%), and ADF (mean = 68.4%), and CP (mean = 75.5%). The urinary concentration of creatinine tended (P = 0.09) to decrease linearly with feeding incremental amounts of RP-His. Estimated urinary volume, and urinary excretion of urea-N (g/d and % of N intake) and total-N (g/d) increased linearly in response to RP-His. In contrast, supplemental RP-His did not affect the output of urea-N expressed as a proportion of total urinary-N excretion (mean = 68.4%) or total urinary-N excretion as a proportion of N intake (mean = 36.8%). Likewise, urinary excretion of uric acid (mean = 39.1 mmol/d), allantoin (mean = 464 mmol/d), and purine derivatives (mean = 503 mmol/d) was not affected by treatments.

Intake and Milk Yield and Composition

Dry matter intake, milk yield, concentrations and yields of milk components, PUN, BW, and BCS from wk 1 to 3 of each experimental period are presented in Supplemental Tables S2.5 to S1.7, and Table 2.8 shows production data during wk 4. Although treatments had no effect on DMI (mean = 22 kg/d), milk yield tended (P = 0.09) to increase linearly in cows fed incremental amounts of RP-His (Table 2.8). Yields of 4% FCM responded to RP-His in a cubic fashion, whereas ECM tended (P = 0.06) to respond cubically. Feed efficiency expressed as milk yield/DMI and 4% FCM yield/DMI increased linearly, and ECM yield/DMI showed a cubic response to elevated RP-His. Concentrations of milk fat, true protein, and lactose did not differ and averaged 3.99, 2.99, and 4.86% across treatments, respectively. Yield of milk true protein increased linearly and tended (P = 0.07) to increase quadratically in cows fed incremental amounts of RP-His. Similarly, milk lactose yield tended (P = 0.06) to increase linearly with feeding various levels of RP-His. Concentrations of both MUN (from 11.5 to 13.2 mg/dL) and

PUN (from 10.7 to 13.0 mg/dL) concentrations increased linearly. There were no treatment effects for BCS (mean = 3.10), BW (mean = 728 kg), and BW change (mean = 0.17 kg/d) in the current experiment.

Temporal Changes of Plasma and Muscle His and His-containing Metabolites

No week or treatment by week interaction effect was observed for the plasma concentration of His in cows fed incremental amounts of RP-His (Figure 1A). Plasma carnosine concentration did not differ between wk 1 and 2 and increased thereafter ($P < 0.001$; Figure 1B); however, no treatment × week interaction was detected. Although no treatment by week interaction was observed for 3-MHis, its plasma concentration was greatest in wk 2, intermediate in wk 1, and lowest in wk 3 and 4 (Figure 1C; $P < 0.01$). No week or treatment × week interaction was observed for muscle His in response to RP-His supplementation (Figure 2A). We did not observe any effect of wk or treatment × week interaction for muscle concentrations of carnosine or anserine (Figures 2B and 2C, respectively).

DISCUSSION

The proportions of His, Met, and Lys of corn silage, mixed mostly grass-legume haylage, and ground corn differed slightly from those reported in the NRC (2001), and larger differences in AA profile were observed for the remaining feedstuffs. The basal diet averaged 15.1% CP and has adequate NFC concentration (mean = 41.4%). The forage NDF concentration averaged 30% due to the high dietary forage-to-concentrate ratio (65:35) in our basal diet.

According to NRC (2001), dietary NE_L supplies were above the requirements for all experimental diets and the NE_L balance ranged from 0.4 Mcal/d in cows fed 246 g/d of RP-His

to 2.4 Mcal/d in those that did not receive RP-His. The supplies of MP varied from 1 to 6% below the requirements, with the greatest MP deficiency (i.e., −136 g/d) observed in cows fed 246 g/d of RP-His. All 4 diets provided adequate RDP (~3% above the requirements), whereas dietary RUP balance varied from 4 to 15% below the requirements and displayed a pattern like that observed for MP balance. An 8% deficiency in dHis relative to requirement was estimated for the control diet (i.e., 0 g/d of RP-His). However, surpluses of 13% (164 g/d of RP-His) and 18% (246 g/d of RP-His) or no deficiency (82 g/d of RP-His) were also observed. Although dMet supplies exceeded the requirements across all 4 diets, dMet balance slightly decreased from +8 to +6 g/d as supplemental RP-His increased from 0 to 246 g/d. The requirements (from 143 to 148 g/d) and supplies (from 144 to 141 g/d) of dLys changed with feeding incremental amounts of RP-His, resulting in a dLys balance that went from slightly positive (+1 g/d; 0 g/d RP-His diet) to negative (−7 g/d; 246 g/d RP-His diet). This may have happened because a source of RP-Lys was not used in the current study or due to the high inclusion of corn silage (i.e., 50%, DM basis) in the basal diet.

A linear response in plasma His was observed in the first week after the beginning of RP-His supplementation, indicating that short-length periods (i.e., 7-d long) in a Latin square design may be suitable to assess the relative bioavailability of His from RP-His supplements as reported for Lys from RP-Lys products (Whitehouse et al., 2017). A sharp elevation in circulating His was observed between 0 and 164 g/d of RP-His supplementation, which was followed by a less pronounced increase from 164 to 246 g/d of RP-His. This was expected because dHis balance averaged −4, 0, +6, and +9 g/d in cows fed 0, 82, 164, and 246 g/d of RP-His, respectively. The increase in plasma His with feeding 246 g/d of RP-His (15 g/d of dHis) ranged from 87% (wk 2) to 99% (wk 3) relative to the control diet. Lapierre et al. (2014) and Ouellet et al. (2014) reported

that the plasma concentration of His increased by 258 and 157%, respectively, after abomasal infusion of 15.2 g/d of His in lactating dairy cows. The lower response in plasma His to supplemental His compared with Lapierre et al. (2014) and Ouellet et al. (2014) is consistent with dietary His supply being less deficient herein (2.06% of MP supply) than in these 2 earlier studies (mean = 1.55% of MP supply), suggesting that excess His was catabolized in the liver with the carbon skeleton used for energy supply and the amino group for urea synthesis. In fact, concentrations of MUN and PUN increased linearly in cows fed various levels of RP-His, which was consistent with both deamination of His and enhanced N intake. Supplementation of RP-His has been shown to increase plasma concentration of His in most (Lee et al., 2012; Giallongo et al., 2016, 2017) but not all studies (Giallongo et al., 2015), likely due to differences in MP balance across experiments. In addition, the methodology used to determine the bioavailability of His from RP-His supplements can result in varied estimations of dHis ultimately affecting the concentration of His in plasma.

Apart from His, incremental amounts of RP-His had no effects on plasma EAA concentrations during wk 4 but changes were observed during wk 1 to 3. These inconsistencies in weekly plasma EAA concentrations are possibly associated with the time required for cows to adapt to a new diet, variation in the AA profile of feeds, stage of lactation, and level of DMI and milk yield. In addition, a quadratic response in the plasma concentration of 3-MHis during wk 3 without changes in wk 1, 2, or 4 suggests transient muscle protein proteolysis. According to Houweling et al. (2012), the catabolism of actin and myosin in skeletal muscles releases 3-MHis, which has been considered a reliable indicator of muscle proteolysis in cattle (Harris and Milne, 1981). Swick and Benevenga (1977) concluded that the breakdown of muscle protein to provide AA for milk protein synthesis is a mechanism of normal metabolic adaptation sensitive to dietary

changes. Alternatively, it cannot be disregarded that some of these inconsistencies seen in our weekly plasma EAA data set may be random and an artifact of large number of AA analyses.

Comparable to our results, Ouellet et al. (2014) reported a linear increase in the plasma concentration of carnosine in cows abomasally infused with incremental amounts of His (0 to 38 g/d). More than 99% of carnosine, which is synthesized by carnosine synthase using His and Ala, is found in skeletal muscles (Maynard et al., 2001; Boldyrev et al., 2013). Therefore, it is conceivable that increased circulating concentrations of carnosine observed herein may be related to its transportation from skeletal muscles to plasma. Everaert et al. (2013) demonstrated that mRNA transcripts of the peptide/His transporter 1 and 2 were found in skeletal muscle samples of mouse and humans even though no data appear to be available for ruminants. Transportation from muscle to plasma may be related to the metabolic functions of carnosine including pH buffering, metal-ion chelation, antioxidant activity, and protection against the formation of advanced glycation and lipoxidation end products (Boldyrev et al., 2013). However, Lee et al. (2012) and Giallongo et al. (2015) reported no effect of 50 g/d of RP-His supplementation (54% estimated bioavailability) on plasma carnosine concentration of dairy cows, which was thus in disagreement with the results from the current study. Differences in the extent of MP deficiency, amount of RP-His fed, bioavailability estimations, and experimental design (changeover vs. continuous) may have all played a role in these discrepant results.

The muscle concentrations of His increased linearly during wk 2 and 4 in cows fed different levels of supplemental RP-His, thus consistent with increased plasma His concentration. In contrast, Giallongo et al. (2015) reported no change in muscle His concentration in dairy cows offered a MP-deficient diet supplemented with 50 g/d of RP-His, possibly because less RP-His was fed in their study than in the current experiment (82 to 246

g/d). Muscle Lys and Met concentrations were not altered by RP-His supplementation, except a linear increase in Lys during wk 2. Giallongo et al. (2015) reported no differences in the muscle concentrations of Lys and Met in lactating dairy cows fed RP-His. Discrepancies in muscle His concentrations between the present study and Giallongo et al. (2015) are potentially explained by animal, dietary, and experimental design factors discussed above.

Muscle carnosine and anserine concentrations were not affected by treatments during wk 4; however, a quadratic effect for anserine and a trend for carnosine were detected in wk 2 with the lowest values observed in cows offered 82 and 164 g/d of RP-His. Lapierre et al. (2014) reported linear and quadratic trends for the muscle concentration of anserine, and a quadratic trend for that of carnosine in lactating dairy cows abomasally infused with increasing amounts of His (0, 7.6, 15.2, and 22.8 g/d) in a Latin square study with 14-d periods. It has been proposed that intramuscular carnosine and anserine, and blood hemoglobin could serve as endogenous sources of His during short-term deficiency (Lapierre et al. (2008). Hemoglobin was not measured and the muscle concentrations of anserine and carnosine responded quadratically only during wk 2, indicating that based on the available data no definite conclusions can be made regarding the use of these 2 His-containing dipeptides as endogenous sources of His under the conditions of our study.

A decoupled response between plasma (increased linearly) and muscle (no change) carnosine concentrations during wk 4 was observed in the present study and agrees with data from Giallongo et al. (2017). Davey (1960) and Maynard et al. (2001) reported significant variations in carnosine concentrations among different skeletal muscles within individual animals and among the same type of muscles across different species, indicating that muscle fiber profile may be involved in these responses. For instance, the concentration of carnosine

increased 5-fold in the predominant oxidative fiber soleus muscle but not in the predominant glycolytic-oxidative fiber red vastus lateralis muscle or predominant oxidative white vastus lateralis muscle of rats fed carnosine (Maynard et al., 2001). The longissimus dorsi muscle contains more glycolytic than oxidative fibers (Kirchofer et al., 2002), which may explain the lack of effect of RP-His on muscle carnosine concentration. While muscle carnosine concentration increased steadily following oral administration of β-alanine to humans (Harris et al., 2006), plasma carnosine did not follow the same pattern due to the high activity of serum carnosinase-1 (Jackson et al., 1991; Everaert et al., 2012; Boldyrev et al., 2013). However, serum carnosinase-1 is not expressed in serum of ruminants (Jackson et al., 1991). Thus, this decouple muscle-plasma carnosine response could be also associated with the lack of serum carnosinase in cattle.

Supplementation of RP-His had no effects on apparent total-tract digestibility of nutrients in the present study. However, the total-tract digestibilities of NDF (mean = 65.6%) and ADF (mean = 68.4%) appear to be overestimated possibly because of uncertainties associated with the adoption of iADF as the internal marker to estimate fecal output of DM. Recently, Velásquez et al. (2018) reported that the fecal recoveries of iADF averaged 147% with 2 fecal grab sampling procedures and 153% with a pooled sample obtained over 3 d of total collection in Holstein cows fed a corn silage-based diet. Fecal recovery greater than 100% can underestimate fecal DM output, resulting in overestimation of apparent total-tract fiber digestibility. Alternatively, cows in the present study consumed a moderate amount of DM (mean = 22 kg/d), which may have slowed down the digesta passage rate leading to increased fiber digestibility. Ferraretto and Shaver (2015) reported in their meta-analysis a range in apparent total-tract digestibility of NDF from 24.2 to 62.5% (mean = 43.8%) in dairy cows consuming diets containing corn silage

harvested from different types of corn hybrids. We obtained an apparent total-tract NDF digestibility of 42% using the prediction equation of de Souza et al. (2018) (i.e., NDF digestibility = 53 + 0.26 × % of grass silage in diet DM − 0.59 × % starch in diet DM + 3.06 × DMI as % of BW − 0.46 × DMI as % of BW^2). This suggests that NDF digestibility may have been overestimated by 23 percentage units when using iADF as the internal marker. Nevertheless, our sampling protocol resulted in 8 fecal grab samples per cow over a 3-d period, thus following literature recommendations for improved estimation accuracy of nutrient digestibility in the total gastrointestinal tract (e.g., Sampaio et al., 2011; Velásquez et al., 2018; Morris et al., 2018).

Urinary excretion of urea N (g/d and % of N intake) and total N (g/d) responded linearly to RP-His supplementation. In contrast, Giallongo et al. (2015) reported no changes in urinary excretion of urea N and total N with feeding 50 g/d of RP-His to lactating dairy cows. Greater amounts of supplemental RP-His in the present study compared with that (84 to 246 g/d vs. 50 g/d) from Giallongo et al. (2015) led to elevated urinary N excretion, suggesting that excess His was deaminated with ammonia being converted to urea in the liver. Alternatively, the bioavailability of His from the RP-His supplement fed in our study may have been underestimated, resulting in excess His supply relative to requirements. Holter et al. (1982) demonstrated that urine volume responded to increased N intake, which agrees with results from the present study. Lee et al. (2012) and Giallongo et al. (2015) did not observe effects of RP-His on urinary excretion of uric acid, allantoin, and purine derivatives also in concordance with our results.

We observed that supplemental RP-His tended to improve milk yield linearly without an effect on DMI, resulting in improved feed efficiency (i.e., milk yield/DMI). In comparison,

Lapierre et al. (2014) reported that DMI tended to increase linearly and milk yield increased linearly with abomasal infusions of His in dairy cows fed a 28% MP-deficient diet. Ouellet et al. (2014) observed linear increases in both DMI and milk yield with various amounts of abomasal infusions of His in 25% MP-deficient diets. Our basal diet was only 1.3% deficient in MP and may explain to a certain extent the discrepant results between the current and previous studies (Lapierre et al., 2014; Ouellet et al., 2014). Giallongo et al. (2016) fed 2% MP-deficient diets supplemented or not with RP-Lys, RP-Met, or RP-His or containing all 3 RP-AA. Specifically, cows fed the MP-deficient diet plus RP-His (not balanced for Lys and Met) showed a tendency for increased DMI and no changes in yields of milk or ECM compared with those offered a MP-deficient ration without RP-AA supplementation (Giallongo et al., 2016). It should be noted that our diets went from slightly adequate to deficient in dLys, which may have affected milk yield responses as Lys together with Met are considered co-limiting AA in typical US dairy diets (Schwab et al., 1976; NRC 2001). Altogether, these results suggest that in addition to MP balance, the ingredient composition of the basal diet, production level, DIM, amount of supplemental His, and status of dLys, dMet, dHis, and possibly other EAA may be also involved in the discrepant results in DMI and milk yield across the literature in cows fed RP-His or receiving postruminal infusions of His.

Yields of 4% FCM and milk fat responded cubically, whereas ECM yield tended to respond cubically in cows fed incremental amounts of RP-His in the present study. Korhonen et al. (2000) reported cubic responses in concentration and yield of milk fat in dairy cows fed grass silage-based diets infused postruminally with 0, 2, 4, or 6 g of His/d. Although these cubic effects are difficult to explain biologically, they may be related to imbalances of nutrient supply at the gut or mammary tissues (Korhonen et al., 2000). In addition, a low number of cows (n = 8)

was used in our study, indicating that milk yield and milk composition data should be interpreted cautiously.

Milk true protein yield averaged 0.93 kg/d from 0 to164 g/d of RP-His and increased to 0.98 kg/d with feeding 246 g/d of RP-His during wk 4 (quadratic trend; $P = 0.07$), suggesting that milk protein synthesis was not stimulated by His supply up to 164 g/d of RP-His. As discussed above, although our diets had adequate dMet status, they were deficient in dLys and this may have affected milk protein synthesis. Giallongo et al. (2016) observed that supplementation of 120 g/d of RP-His did not modify milk true protein yield in dairy cows fed a MP-deficient diet with negative dLys and dMet balances. However, milk true protein increased significantly when RP-Lys, RP-Met, and RP-His were all supplemented to a MP-deficient diet in their experiment (i.e., Giallongo et al., 2016), thereby in agreement with data from Lee at al. (2012). No changes in plasma EAA concentrations apart from His were observed during wk 4 in cows receiving supplemental RP-His in the current study despite the quadratic trend for increased milk true protein yield. Milk yield was moderate in the present study and MP balance was slightly negative so that the EAA requirements for milk protein synthesis were lowered if compared with those of high-producing dairy cows. According to Patton et al. (2015), concentrations of circulating EAA are the product of duodenal flows of EAA, digestibility, and EAA utilization in different tissues. Although EAA can be removed by hepatic (Raggio et al., 2014), peripheral (Dalbach et al., 2011), and mammary tissues (Raggio et al., 2006), Patton et al. (2015) demonstrated that the plasma concentrations of EAA are mostly affected by duodenal flows of AA. Patton et al. (2015) also showed that prediction of plasma AA concentrations was not improved even when expressing supply relative to milk true protein output. They concluded

that the plasma concentrations of EAA appear to be tightly controlled elevating steadily in response to increased amounts of EAA reaching the small intestine.

No treatment by week interactions were observed for the plasma and muscle concentrations of His, His-containing dipeptides, and 3-MHis despite some significant week effects. For instance, plasma carnosine concentration did not change from wk 1 to 2 but increased from wk 2 to 4 (Figure 1B), which may be attributed to exportation of carnosine from muscle to blood as discussed in detail above. We also observed that the concentration of 3-MHis in plasma was greatest during wk 2, suggesting increased muscle proteolysis after cows have been on a given diet for about 14 d (Figure 1C). However, we do not have a definite explanation for this change in plasma 3-MHis concentration based on data available. Overall, His deficiency may not have been pronounced enough to trigger major temporal changes in plasma and muscle His and His-derived metabolites in the current study.

CONCLUSIONS

Our hypothesis that the concentrations of plasma and muscle His would increase linearly in response to incremental amounts of RP-His was confirmed. Furthermore, milk true protein yield tended to increase in a quadratic manner in dairy cows fed increasing levels of RP-His. Specifically, milk true protein yield averaged 0.93 kg/d from 0 to 164 g/d of RP-His and increased to 0.98 kg/d with feeding 246 g/d of RP-His. This suggests that milk protein synthesis was not stimulated by His supply up to 164 g/d of RP-His (i.e., 10 g/d of dHis) under the conditions of the present study. It is important to note that our diets went from slightly adequate to deficient in predicted dLys balance, which may have affected yields of milk and milk true protein despite adequate dMet status. Concentrations of MUN and PUN, as well as urinary

excretion of urea N (g/d and % of N intake) and total N (g/d), increased linearly with the greatest values observed in cows offered 164 or 246 g of RP-His daily. Intriguingly, the threshold amount of RP-His that stimulated milk protein synthesis (i.e., ≥ 164 g/d or ≥ 10 g/d of dHis) also led to increased MUN, PUN, and urinary N excretion. These conflicting results could not be sorted out using our data, so future research is warranted to accurately determine the bioavailability of RP-His supplements and His requirements for milk protein synthesis. Continuous, dose-response production studies are particularly needed to provide further insights regarding His metabolism in high-producing dairy cows.

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Table 2.1. Nutrient composition of dietary ingredients (mean ± SD) used in the experimental diets (% of DM, unless otherwise noted)

Item	Corn silage	Haylage[1]	Ground corn	Beet pulp	Soybean meal	Steam-flaked corn	Canola meal	Liquid molasses	DDGS[2]
No. of samples	4	4	2	2	2	2	2	2	2
DM, % of fresh matter	30.5 ± 0.97	27.7 ± 2.89	84.7 ± 1.63	86.6 ± 2.30	85.3 ± 0.23	80.7 ± 2.21	86.1 ± 0.20	59.3 ± 0.92	82.1 ± 0.32
CP	8.70 ± 0.29	17.4 ± 2.91	9.05 ± 0.92	8.65 ± 0.21	53.5 ± 0.99	8.30 ± 0.28	41.5 ± 0.00	7.00 ± 0.14	31.3 ± 0.49
Soluble protein, % of CP	61.5 ± 1.00	58.3 ± 1.26	26.0 ± 2.83	18.0 ± 1.41	31.5 ± 9.19	12.5 ± 0.71	21.5 ± 0.71	-	16.0 ± 1.41
NDICP[3]	1.05 ± 0.21	3.00 ± 0.77	0.85 ± 0.21	5.65 ± 0.21	10.8 ± 0.64	1.30 ± 0.28	7.15 ± 1.06	-	5.75 ± 0.64
ADICP[4]	0.58 ± 0.13	1.75 ± 0.49	0.30 ± 0.00	3.85 ± 0.21	3.05 ± 1.06	0.55 ± 0.35	2.55 ± 0.07	-	1.85 ± 0.35
aNDFom[5]	44.0 ± 4.59	53.9 ± 6.61	7.60 ± 1.14	33.8 ± 1.84	11.4 ± 0.99	6.40 ± 0.42	27.8 ± 0.64	-	27.4 ± 0.71
ADF	26.5 ± 3.19	39.0 ± 4.76	3.15 ± 1.48	31.9 ± 3.11	7.75 ± 0.21	2.75 ± 0.78	23.2 ± 0.57	-	21.5 ± 0.71
ADL	3.30 ± 0.74	8.40 ± 1.27	0.75 ± 0.64	8.50 ± 2.55	0.95 ± 0.21	1.35 ± 0.49	11.7 ± 1.91	-	4.95 ± 0.07
NFC[6]	40.0 ± 4.04	14.9 ± 2.81	77.9 ± 3.25	42.1 ± 0.42	27.2 ± 0.21	81.9 ± 0.85	18.7 ± 0.28	-	18.6 ± 1.34
Starch	32.4 ± 3.58	0.93 ± 0.49	72.4 ± 4.17	0.20 ± 0.07	0.65 ± 0.07	79.5 ± 1.98	0.50 ± 0.14	-	1.25 ± 0.21
ESC[7]	2.03 ± 0.92	3.50 ± 0.76	1.35 ± 0.07	5.35 ± 0.35	14.1 ± 2.12	1.40 ± 0.14	7.10 ± 0.57	-	5.70 ± 0.14
Ether extract	3.28 ± 0.22	4.83 ± 0.46	3.90 ± 0.57	1.25 ± 0.07	1.15 ± 0.35	2.35 ± 0.21	4.40 ± 0.42	1.50 ± 0.14	17.3 ± 0.07
NE_L, Mcal/kg of DM	1.60 ± 0.09	1.22 ± 0.12	2.10 ± 0.02	1.25 ± 0.06	1.90 ± 0.02	2.04 ± 0.02	1.61 ± 0.03	1.77 ± 0.05	2.33 ± 0.00
Ash	3.31 ± 1.54	9.02 ± 0.72	1.54 ± 0.39	14.2 ± 1.09	6.81 ± 0.10	1.00 ± 0.04	7.64 ± 0.06	16.0 ± 1.68	5.53 ± 0.01
Ca	0.22 ± 0.01	0.66 ± 0.14	0.02 ± 0.01	1.67 ± 0.18	0.33 ± 0.01	0.01 ± 0.00	0.84 ± 0.01	1.15 ± 0.01	0.04 ± 0.01
P	0.29 ± 0.01	0.38 ± 0.04	0.31 ± 0.03	0.09 ± 0.00	0.77 ± 0.01	0.19 ± 0.01	1.28 ± 0.02	0.07 ± 0.00	1.09 ± 0.03
Mg	0.17 ± 0.01	0.28 ± 0.08	0.12 ± 0.01	0.31 ± 0.03	0.33 ± 0.00	0.07 ± 0.00	0.64 ± 0.03	0.46 ± 0.00	0.40 ± 0.01
K	1.17 ± 0.03	2.89 ± 0.24	0.38 ± 0.04	0.30 ± 0.01	2.36 ± 0.08	0.27 ± 0.01	1.24 ± 0.04	4.97 ± 0.13	1.24 ± 0.01
Na	0.01 ± 0.00	0.06 ± 0.02	0.00 ± 0.00	0.05 ± 0.00	0.01 ± 0.01	0.01 ± 0.00	0.09 ± 0.00	0.12 ± 0.00	0.03 ± 0.00
S	0.12 ± 0.00	0.26 ± 0.06	0.11 ± 0.00	0.45 ± 0.08	0.46 ± 0.00	0.10 ± 0.00	0.82 ± 0.02	1.12 ± 0.01	0.38 ± 0.00
Cl	0.30 ± 0.01	0.64 ± 0.09	0.08 ± 0.01	0.05 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.08 ± 0.01	-	0.21 ± 0.00
Fe, mg/kg of DM	136 ± 57.3	267 ± 129	42.0 ± 17.0	2,195 ± 49.5	117 ± 12.0	23.5 ± 6.36	210 ± 21.2	135 ± 17.0	108 ± 5.66
Zn, mg/kg of DM	23.3 ± 1.26	31.5 ± 2.65	22.5 ± 2.12	33.0 ± 0.00	51.5 ± 3.54	14.5 ± 0.71	65.0 ± 1.41	10.5 ± 0.71	72.5 ± 0.71
Cu, mg/kg of DM	6.25 ± 0.50	10.5 ± 1.73	3.00 ± 1.41	14.0 ± 0.00	16.5 ± 0.71	2.00 ± 0.00	8.00 ± 0.00	12.0 ± 1.41	11.0 ± 1.41
Mn, mg/kg of DM	12.0 ± 1.14	51.8 ± 10.2	7.00 ± 4.24	128 ± 6.36	36.0 ± 7.07	3.50 ± 0.71	70.0 ± 0.00	10.0 ± 1.41	15.0 ± 0.00
Mo, mg/kg of DM	1.05 ± 0.13	3.45 ± 1.13	0.45 ± 0.07	2.00 ± 0.00	6.85 ± 0.92	0.60 ± 0.14	1.40 ± 0.14	1.25 ± 0.07	1.10 ± 0.00

[1]Mixed-mostly grass-legume haylage.
[2] DDGS = Dried corn distillers grains with solubles.
[3]NDICP = neutral detergent insoluble CP.
[4]ADICP = acid detergent insoluble CP.
[5]aNDFom = amylase-treated, ash-free NDF.

[6]NFC = 100 – [(CP – NDICP) + NDF + ether extract + ash]
[7]ESC = ethanol soluble carbohydrates (Hall et al., 1999).

Table 2.2. Amino acid composition of dietary ingredients used in the experimental diets (n = 1 composited sample/feedstuff)

Item	Corn silage	Haylage[1]	Ground corn	Beet pulp	Soybean meal	Steam-flaked corn	Canola meal	Liquid molasses	DDGS[2]
EAA, % CP									
Arg	2.18	3.04	4.95	1.82	7.39	4.31	6.45	0.38	4.83
His	1.51	1.77	2.92	3.07	2.66	2.96	2.94	0.38	2.82
Ile	4.53	5.49	3.81	5.03	4.92	3.90	4.57	2.29	4.56
Leu	11.6	9.20	11.3	7.40	7.90	12.1	7.68	2.29	13.0
Lys	3.02	5.40	3.81	2.65	6.57	3.36	6.22	0.76	3.28
Met	2.01	1.86	2.03	2.51	1.44	2.15	2.19	0.00	2.09
Phe	4.70	5.65	4.95	4.75	5.32	5.11	4.43	1.53	5.56
Thr	3.52	4.73	3.68	5.31	3.97	3.63	4.65	2.29	4.10
Trp	0.67	1.18	0.89	2.51	1.52	1.08	1.46	0.76	0.97
Val	6.04	7.00	5.08	7.12	5.03	4.98	5.72	4.20	5.53
NEAA, % CP									
Ala	13.3	9.54	7.23	5.03	4.39	7.40	4.77	8.78	7.26
Asp	5.70	8.95	7.49	10.6	11.5	7.13	7.77	48.9	6.65
Cys	1.68	1.10	2.41	2.09	1.56	2.42	2.83	0.76	2.28
Gly	5.03	5.74	4.31	4.89	4.37	4.04	5.49	2.29	3.67
Glu	12.3	9.28	17.5	9.36	18.0	18.2	17.8	10.3	13.9
Orn	0.67	1.27	0.13	0.28	0.08	0.13	0.08	0.00	0.12
Pro	7.38	5.65	7.87	4.47	4.73	8.21	6.14	1.53	8.31
Ser	3.02	3.38	4.44	4.47	4.35	4.58	3.90	3.05	4.68
Tyr	2.35	3.12	2.66	6.28	3.78	2.29	3.14	2.29	4.37
Tau	2.01	0.93	2.03	3.63	0.23	1.62	0.25	4.20	0.23

[1]Mixed mostly grass-legume haylage.
[2]Corn dried distillers grains with solubles

Table 2.3. Ingredient and nutrient composition (% of DM, unless otherwise noted) of the basal diet used in the experimental treatments

Item	Diet
Ingredient, % DM	
Corn silage	50.0
Mixed-mostly grass-legume haylage	15.2
Ground corn	9.17
Beet pulp	6.79
Soybean meal, 48% CP	5.54
Steam-flaked corn	3.04
BergaFat F100[1]	3.00
Minerals and vitamins premix[2]	3.00
Canola meal	1.75
Liquid molasses	1.00
Urea[3]	0.83
Corn dried distillers grains with solubles	0.58
Smartamine® M[4]	0.05
Nutrient composition	
DM, % of fresh matter	41.6
CP	15.1
aNDFom[5]	34.8
Forage NDF	30.2
ADF	22.7
NFC	41.4
Ether extract	6.10
NE_L, Mcal/kg of DM	1.62
Ca	0.80
P	0.40

[1]BergaFat F100 is a product containing palmitic acid (Berg+Schimidt GmbH & Co., Hamburg, Germany).
[2]Contained (as-fed basis) 297 mg/kg of monensin sodium (Rumensin; Elanco, Greenfield, IN), 11.3% Ca, 1.76% P, 5.98% Mg, 6% K, 3% S, 15 mg/kg of Co, 650 mg/kg of Cu, 50 mg/kg of I, 1,200 mg/kg of Mn, 8.97 mg/kg of Se, 3,700 mg/kg of Zn, and 87.1 kIU/kg of vitamin A.
[3]Urea consists of 95.2% DM and 283% CP.
[4]Smartamine® M is a rumen-protected Met supplement (Adisseo USA Inc., Alpharetta, GA).
[5]aNDFom = amylase-treated, ash-free NDF.

Table 2.4. NRC (2001) evaluation of the experimental diets containing incremental amounts of rumen protected-His (RP-His)[1]

	RP-His			
Item[2]	0 g/d	82 g/d	164 g/d	246 g/d
NE_L, Mcal/d				
Requirement	34.0	34.4	34.2	35.3
Supply	36.4	35.8	36.3	35.7
Balance	2.4	1.4	2.1	0.4
MP, g/d				
Requirement	2,170	2,184	2,190	2,243
Supply	2,141	2,107	2,141	2,107
Balance	−29	−77	−49	−136
RDP, g/d				
Requirement	2,256	2,218	2,247	2,210
Supply	2,327	2,284	2,316	2,276
Balance	70	67	69	66
RUP, g/d				
Requirement	1,068	1,115	1,101	1,198
Supply	1,030	1,014	1,024	1,017
Balance	−38	−101	−77	−181
dHis,[3] g/d				
Requirement[4]	48	48	48	49
Supply from the diet	44	43	44	43
Supply from RP-His	0	5	10	15
Balance	−4	0	6	9
dMet,[3] g/d				
Requirement[4]	48	48	48	49
Supply from the diet	49	49	49	48
Supply from RP-Met[5]	7	7	7	7
Balance	8	8	7	6
dLys,[3] g/d				
Requirement[4]	143	144	145	148
Supply	144	141	143	141
Balance	1	−3	−2	−7

[1]RP-His is a prototype supplement (Ajinomoto Co. Inc.; Kawasaki-shi, Japan).
[2]All values were estimated using the NRC (2001) based on actual DMI, DIM, milk yield and composition, and BW of the cows.
[3]dHis, dMet, and dLys represents digestible His, Met, and Lys, respectively.
[4]Requiements of dHis, dMet, and dLys were calculated as 2.2, 2.2, and 6.6% of MP requirements, respectively.
[5]RP-Met is Smartamine® M (Adisseo USA Inc., Alpharetta, GA).

Table 2.5. Plasma concentrations of AA and His-containing metabolites in mid-lactation dairy cows fed incremental amounts of rumen protected-His (RP-His)[1] during wk 4 of each experimental period[2]

	RP-His					*P*-value[3]		
Item	0 g/d	82 g/d	164 g/d	246 g/d	SEM	Linear	Quadratic	Cubic
EAA, μ*M*								
Arg	64.7	60.8	64.6	64.1	4.03	0.90	0.68	0.51
His	33.2	44.9	59.1	63.1	3.27	<0.001	0.13	0.25
Ile	90.5	83.2	92.3	93.6	5.83	0.34	0.32	0.22
Leu	106	98.0	104	101	5.23	0.63	0.58	0.31
Lys	63.2	57.0	65.2	63.2	3.97	0.62	0.57	0.16
Met	26.6	26.5	27.0	28.2	1.64	0.41	0.65	0.97
Phe	41.1	40.5	41.9	41.3	1.70	0.81	0.99	0.60
Thr	74.6	73.0	76.3	74.0	5.35	0.94	0.93	0.57
Trp	32.8	31.5	31.9	30.4	1.92	0.24	0.94	0.51
Val	169	156	164	162	9.05	0.67	0.38	0.27
NEAA, μ*M*								
Ala	245	246	258	244	12.9	0.83	0.39	0.36
Asn	43.1	42.8	45.8	45.5	2.65	0.38	0.99	0.57
Asp	2.63	2.57	2.82	2.95	0.14	0.04	0.45	0.45
Cit	94.9	93.7	94.6	93.2	6.34	0.85	0.97	0.85
Cys	17.4	17.5	17.8	17.6	1.04	0.49	0.52	0.65
Gln	253	254	265	258	14.2	0.59	0.72	0.59
Glu	46.0	43.2	46.0	45.3	2.30	0.94	0.63	0.34
Gly	301	309	310	293	10.8	0.57	0.19	0.79
Orn	34.4	33.3	36.3	34.0	1.94	0.85	0.76	0.29
Pro	74.7	72.4	78.5	74.4	3.42	0.58	0.66	0.06
Ser	70.7	67.3	67.9	65.8	5.45	0.51	0.90	0.76
Tau	29.7	28.7	33.4	33.1	2.85	0.08	0.84	0.20
Tyr	38.4	35.8	37.6	36.4	3.33	0.75	0.82	0.58
Sum and ratio of AA								
∑ EAA, μ*M*	702	671	726	721	31.2	0.38	0.66	0.26
∑ NEAA, μ*M*	1,251	1,247	1,293	1,243	47.4	0.89	0.53	0.38
∑ BCAA,[4] μ*M*	366	337	360	356	17.2	0.95	0.39	0.24

Total AA,[5] µ*M*	1,953	1,918	2,019	1,964	70.3	0.62	0.86	0.28
Lys:Met	2.36	2.18	2.51	2.25	0.17	1.00	0.76	0.05
His-containing metabolites, µ*M*								
Carnosine	29.3	32.0	32.6	33.5	1.20	0.02	0.49	0.67
3-MHis[6]	3.18	3.46	3.38	3.24	0.28	0.91	0.21	0.67

[1]The RP-His used is a prototype supplement (Ajinomoto Co. Inc., Kawasaki-shi, Japan).
[2]Wk 4 = d 22 to 28 of periods 1 to 4.
[3]Orthogonal polynomials were used to test linear, quadratic, and cubic effects; significance was declared at $P \leq 0.05$ and trends at $0.05 < P \leq 0.10$.
[4]BCAA = branched-chain AA.
[5]Total AA = EAA + NEAA.
[6]3-MHis = 3-methylhistidine.

Table 2.6. Muscle concentrations of AA and His-containing dipeptides in mid-lactation dairy cows fed incremental amounts of rumen protected-His (RP-His)[1] during wk 4 of each experimental period[2]

	RP-His					*P*-value[3]		
Item	0 g/d	82 g/d	164 g/d	246 g/d	SEM	Linear	Quadratic	Cubic
EAA, µg/g								
Arg	5.67	5.46	5.22	6.00	0.58	0.77	0.39	0.69
His	3.15	4.31	4.69	5.51	0.31	<0.001	0.60	0.39
Ile	3.74	3.69	3.49	3.72	0.25	0.74	0.44	0.46
Leu	4.94	4.91	4.82	4.94	0.36	0.93	0.74	0.80
Lys	3.99	4.41	4.60	4.76	0.55	0.27	0.79	0.92
Met	2.20	2.28	2.07	1.94	0.23	0.31	0.62	0.69
Phe	2.49	2.71	2.58	2.67	0.13	0.46	0.63	0.33
Thr	4.12	4.29	4.47	5.41	0.48	0.08	0.43	0.73
Val	5.40	5.60	5.85	5.68	0.43	0.67	0.58	0.75
NEAA, µg/g								
Ala	37.5	41.4	41.1	40.3	2.72	0.52	0.41	0.78
Asn	3.25	3.02	3.16	4.86	0.78	0.17	0.23	0.74
Asp	17.3	18.2	19.5	20.0	1.20	0.09	0.86	0.86
Cit	2.57	2.37	2.57	2.88	0.32	0.34	0.33	0.81
Gln	97.7	91.3	96.1	122	12.6	0.18	0.21	0.86
Glu	66.1	69.4	68.9	79.8	5.71	0.13	0.52	0.56
Gly	21.9	21.2	21.2	25.7	3.66	0.44	0.45	0.79
Orn	2.32	2.17	2.52	2.55	0.24	0.33	0.70	0.43
Pro	5.51	5.64	6.17	6.79	0.64	0.13	0.69	0.91
Ser	7.78	7.56	6.87	7.44	0.88	0.66	0.65	0.65
Tau	43.4	49.1	46.5	61.3	6.32	0.08	0.48	0.38
Tyr	3.62	3.65	3.21	3.53	0.23	0.44	0.48	0.20
Sum and ratio of AA								
∑ EAA, µg/g	35.7	37.7	37.8	40.6	2.35	0.11	0.82	0.61
∑ NEAA, µg/g	309	315	318	378	29.3	0.13	0.37	0.65
∑ BCAA,[4] µg/g	14.1	14.2	14.2	14.3	0.98	0.82	0.96	0.90
Total AA,[5] µg/g	345	353	356	418	30.1	0.11	0.38	0.64
Lys:Met	1.96	1.97	2.30	2.42	0.25	0.08	0.79	0.57

His-containing dipeptides, µg/g								
Anserine	139	150	134	141	11.0	0.78	0.83	0.26
Carnosine	952	1,085	970	925	77.8	0.56	0.24	0.34

[1]The RP-His used is a prototype supplement (Ajinomoto Co. Inc., Kawasaki-shi, Japan).
[2]Wk 4 = d 22 to 28 of periods 1 to 4.
[3]Orthogonal polynomials were used to test linear, quadratic, and cubic effects; significance was declared at $P \leq 0.05$ and trends at $0.05 < P \leq 0.10$.
[4]BCAA = branched-chain AA.
[5]Total AA = EAA + NEAA.

Table 2.7. Apparent total-tract digestibility of nutrients and urinary excretion of nitrogenous compounds in mid-lactation dairy cows fed incremental amount of rumen protected-His (RP-His)[1] during wk 4 of each experimental period[2]

	RP-His					*P*-value[3]		
Item	0 g/d	82 g/d	164 g/d	246 g/d	SEM	Linear	Quadratic	Cubic
Apparent total-tract digestibility								
DM, % of DMI	75.2	75.4	75.5	75.1	0.58	0.94	0.63	0.85
OM, % of OM intake	76.7	76.9	77.0	76.4	0.62	0.69	0.55	0.81
CP, % of CP intake	74.9	75.9	75.8	75.5	0.80	0.61	0.37	0.79
NDF, % of NDF intake	65.4	65.2	65.7	66.0	1.15	0.68	0.83	0.88
ADF, % of ADF intake	68.0	68.0	68.6	69.0	1.01	0.45	0.84	0.88
N intake and urinary excretion								
N intake, g/d	562	563	574	574	25.6	0.04	0.89	0.39
Creatinine, m*M*	6.48	6.39	5.42	5.90	0.41	0.09	0.40	0.13
Volume, L/d	30.0	29.4	35.4	34.2	2.28	0.02	0.85	0.08
Urea-N, g/d	130	129	145	153	8.71	0.02	0.54	0.47
Total-N, g/d	191	198	219	215	13.5	0.03	0.54	0.30
Urea-N, % of total-N	67.3	66.6	66.8	73.0	3.86	0.33	0.39	0.77
Urea-N, % of N intake	23.7	23.3	25.8	27.0	1.60	0.04	0.53	0.48
Total-N, % of N intake	35.0	35.9	38.7	37.5	1.96	0.11	0.44	0.33
Uric acid, mmol/d	39.2	36.2	39.1	41.8	5.54	0.31	0.24	0.56
Allantoin, mmol/d	457	424	484	490	38.4	0.17	0.43	0.20
Purine derivatives, mmol/d	496	460	523	532	41.2	0.17	0.40	0.21

[1]The RP-His used is a prototype supplement (Ajinomoto Co. Inc., Kawasaki-shi, Japan).
[2]Wk 4 = d 22 to 28 of periods 1 to 4.
[3]Orthogonal polynomials were used to test linear, quadratic, and cubic effects; significance was declared at $P \leq 0.05$ and trends at $0.05 < P \leq 0.10$.

Table 2.8. Dry matter intake, milk yield, concentrations and yields of milk components, feed efficiency, concentration of plasma urea N (PUN), BW, and BCS in mid-lactation dairy cows fed incremental amounts of rumen protected-His (RP-His)[1] during wk 4 of each experimental period[2]

	RP-His					*P*-value[3]		
Item	0 g/d	82 g/d	164 g/d	246 g/d	SEM	Linear	Quadratic	Cubic
DMI, kg/d	22.2	21.8	22.1	21.8	0.54	0.11	0.95	0.14
Milk yield, kg/d	31.2	31.6	31.1	32.7	1.34	0.09	0.24	0.20
Milk yield/DMI, kg/kg	1.40	1.44	1.43	1.50	0.06	0.02	0.48	0.22
4% FCM,[4] kg/d	31.3	31.8	30.7	32.3	1.54	0.34	0.28	0.04
4% FCM/DMI, kg/kg	1.40	1.45	1.41	1.49	0.05	0.01	0.44	0.02
ECM,[5] kg/d	33.5	34.0	33.1	34.7	1.58	0.20	0.19	0.06
ECM/DMI, kg/kg	1.50	1.55	1.51	1.60	0.05	<0.01	0.33	0.03
Milk fat, %	4.03	4.08	3.92	3.91	0.08	0.13	0.67	0.24
Milk fat, kg/d	1.25	1.28	1.22	1.28	0.07	0.80	0.43	0.04
Milk true protein, %	2.99	2.97	3.00	2.98	0.05	0.96	0.97	0.43
Milk true protein, kg/d	0.93	0.93	0.93	0.98	0.04	0.03	0.07	0.42
Milk lactose, %	4.85	4.83	4.90	4.86	0.03	0.35	0.79	0.10
Milk lactose, kg/d	1.52	1.53	1.53	1.59	0.06	0.06	0.31	0.37
Milk N, % of N intake	27.5	27.6	27.0	28.1	1.00	0.50	0.23	0.18
MUN, mg/dL	11.5	11.3	12.2	13.2	1.02	0.01	0.21	0.61
PUN, mg/dL	10.7	10.4	11.5	13.0	0.76	<0.01	0.14	0.71
BCS	3.06	3.06	3.13	3.16	0.10	0.23	0.80	0.74
BW, kg	730	724	730	729	7.28	0.91	0.66	0.45
BW change, kg/d	0.18	0.10	0.20	0.20	0.17	0.83	0.78	0.70

[1]The RP-His used is a prototype supplement (Ajinomoto Co. Inc., Kawasaki-shi, Japan).
[2]Wk 4 = d 22 to 28 of periods 1 to 4.
[3]Orthogonal polynomials were used to test linear, quadratic, and cubic effects; significance was declared at $P \leq 0.05$ and trends at $0.05 < P \leq 0.10$.
[4]4% FCM = (0.4 × kg of milk) + (15 × kg of milk fat); Gaines and Davidson (1923).
[5]ECM = (0.327 × kg of milk) + (12.95 × kg of milk fat) + (7.65 × kg of milk protein); Tyrrell and Reid (1965).

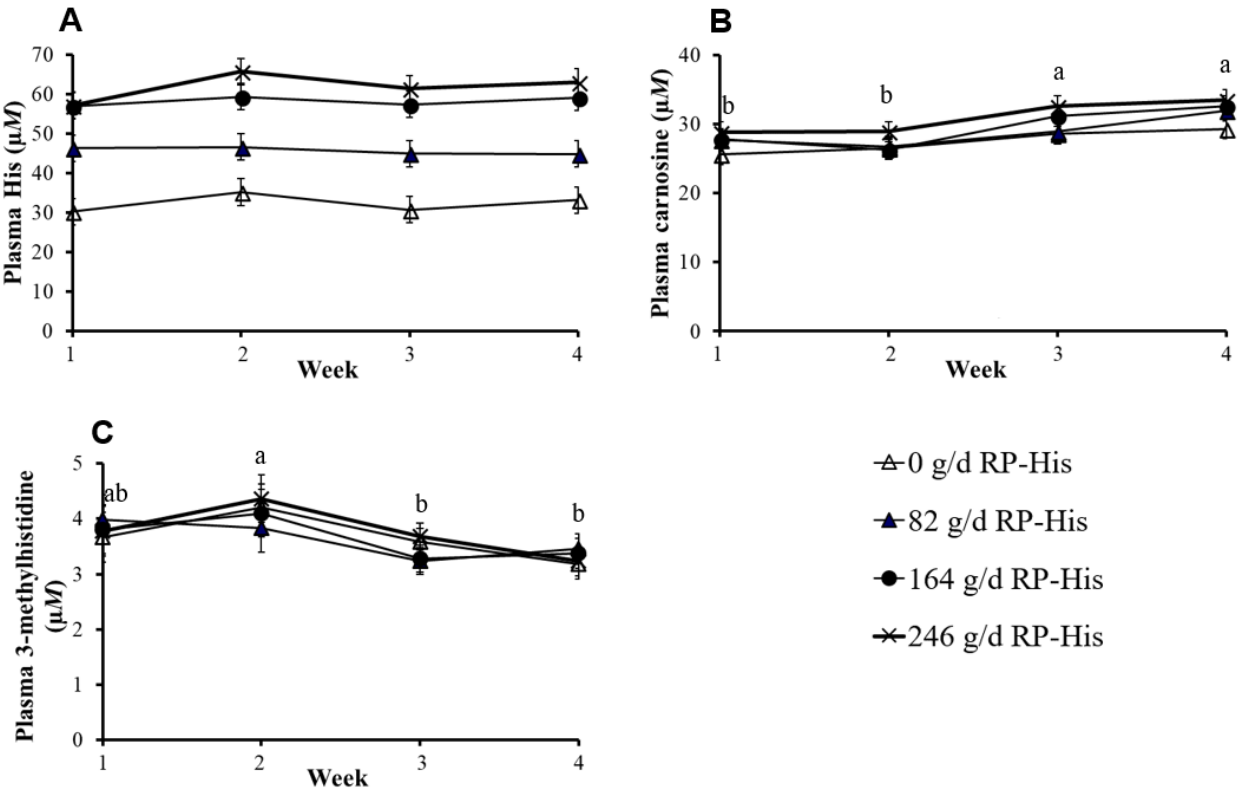

Figure 2.1. Plasma concentrations of (A) His (treatment: $P < 0.001$; wk: $P = 0.13$; treatment × wk: $P = 0.93$; SEM = 2.54), (B) carnosine (treatment: $P < 0.001$; wk: $P < 0.001$; treatment × wk: $P = 0.89$; SEM = 0.73), and (C) 3-methylhistidine (treatment: $P = 0.93$; wk: $P < 0.01$; treatment × wk: $P = 0.76$; SEM = 0.23) in mid-lactation dairy cows fed incremental amounts of rumen protected-His (RP-His). Wk 1 (d 1 to 7), wk 2 (d 8 to 14), wk 3 (d 15 to 21), and wk 4 (d 21 to 28) of periods 1, 2, 3, and 4, respectively. Data are presented as LSM ± SEM. [a,b]Means with different superscripts across wk differ at $P \leq 0.05$. The RP-His used is a prototype supplement (Ajinomoto Co. Inc., Kawasaki-shi, Japan).

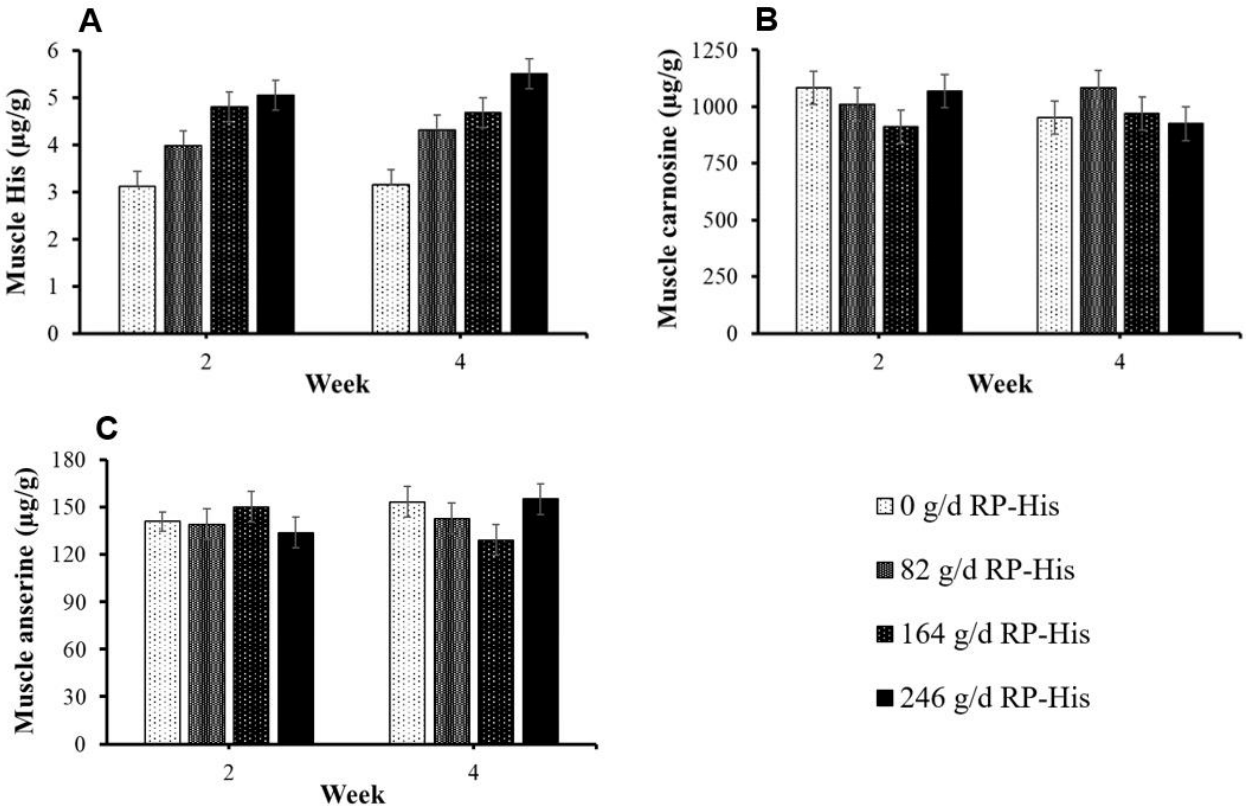

Figure 2.2. Muscle concentrations of (A) His (treatment: $P < 0.001$; wk: $P = 0.44$; treatment × wk: $P = 0.78$; SEM = 0.23), (B) carnosine (treatment: $P = 0.48$; wk: $P = 0.46$; treatment × wk: $P = 0.23$; SEM = 55.2), and (C) anserine (treatment: $P = 0.21$; wk: $P = 0.50$; treatment × wk: $P = 0.43$; SEM = 7.56) in mid-lactation dairy cows fed incremental amounts of rumen protected-His (RP-His). Wk 1 (d 1 to 7), wk 2 (d 8 to 14), wk 3 (d 15 to 21), and wk 4 (d 21 to 28) of periods 1, 2, 3, and 4, respectively. Data are presented as LSM ± SEM. The RP-His used is a prototype supplement (Ajinomoto Co. Inc., Kawasaki-shi, Japan).

Supplemental Table S2.1. Plasma concentrations of AA and His-containing metabolites in mid-lactation dairy cows in fed incremental amounts of rumen protected-His (RP-His)[1] during wk 1 of each experimental period[2]

	RP-His					*P*-value[3]		
Item	0 g/d	82 g/d	164 g/d	246 g/d	SEM	Linear	Quadratic	Cubic
EAA, μ*M*								
Arg	65.5	63.8	73.5	58.6	4.45	0.49	0.08	0.03
His	30.3	46.3	57.0	57.2	3.11	<0.001	<0.01	0.64
Ile	90.2	92.3	105	88.1	6.88	0.73	0.04	0.05
Leu	105	108	115	99.0	5.61	0.64	0.08	0.26
Lys	58.9	62.7	72.1	55.5	4.28	0.97	0.01	0.07
Met	25.5	26.4	28.7	23.8	1.57	0.67	0.05	0.19
Phe	42.8	45.0	46.7	40.4	1.55	0.43	0.01	0.30
Thr	75.9	76.2	87.0	70.7	4.86	0.77	0.03	0.03
Trp	31.2	32.7	35.2	31.4	1.51	0.63	0.05	0.22
Val	176	177	194	166	10.2	0.74	0.12	0.12
NEAA, μ*M*								
Ala	243	254	274	228	16.2	0.62	0.02	0.14
Asn	37.7	42.1	46.1	37.2	2.43	0.80	<0.01	0.23
Asp	2.72	3.20	2.83	2.61	0.33	0.63	0.28	0.49
Cit	92.1	84.4	92.6	94.9	6.42	0.46	0.32	0.33
Cys	16.8	18.0	18.9	17.4	1.01	0.09	<0.01	0.23
Gln	252	271	268	253	15.1	0.99	0.15	0.86
Glu	44.0	43.4	42.1	40.4	2.44	0.12	0.74	0.98
Gly	292	324	308	317	12.1	0.23	0.31	0.14
Orn	32.9	32.3	36.3	30.6	2.17	0.69	0.14	0.07
Pro	73.8	76.9	78.6	72.1	3.33	0.74	0.05	0.50
Ser	65.1	70.0	69.6	64.2	3.52	0.85	0.16	0.98
Tau	31.5	32.8	30.6	30.7	2.61	0.62	0.79	0.53
Tyr	35.9	37.0	41.6	30.8	2.45	0.34	0.02	0.10
Sum and ratio of AA								
∑ EAA, μ*M*	702	730	815	691	33.8	0.70	0.02	0.07
∑ NEAA, μ*M*	1,219	1,289	1,310	1,219	36.6	0.89	0.02	0.65
∑ BCAA,[4] μ*M*	372	377	415	353	20.3	0.83	0.07	0.12

Total AA,[5] μ*M*	1,921	2,019	2,124	1,910	60.0	0.77	<0.01	0.18
Lys:Met	2.33	2.40	2.51	2.38	0.18	0.61	0.37	0.58
His-containing metabolites, μ*M*								
Carnosine	25.6	27.7	27.8	28.9	0.73	<0.01	0.41	0.28
3-MHis[6]	3.67	3.98	3.81	3.77	0.19	0.84	0.25	0.36

[1]The RP-His used is a prototype supplement (Ajinomoto Co. Inc., Kawasaki-shi, Japan).
[2]Wk 1 = d 1 to 7 of periods 1 to 4.
[3]Orthogonal polynomials were used to test linear, quadratic, and cubic effects; significance was declared at $P \leq 0.05$ and trends at $0.05 < P \leq 0.10$.
[4]BCAA = branched-chain AA.
[5]Total AA = EAA + NEAA.
[6]3-MHis = 3-methylhistidine.

Supplemental Table S2.2. Plasma concentrations of AA and His-containing metabolites in mid-lactation dairy cows fed incremental amounts of rumen protected-His (RP-His)[1] during wk 2 of each experimental period[2]

	RP-His					*P*-value[3]		
Item	0 g/d	82 g/d	164 g/d	246 g/d	SEM	Linear	Quadratic	Cubic
EAA, μ*M*								
Arg	76.1	74.8	64.1	76.3	5.15	0.57	0.11	0.09
His	35.2	46.6	59.3	65.7	3.73	<0.001	0.41	0.57
Ile	107	103	94.1	99.9	6.14	0.24	0.36	0.45
Leu	122	115	104	116	7.33	0.34	0.16	0.39
Lys	72.5	71.5	62.2	83.6	8.01	0.46	0.14	0.24
Met	29.7	28.8	28.5	29.8	1.70	0.99	0.48	0.89
Phe	47.3	46.5	43.0	45.4	2.12	0.33	0.44	0.37
Thr	84.8	83.7	76.2	83.0	7.32	0.51	0.37	0.29
Trp	34.3	32.0	31.0	34.2	2.13	0.87	0.10	0.69
Val	190	181	167	182	11.9	0.28	0.11	0.33
NEAA, μ*M*								
Ala	275	272	254	268	17.2	0.40	0.41	0.35
Asn	45.3	48.5	42.2	45.4	3.41	0.62	1.00	0.13
Asp	2.78	2.84	2.62	2.69	0.16	0.42	0.96	0.35
Cit	99.3	98.0	92.5	113	5.35	0.03	<0.01	0.07
Cys	17.6	17.1	18.1	18.3	1.05	0.26	0.55	0.40
Gln	246	273	265	265	13.1	0.29	0.21	0.35
Glu	47.0	46.5	46.2	42.8	1.98	0.16	0.46	0.71
Gly	304	313	300	319	10.4	0.48	0.63	0.26
Orn	36.1	37.4	33.8	38.3	2.76	0.72	0.38	0.13
Pro	77.9	80.9	70.5	79.5	4.25	0.67	0.31	0.02
Ser	74.5	74.1	67.1	71.8	4.96	0.44	0.57	0.36
Tau	31.8	35.1	33.6	35.2	2.56	0.15	0.51	0.20
Tyr	43.0	43.3	35.7	40.8	3.25	0.22	0.36	0.09
Sum and ratio of AA								
∑ EAA, μ*M*	799	782	730	816	44.6	1.00	0.15	0.27
∑ NEAA, μ*M*	1,300	1,342	1,262	1,340	42.6	0.78	0.57	0.07
∑ BCAA,[4] μ*M*	419	398	365	399	23.8	0.27	0.16	0.36

Total AA,[5] μ*M*	2,099	2,123	1,991	2,157	77.2	0.89	0.28	0.13
Lys:Met	2.43	2.49	2.24	2.86	0.27	0.30	0.22	0.25
His-containing metabolites, μ*M*								
Carnosine	26.5	26.7	26.3	28.9	1.22	0.21	0.32	0.50
3-MHis[6]	4.20	3.83	4.10	4.36	0.35	0.63	0.37	0.69

[1]The RP-His used is a prototype supplement (Ajinomoto Co. Inc., Kawasaki-shi, Japan).
[2]Wk 2 = d 8 to 14 of periods 1 to 4.
[3]Orthogonal polynomials were used to test linear, quadratic, and cubic effects; significance was declared at $P \leq 0.05$ and trends at $0.05 < P \leq 0.10$.
[4]BCAA = branched-chain AA.
[5]Total AA = EAA + NEAA.
[6]3-MHis = 3-methylhistidine.

Supplemental Table S2.3. Plasma concentrations of AA and His-containing metabolites in mid-lactation dairy cows fed incremental amounts of rumen protected-His (RP-His)[1] during wk 3 of each experimental period[2]

	RP-His					*P*-value[3]		
Item	0 g/d	82 g/d	164 g/d	246 g/d	SEM	Linear	Quadratic	Cubic
EAA, μ*M*								
Arg	71.4	68.7	62.0	63.0	5.54	0.05	0.60	0.45
His	30.8	44.9	57.4	61.4	3.10	<0.001	0.08	0.59
Ile	94.3	97.0	88.0	84.5	5.63	0.06	0.47	0.38
Leu	113	108	99.6	97.8	6.24	<0.01	0.67	0.60
Lys	70.0	67.6	61.4	58.0	4.65	0.01	0.89	0.68
Met	28.2	28.2	27.1	28.0	1.10	0.73	0.68	0.51
Phe	44.9	42.3	42.1	40.5	1.81	0.05	0.73	0.56
Thr	78.9	75.1	69.7	75.6	4.03	0.39	0.24	0.48
Trp	33.0	29.2	29.8	31.5	1.79	0.56	0.08	0.62
Val	172	166	156	153	9.69	0.02	0.71	0.68
NEAA, μ*M*								
Ala	258	247	232	235	13.4	0.05	0.45	0.62
Asn	43.6	45.4	39.6	41.2	2.13	0.19	0.97	0.13
Asp	2.75	2.81	2.60	2.62	0.15	0.36	0.90	0.46
Cit	90.1	88.4	87.5	97.8	6.93	0.28	0.20	0.61
Cys	17.6	17.4	17.4	17.2	1.15	0.71	0.94	0.89
Gln	263	245	260	259	12.7	0.95	0.42	0.31
Glu	47.0	48.8	41.7	41.2	2.40	<0.001	0.34	<0.01
Gly	303	295	290	300	9.84	0.74	0.33	0.78
Orn	35.9	35.2	31.7	32.7	2.37	0.19	0.70	0.43
Pro	72.1	72.0	68.9	70.2	3.19	0.53	0.82	0.60
Ser	71.6	66.4	65.9	64.9	3.40	0.10	0.44	0.67
Tau	29.7	31.8	30.1	32.7	3.19	0.42	0.91	0.37
Tyr	40.9	38.2	36.2	36.9	1.77	0.08	0.32	0.77
Sum and ratio of AA								
∑ EAA, μ*M*	736	726	693	694	35.5	0.14	0.82	0.59
∑ NEAA, μ*M*	1,275	1,233	1,204	1,231	30.6	0.25	0.27	0.75
∑ BCAA,[4] μ*M*	379	370	343	336	19.5	0.01	0.96	0.52

Total AA,[5] μ*M*	2,011	1,960	1,897	1,924	54.1	0.18	0.50	0.16
Lys:Met	2.51	2.40	2.24	2.10	0.17	<0.01	0.90	0.87
His-containing metabolites, μ*M*								
Carnosine	28.6	28.9	31.2	32.6	1.26	0.02	0.64	0.62
3-MHis[6]	3.58	3.24	3.27	3.68	0.21	0.65	0.03	0.98

[1]The RP-His used is a prototype supplement (Ajinomoto Co. Inc., Kawasaki-shi, Japan).
[2]Wk 3 = d 15 to 21 of periods 1 to 4.
[2]Orthogonal polynomials were used to test linear, quadratic, and cubic effects; significance was declared at $P \leq 0.05$ and trends at $0.05 < P \leq$ 0.10.
[3]BCAA = branched-chain AA.
[4]Total AA = EAA + NEAA.
[5]3-MHis = 3-methylhistidine.

Supplemental Table S2.4. Muscle concentrations of AA and His-containing dipeptides in mid-lactation dairy cows fed incremental amounts of rumen protected-His (RP-His)[1] during wk 2 of each experimental period[2]

	RP-His					*P*-value[3]		
Item	0 g/d	82 g/d	164 g/d	246 g/d	SEM	Linear	Quadratic	Cubic
EAA, µg/g								
Arg	5.23	6.42	5.24	6.23	0.62	0.48	0.87	0.09
His	3.12	3.98	4.81	5.05	0.29	<0.001	0.30	0.68
Ile	4.13	4.17	4.15	1.45	0.22	0.98	0.92	0.95
Leu	5.35	5.33	5.43	5.38	0.31	0.87	0.95	0.82
Lys	3.24	4.92	4.34	5.29	0.56	0.04	0.52	0.14
Met	2.45	2.43	2.16	2.50	0.23	0.88	0.23	0.19
Phe	2.87	2.92	2.79	2.75	0.13	0.40	0.73	0.64
Thr	4.24	4.37	4.02	4.30	0.25	0.85	0.74	0.26
Val	5.78	5.66	5.65	5.70	0.37	0.81	0.73	0.95
NEAA, µg/g								
Ala	40.9	39.2	36.2	39.1	2.56	0.46	0.37	0.52
Asn	2.64	3.31	2.48	2.03	0.50	0.22	0.25	0.37
Asp	18.0	17.7	17.6	18.0	0.95	0.98	0.69	0.96
Cit	2.38	2.67	2.52	2.47	0.33	0.88	0.36	0.51
Gln	84.7	94.2	79.9	89.1	6.54	0.96	0.97	0.10
Glu	56.4	60.4	50.9	58.8	5.16	0.92	0.71	0.20
Gly	20.3	20.3	14.9	16.5	2.89	0.21	0.79	0.34
Orn	2.07	2.39	2.35	2.24	0.17	0.56	0.22	0.69
Pro	5.28	5.22	4.52	5.34	0.49	0.80	0.34	0.29
Ser	6.63	6.99	6.16	6.71	0.40	0.72	0.78	0.13
Tau	37.1	38.7	31.9	34.1	2.77	0.16	0.92	0.12
Tyr	3.71	3.91	3.68	3.73	0.19	0.86	0.70	0.41
Sum and ratio of AA								
∑ EAA, µg/g	36.4	40.2	38.6	41.3	1.83	0.12	0.78	0.25
∑ NEAA, µg/g	280	295	253	278	17.2	0.54	0.78	0.12
∑ BCAA,[4] µg/g	15.3	15.2	15.2	15.2	0.86	0.99	0.95	0.93
Total AA,[5] µg/g	316	335	292	319	17.4	0.66	0.80	0.10

Lys:Met	1.40	2.04	2.23	2.21	0.28	0.04	0.22	0.84
His-containing dipeptides, µg/g								
Anserine	154	143	129	155	9.04	0.84	0.05	0.29
Carnosine	1,084	1,010	912	1,070	68.8	0.63	0.08	0.33

[1]The RP-His used is a prototype supplement (Ajinomoto Co. Inc., Kawasaki-shi, Japan).
[2]Wk 2 = d 8 to 14 of periods 1 to 4.
[3]Orthogonal polynomials were used to test linear, quadratic, and cubic effects; significance was declared at $P \leq 0.05$ and trends at $0.05 < P \leq 0.10$.
[4]BCAA = branched-chain AA.
[5]Total AA = EAA + NEAA.

Supplemental Table S2.5. Dry matter intake, milk yield, concentrations and yields of milk components, feed efficiency, concentration of plasma urea N (PUN), BW, and BCS in mid-lactation dairy cows fed incremental amounts of rumen protected-His (RP-His)[1] during wk 1 of each experimental period[2]

	RP-His					*P*-value[3]		
Item	0 g/d	82 g/d	164 g/d	246 g/d	SEM	Linear	Quadratic	Cubic
DMI, kg/d	23.4	23.1	23.0	23.4	0.58	0.96	0.29	0.89
Milk yield, kg/d	33.4	33.2	32.8	32.9	1.16	0.33	0.77	0.75
Milk yield/DMI, kg/kg	1.43	1.44	1.44	1.43	0.06	0.99	0.65	0.90
4% FCM,[4] kg/d	33.9	33.9	33.9	34.9	1.52	0.48	0.59	0.76
4% FCM/DMI, kg/kg	1.44	1.47	1.48	1.51	0.06	0.18	0.91	0.77
ECM,[5] kg/d	36.1	36.2	36.2	36.9	1.52	0.49	0.72	0.81
ECM/DMI, kg/kg	1.54	1.57	1.58	1.60	0.06	0.19	0.66	0.83
Milk fat, %	4.12	4.15	4.22	4.38	0.16	0.22	0.67	0.93
Milk fat, kg/d	1.37	1.38	1.38	1.45	0.08	0.33	0.61	0.80
Milk true protein, %	2.93	2.97	3.01	2.95	0.04	0.19	0.01	0.27
Milk true protein, kg/d	0.97	0.98	0.99	0.97	0.04	0.98	0.25	0.73
Milk lactose, %	4.92	4.84	4.87	4.85	0.03	0.13	0.30	0.17
Milk lactose, kg/d	1.64	1.61	1.60	1.59	0.05	0.19	0.75	0.86
Milk N, % of N intake	27.4	27.6	27.6	26.1	1.00	0.12	0.10	0.59
MUN, mg/dL	12.8	12.2	12.5	13.0	1.07	0.76	0.42	0.83
PUN, mg/dL	11.3	11.2	11.9	11.9	0.82	0.23	0.98	0.48

[1]The RP-His used is a prototype supplement (Ajinomoto Co. Inc., Kawasaki-shi, Japan).
[2]Wk 1 = d 1 to 7 of periods 1 to 4.
[3]Orthogonal polynomials were used to test linear, quadratic, and cubic effects; significance was declared at $P \leq 0.05$ and trends at $0.05 < P \leq 0.10$.
[4]4 % FCM = (0.4 × kg of milk) + (15 × kg of milk fat); Gaines and Davidson (1923).
[5]ECM = (0.327 × kg of milk) + (12.95 × kg of milk fat) + (7.65 × kg of milk protein); Tyrrell and Reid (1965).

Supplemental Table S2.6. Dry matter intake, milk yield, concentrations and yields of milk components, feed efficiency, concentration of plasma urea N (PUN), BW, and BCS in mid-lactation dairy cows fed incremental amounts of rumen protected-His (RP-His)[1] during wk 2 of each experimental period[2]

	RP-His					*P*-value[3]		
Item	0 g/d	82 g/d	164 g/d	246 g/d	SEM	Linear	Quadratic	Cubic
DMI, kg/d	21.9	21.9	21.9	22.1	0.51	0.50	0.59	0.89
Milk yield, kg/d	31.6	32.5	32.2	33.5	1.35	0.04	0.70	0.25
Milk yield/DMI, kg/kg	1.44	1.48	1.48	1.52	0.06	0.08	0.95	0.54
4% FCM,[4] kg/d	32.4	32.2	32.5	33.7	1.50	0.03	0.11	0.93
4% FCM/DMI, kg/kg	1.47	1.46	1.50	1.53	0.05	0.10	0.45	0.66
ECM,[5] kg/d	34.6	34.6	35.0	36.3	1.60	0.02	0.15	0.77
ECM/DMI, kg/kg	1.58	1.58	1.61	1.64	0.06	0.09	0.53	0.76
Milk fat, %	4.14	3.90	4.14	4.06	0.13	0.95	0.37	0.06
Milk fat, kg/d	1.32	1.28	1.31	1.36	0.06	0.09	0.04	0.43
Milk true protein, %	2.98	2.99	3.06	3.03	0.05	0.06	0.39	0.08
Milk true protein, kg/d	0.95	0.97	0.98	1.02	0.05	0.02	0.62	0.42
Milk lactose, %	4.89	4.87	4.91	4.87	0.03	0.86	0.68	0.16
Milk lactose, kg/d	1.56	1.60	1.57	1.64	0.06	0.15	0.69	0.29
Milk N, % of N intake	28.6	28.9	28.5	28.8	1.00	1.00	1.00	0.61
MUN, mg/dL	12.4	13.0	13.2	13.5	0.89	0.14	0.83	0.88
PUN, mg/dL	12.5	12.0	11.6	13.8	1.06	0.27	0.08	0.46

[1]The RP-His used is a prototype supplement (Ajinomoto Co. Inc., Kawasaki-shi, Japan).
[2]Wk 2 = d 8 to 14 of periods 1 to 4.
[3]Orthogonal polynomials were used to test linear, quadratic, and cubic effects; significance was declared at $P \leq 0.05$ and trends at $0.05 < P \leq 0.10$.
[4]4% FCM = (0.4 × kg of milk) + (15 × kg of milk fat); Gaines and Davidson (1923).
[5]ECM = (0.327 × kg of milk) + (12.95 × kg of milk fat) + (7.65 × kg of milk protein); Tyrrell and Reid (1965).

Supplemental Table S2.7. Dry matter intake, milk yield, concentrations and yields of milk components, feed efficiency, concentration of plasma urea N (PUN), BW, and BCS in mid-lactation dairy cows fed incremental amounts of rumen protected-His (RP-His)[1] during wk 3 of each experimental period[2]

	RP-His					*P*-value[3]		
Item	0 g/d	82 g/d	164 g/d	246 g/d	SEM	Linear	Quadratic	Cubic
DMI, kg/d	21.4	21.5	21.6	21.9	0.59	0.30	0.85	0.88
Milk yield, kg/d	30.4	31.1	30.9	33.1	1.40	<0.01	0.17	0.17
Milk yield/DMI, kg/kg	1.41	1.44	1.45	1.53	0.06	<0.01	0.30	0.37
4% FCM,[4] kg/d	30.2	30.8	31.0	33.0	1.57	<0.01	0.23	0.44
4% FCM/DMI, kg/kg	1.40	1.43	1.45	1.51	0.05	<0.01	0.50	0.83
ECM,[5] kg/d	32.4	33.1	33.4	35.6	1.65	<0.01	0.24	0.43
ECM/DMI, kg/kg	1.50	1.53	1.56	1.63	0.05	<0.01	0.47	0.73
Milk fat, %	4.01	3.96	4.05	3.91	0.10	0.46	0.45	0.21
Milk fat, kg/d	1.20	1.22	1.24	1.32	0.07	<0.01	0.31	0.69
Milk true protein, %	2.98	3.00	3.03	2.98	0.04	0.77	0.09	0.36
Milk true protein, kg/d	0.90	0.93	0.93	1.00	0.04	<0.01	0.32	0.39
Milk lactose, %	4.83	4.81	4.84	4.84	0.03	0.50	0.60	0.45
Milk lactose, kg/d	1.47	1.50	1.50	1.62	0.06	<0.01	0.11	0.23
Milk N, % of N intake	27.8	27.9	28.0	28.6	1.00	0.15	0.55	0.79
MUN, mg/dL	11.9	12.1	12.2	12.6	0.84	0.22	0.71	0.78
PUN, mg/dL	10.9	11.3	11.4	11.5	0.65	0.31	0.79	0.94

[1]The RP-His used is a prototype supplement (Ajinomoto Co. Inc., Kawasaki-shi, Japan).
[2]Wk 3 = d 15 to 21 of periods 1 to 4.
[3]Orthogonal polynomials were used to test linear, quadratic, and cubic effects; significance was declared at $P \leq 0.05$ and trends at $0.05 < P \leq 0.10$.
[4]4% FCM = (0.4 × kg of milk) + (15 × kg of milk fat); Gaines and Davidson (1923).
[5]ECM = (0.327 × kg of milk) + (12.95 × kg of milk fat) + (7.65 × kg of milk protein); Tyrrell and Reid (1965).

CHAPTER III: INTERRELATIONSHIPS BETWEEN DIETARY STARCH LEVEL AND RUMEN-PROTECTED METHIONINE, LYSINE AND HISTIDINE ON MILK PRODUCTION AND NUTRIENT UTILIZATION IN DAIRY COWS FED METABOLIZABLE PROTEIN-DEFICIENT DIETS

ABSTRACT

Metabolizable protein (MP)-deficient diets have been shown to improve N utilization in dairy cows but may limit synthesis of milk and milk protein. Addition of rumen-protected (RP) Met, Lys, and His (MLH) and increased energy supply have been used independently to improve milk yield in dairy cows fed MP-deficient diets. Our objective was to investigate the interactions between starch level and RP-MLH on milk production and nutrient utilization when feeding MP-deficient diets. Sixteen multiparous Holstein cows were used in a replicated 4 × 4 Latin square with 2 × 2 factorial arrangement of treatments. Each period lasted 21 d with 14 d for diet adaptation and 7 d for data and sample collection. Dietary treatments were high-starch (HS), HS + RP-MLH, reduced-starch (RS), and RS + RP-MLH. The basal diets consisted (dry matter basis) of 35.7% corn silage, 14.7% haylage, and 49.6% concentrate. Dietary starch level varied by replacing 30% ground corn with 20% beet pulp and 10% soyhulls, and was 34.4 and 12.3% for HS and RS diets, respectively. Smartamine® M (25 g/d), AjiPro®-L (76 g/d), and an Ajinomoto prototype RP-His (110 g/d) were supplemented to meet digestible MLH requirements, respectively. Data were analyzed using the MIXED procedure of SAS. Compared with RS diets, feeding HS diets increased yields of milk (37.9 vs. 40.1 kg/d) and milk true protein (1.07 vs. 1.16 kg/d) and decreased dry matter intake (25.4 vs. 24.7 kg/d). Milk N efficiency were greater with feeding HS versus RS diets (29.2 vs. 25.4%, respectively). Both milk and plasma urea N decreased in cows fed HS than RS diets. Milk true protein content was improved by RP-MLH. Further, starch level × RP-MLH interactions were observed for plasma concentrations of Arg and Lys, with greater increases with RP-MLH in RS cows compared with HS cows. Replacing fibrous byproducts with ground corn reduced plasma concentrations of all essential AA except Met and Thr. Plasma His and Met were increased by RP-MLH. Apparent

total-tract digestibilities of neutral and acid detergent fiber, as well as urinary urea N excretion were lower for HS compared with RS diets. Daily enteric CH_4 production (434 vs. 545 g/d), CH_4 yield (17.7 vs. 21.6 g/kg of dry matter intake), and CH_4 intensity (10.7 vs. 13.6 g/kg of energy corrected milk) all decreased with feeding HS versus RS diets. Gross energy and digestible energy intakes increased and CH_4 energy decreased in cows fed HS versus RS diets. Although milk energy ouput tended to increase, milk energy efficiency expressed as a proportion of gross energy intake, decreased for HS versus RS cows. Supplementation with RP-MLH had no impact on energy utilization in dairy cows. Overall, elevated dietary starch level improved yields of milk and milk protein possibly by increased energy intake and mammary uptake of essential AA, and lower energy losses as CH_4.

Key words: energy balance, essential amino acid, ground corn, methane

INTRODUCTION

It is well known that milk N efficiency (i.e., milk N/N intake) is low in lactating dairy cows, and on average, only about 24.7% of dietary N was converted to milk protein when feeding typical North America diets (Huhtanen and Hristov, 2009). Dietary N not captured in milk protein is excreted in urine and feces, thus contributing to environmental pollution (Castillo et al., 2000). Additionally, N losses may shrink the profit margin of dairy producers due to costly protein sources. Broderick et al. (2009) and Lee et al. (2011) reported that MP-deficient diets improved milk N efficiency and reduced urinary N excretion, but also decreased yields of milk and milk protein. Accordingly, supplementation of MP-deficient diets with rumen-protected (**RP**)-AA or fermentable energy have been evaluated to enhance milk production without decreasing efficiency of dietary N use (Broderick, 2003; Rius et al., 2010b; Giallongo et al., 2016).

Despite the positive effects on N utilization (Broderick et al., 2009; Lee et al., 2011), MP-deficient diets may not be able to supply adequate EAA, particularly Met, Lys, and His (**MLH**; Bequette et al., 2000). Supplementation of RP-AA is a common strategy to alleviate shortages of EAA and can increase circulating MLH in the blood (Lee et al., 2012a; Giallongo et al., 2016). Elevated plasma concentrations of EAA stimulated their uptake by the mammary gland, which in turn improved yields of milk and milk protein (Doepel and Lapierre, 2010). Lee et al. (2012a) reported that yields of milk and milk protein increased in dairy cows fed a MP-deficient diet supplemented with RP-MLH. Giallongo et al. (2016) also observed a similar pattern on milk protein yield with feeding a MP-deficient diet plus RP-MLH. Additionally, enhanced supply of fermentable energy via starch-based sources is an alternative approach to mitigate milk yield losses in dairy cows fed MP-deficient diets. For instance, Boerman et al. (2015) observed that

yields of milk and milk protein increased when soyhulls were replaced with ground corn at 30% of diet DM. Similarly, yields of milk components, except milk fat, improved when dairy cows were offered a high-energy, low-protein diet compared with a low-energy, low-protein counterpart (Rius et al., 2010b).

Therefore, it is conceivable that increased supply of ruminally fermentable energy could synergistically interact with RP-MLH to improve milk yield and N utilization in dairy cows fed MP-deficient diets. Elevated supply of ruminally fermentable energy can be achieved by replacing fibrous byproducts with ground corn. Although numerous studies in which fibrous byproducts replaced cereal grains have been published (Ipharraguerre and Clark, 2003; Fredin et al., 2015a,b), there is scarce information regarding how different dietary proportions of ground corn and fibrous byproducts affect energy utilization and enteric CH_4 emissions in MP-deficient diets. Dairy practices account for around 4% of total greenhouse gas emissions and about 25% of enteric CH_4 in the United States (Chase, 2014; FAO, 2019; USEPA, 2019). The global warming potential of CH_4 is 28-36 times greater than that of CO_2 (USEPA, 2020), and CH_4 production also represents 2-12% dietary energy losses (Johnson and Johnson, 1995; Niu et al., 2018), thus justifying the need to mitigate CH_4 emissions in dairy cows.

To our knowledge, the potential interactions between dietary starch level (**SL**) and RP-MLH on production and nutrient utilization have not been evaluated to date. We hypothesized that a high-starch, low-protein diet supplemented with RP-MLH would improve milk production and nutrient utilization in dairy cows. Our objective was to investigate the interrelationships between dietary SL, varied by replacing beet pulp and soyhulls with ground corn, and RP-MLH supplementation on yields of milk and milk components, plasma AA concentrations, apparent

total-tract digestibility of nutrients, and energy and N utilization in dairy cows fed MP-deficient diets.

MATERIALS AND METHODS

All experimental procedures were approved by the Institutional Animal Care and Use Committee (protocol no. 180305) of the University of New Hampshire (Durham). The experiment was conducted at the University of New Hampshire Fairchild Dairy Teaching and Research Center (Durham) from June 11 to August 26, 2018.

Cows, Experimental Design, and Treatments

Sixteen multiparous Holstein cows averaging (mean ± SD) 138 ± 46 DIM, 46 ± 6 kg/d of milk, and 700 ± 55 kg of BW were selected at the beginning of the study. Animals were housed in a tie-stall barn equipped with water bowels for free access to water and feed tubs for individual feeding. Cows were milked twice a day at 0530 and 1630 h, and milk yield was recorded automatically at each milking throughout the experiment. Animals were weighed (Northeast Scale Co. In., Hooksett, NH) immediately after the afternoon milking during 3 consecutive days before the beginning of the study and at the end of each period to compute BW change. Body condition score was assigned by 3 trained investigators before the start of the experiment and on the last day of each period following the procedures outlined by Wildman et al. (1982).

Cows were blocked by milk yield and randomly assigned to treatment sequences in a replicated 4 × 4 Latin square design with a 2 × 2 factorial arrangement of treatments. Squares were balanced for potential first-order carryover effects in subsequent periods as each treatment immediately preceded and followed every other exactly once in each square (Williams, 1949;

Kim and Stein, 2009). Each experimental period lasted 21 d, including 14 d for diet adaptation and 7 d for data and sample collection. Dietary treatments were: (1) high-starch diet (**HS**); (2) HS + RP-MLH (**HS/MLH**); (3) reduced-starch diet (**RS**); and (4) RS + RP-MLH (**RS/MLH**). The basal diets were formulated to meet the nutrient requirements, except MP, of a lactating dairy cow averaging 700 kg of BW, 138 DIM, 42 kg/d of milk, 3.5% of milk fat, 3% of milk true protein, 4.8% of milk lactose, and 25 kg/d of DMI using the NRC (2001) evaluation software (v.1.1.9), and contained (DM basis) 35.7% corn silage, 14.7% mixed-mostly grass-legume haylage, and 49.6% concentrate. Dietary SL varied by replacing 30% ground corn with 20% beet pulp and 10% soyhulls. The RP-MLH supplements were top-dressed on TMR to meet the requirements of digestible MLH (Schwab et al., 2005). The amounts of RP-Met (Smartamine® M; Adisseo USA Inc., Alpharetta, GA), RP-Lys (Aji-Pro L; Ajinomoto Heartland Inc., Eddyville, IA), and RP-His (Ajinomoto prototype supplement; Ajinomoto Co. Inc., Kawasaki-shi, Japan) fed were 25, 76, and 110 g/d, respectively. The RP-Met, RP-Lys, and RP-His supplements contained 75% DL-Met with 80% bioavailability (Chirgwin et al., 2015), 40% Lys with 54% bioavailability (Giallongo et al., 2016), and 44% His with 14% bioavailability (according to the manufacturer), respectively. These RP-AA supplements (Smartamine® M, Aji-Pro L, and Ajinomoto prototype product) were expected to provide 15, 16, and 6.78 g/d of digestible Met, Lys, and His, respectively.

Dietary ingredients were mixed and offered as TMR twice daily at 0600 and 1700 h using a Super Data Ranger mixer (American Calan Inc., Northwood, NH). Orts were collected and weighed once daily before the afternoon feeding. Feed offered was adjusted daily to achieve 5 to 10% orts. Feed intake was recorded throughout the experiment.

Feed Sampling and Analyses

Samples of corn silage, mixed mostly grass-legume haylage, TMR, and orts were collected thrice weekly and composited by week. The composite samples were dried (55°C, 48 h) in a forced-air oven (VWR Scientific, Radnor, PA) for determination of DM to adjust the TMR on an as-fed basis and to calculate DMI. Samples of forages, concentrates (i.e., ground corn, beet pulp, soyhulls, soybean meal, canola meal, corn dried distillers grains with solubles, and urea), TMR, and orts were collected thrice during the sampling week of each period and composited by week. Weekly ingredients, TMR, and orts were lyophilized for 48 h (Labconco Inc., Kansas City, MO), ground with a Wiley mill (A. H. Thomas Co., Swedesboro, NJ) to pass through a 1-mm screen, and stored in air-tight glass jars until nutritional analysis.

Lyophilized and ground samples of dietary ingredients were shipped to Dairy One Cooperative Inc. (Ithaca, NY) and analyzed for DM, CP, soluble protein, aNDF, ADF, ADL, starch, ether extract, ash, and individual minerals (Ca, P, Mg, K, Na, S, Fe, Zn, Cu, Mn, and Mo) using the procedures described by Dairy One Cooperative Inc. (https://dairyone.com/download/forage-forage-lab-analytical-procedures; Accessed Mar. 18, 2020). In addition, TMR and orts were analyzed for CP, aNDF, ADF, and ash at Dairy One Cooperative Inc. laboratory. Samples of dietary ingredients were further ground (Wiley mill; A. H. Thomas Co.) to pass through a 0.5-mm screen and used for determination of AA by cation exchange chromatography (cIEC-HPLC) coupled with postcolumn ninhydrin derivatization with norleucine as the internal standard (method 982.30; AOAC International, 2016) at the University of Missouri Agricultural Experiment Station Chemical Laboratory (Columbia, MO). Tryptophan was determined after alkaline hydrolysis and sulfur AA were analyzed after performic acid oxidation (method 988.15; AOAC International, 2016). Gross energy of TMR samples was

determined using an adiabatic oxygen bomb calorimeter (model 1241; Parr Instrument Co., Moline, IL).

Milk and Blood Sampling and Analyses

Milk samples were collected using automatic samplers during 4 consecutive milkings starting in the p.m. milking of d 15 of each period. Milk samples were transferred into tubes preserved with 2-bromo-2-nitropropane-1,3 diol (Broad Spectrum Microtabs II; Advanced Instruments Inc., Norwood, MA) and stored at 4°C, before being transported overnight to Dairy One Cooperative Inc. laboratory. At the laboratory, milk samples were analyzed for concentrations of fat, true protein, lactose, and MUN by Fourier transform infrared spectroscopy using a MilkoScan FT+ (Foss Inc., Hillerød, Denmark).

Blood samples were collected into vacutainer 15% EDTA tubes (Monoject, Mansfield, MA) from coccygeal vessels approximately 4 h after the morning feeding on d 16 and 17 of each period. Tubes were immediately placed in a chill bucket with beads (Chemglass Life Sciences, Vineland, NJ) and transported to the laboratory for centrifugation (2,155 × g, 20 min, 4ºC) using an Eppendorf centrifuge (model 5810; Eppendorf, Hamburg, Germany). Plasma samples were composited by cow and period, and the composite samples were used to determine the concentrations of AA, His-containing metabolites, and urea N (**PUN**) at Ajinomoto Co. Inc. using a High-Speed AA analyzer L-8900 (Hitachi High-Technologies Co., Tokyo, Japan) following the procedures stated by the manufacturer (https://www.hitachi-hightech.com/us/library/literature/brochure-l-8900-amino-acid-analyzer.html; Accessed March 18, 2020). Codified plasma samples were sent to Ajinomoto Co. Inc. to blind treatments identity.

Fecal and Urinary Sampling and Analyses

Fecal grab samples were taken directly from the rectum or during voluntary defecation at 8 time points (0600 h and 1500 h on d 18; 0900 h, 1200 h, and 1800 h on d 19; and 0000 h and 0300 h and 2100 h on d 20) during the sampling week of each period. Fecal samples (~200 g/sampling) were collected into 100-mL specimen containers and transferred into 4-L plastic bags to generate composited samples by cow per period. Next, samples were dried in a forced-air oven (VWR Scientific) at 55°C for approximately 72 h and ground (Wiley mill; A. H. Thomas Co.) to pass through a 1-mm screen. Fecal samples were analyzed for DM, CP, aNDF, ADF, and ash at Dairy One Cooperative Inc. as done for dietary ingredients and TMR. Triplicate samples (~0.5 g) of feces, TMR, and orts were weighed into Ankom F57 bags (25 µm pore size; Ankom Technology, Macedon, NY), placed in a larger laundry nylon bag, and inserted in the rumen of 1 ruminally cannulated late-lactation Holstein cow fed a corn silage and grass silage-based diet with a forage-to-concentrate ratio of 50:50 for 12 d. After removal from the rumen, bags were rinsed with tap water and analyzed in-house for aNDF using an Ankom^{2000} fiber analyzer (Ankom Technology). Indigestible NDF was used as the internal marker to estimate fecal output of DM and apparent total-tract digestibility of nutrients (Cochran et al., 1986; Huhtanen et al., 1994). Gross energy of fecal samples was determined using an adiabatic oxygen bomb calorimeter (model 1241; Parr Instrument Co.).

Spot urine samples were collected concurrently with fecal samples into 100-mL specimen containers through stimulation of the pudendal nerve by massaging the area below the vulva or during voluntary urination. After each sampling, 1 mL of urine was pipetted into 50-mL centrifuge tubes containing 32 mL of 0.072 *N* H_2SO_4 to obtain composited urine samples by cow per period. Urine samples were stored at −20°C until analyses of nitrogenous metabolites. After

thawing at room temperature, samples were analyzed for concentrations of creatinine (assay kit no. 500701, Cayman Chemical Co., Ann Arbor, MI) using a chromate microplate reader set at a wavelength of 492 nm (Awareness Technology Inc., Palm City, FL), allantoin (Chen et al., 1992), uric acid (assay kit no. 1045–225; Stanbio Laboratory, Boerne, TX), urea N (Stanbio Urea Nitrogen Kit 580; Stanbio Laboratory Inc.), and total N (micro-Kjeldahl analysis, AOAC, 1990; Dairy One Cooperative Inc.). Allantoin, uric acid, and urea N were determined at wavelengths of 522, 520, and 520 nm, respectively, in a UV/visible spectrophotometer (Beckman Coulter Inc., Pasadena, CA). Daily urine volume was estimated from urinary creatinine concentration assuming a constant creatinine excretion rate of 29 mg/kg of BW (Valadares et al., 1999). Urinary excretion of urea N, total N, allantoin, uric acid, and purine derivatives (allantoin + uric acid) were calculated by multiplying the concentration of each of these metabolites by the urinary volume.

Measurements of Gaseous Fluxes

Emissions of CO_2 and enteric CH_4 were measured at 8 timepoints (0200 h and 1400 h on d 15; 0500 h and 1700 h on d 16; 0800 h and 2000 h on d 17; and 1100 h and 2300 h on d 18) to account for diurnal variation in gaseous fluxes using the GreenFeed system (C-Lock Inc., Rapid City, SD) during the sampling week of each period (Harper et al., 2017). The GreenFeed unit was placed in front of each cow for approximately 5 min to sample breath gases and then moved to the alley for 2 min to sample background gases. The unit was moved from cow to cow in a sequential manner. About 2 weeks prior to the beginning of the study, cows were trained to have access to the GreenFeed unit by using a bait feed (Hi-Line 16% Dairy/Beef Pellet, Poulin Grain Inc., Newport, VT). The bait feed was a pelleted product, and contained 16% CP, 6.2% crude

fiber, 31% NFC, 19% starch, and 3.9% ether extract. Approximately 25 g of pellets were dropped from the dispenser of the GreenFeed unit every 15 sec, resulting in a total consumption of ~0.5 kg of pellets per sampling (20 drops in total). One cow refused to consistently access the unit and her data were not included in the statistical analyses. Data were averaged by cow per period. A complete description of the gaseous sampling protocol and calculations used herein has been reported elsewhere (Dorich et al., 2015).

Calculations and Statistical Analyses

Yields of milk components were calculated using milk yield and concentrations of milk components at each milking, summed for daily total, and averaged by period. Digestible energy (**DE**) was calculated by subtracting fecal energy from GE, and ME was estimated as the sum of heat production (**HP**), milk energy output, and tissue energy balance (NRC, 2001). Urinary energy was calculated as 6.5% of estimated ME according to Ferris et al. (1999) and Ferrell and Oltjen (2008). Energy losses as CH_4 was calculated by multiplying CH4 emissions (g/d) by a constant of 0.0132 Mcal/g (Judy et al., 2019). Heat production was calculated using the equation reported by Bayat et al. (2019): HP (Mcal/d) = [0.0184 × CO2 (L/d) + 7.50] ÷ 4.184. According to NRC (2001), milk energy output was calculated using the equation: milk energy (Mcal/d) = [(0.0929 × milk fat%) + (0.0563 × milk true protein%) + (0.0395 × milk lactose%)] × milk yield (kg/d), and tissue energy balance was estimated using the equation: tissue energy (Mcal/d) = (body fat% × 9.4 + body protein% × 5.55) × BW change (kg/d), in which body fat% and body protein% were determined by BCS of cows when measurements were taken.

Data were analyzed using the MIXED procedure of SAS (version 9.4; SAS Institute Inc., Cary, NC) according to a replicated 4 × 4 Latin square design with a 2 × 2 factorial arrangement of treatments. The following model was used:

$$Y_{ijklm} = \mu + S_i + C_{j(i)} + P_k + SL_l + MLH_m + SL_l \times MLH_m + e_{ijklm}$$

where, Y_{ijklm} = dependent variable, μ = overall mean, S_i = fixed effect of square (i = 1 to 4), $C_{j(i)}$ = random effect of cow nested within square, P_k = fixed effect of period (k = 1 to 4), SL_l = fixed effect of SL (l = HS or RS), MLH_m = fixed effect of supplemental RP-MLH (m = yes or no), $SL_l \times MLH_m$ = interaction between dietary SL and supplemental RP-MLH, and e_{ijklm} = residual error. Normality of residuals was checked with normal probability and box plots and homogeneity of variances with plots of residual versus predicted values. Outliers were removed from statistical analyses when studentized residuals were >3.0 or < −3.0. All results were expressed as least squares means and standard errors. Significance was declared at $P \leq 0.05$ and trends at $0.05 < P \leq 0.10$. The PDIFF option of SAS was used for mean comparisons when interaction between SL and RP-MLH was significant.

RESULTS

The nutritional composition and AA profile (% of CP) of the dietary ingredients are presented in Tables 3.1 and 3.2, respectively, while the ingredient and nutritional composition of the basal diets are listed in Table 3.3. The basal diets contained (DM basis) 35.7% corn silage, 14.7% mixed mostly grass-legume haylage, and 49.6% concentrate. Specifically, the HS basal diet was obtained by replacing 20% beet pulp and 10% soyhulls in the RS basal diet with 30% ground corn. The CP concentration was slightly lower for the HS basal diet compared with the RS basal diet (16.4 vs. 16.0%). Substituting beet pulp and soyhulls with ground corn decreased

the dietary concentrations of aNDF and ADF and did not change the proportion of forage NDF. As expected, the HS basal diet had greater levels of NFC, starch, and NE_L than the RS basal diet (47%, 34.4%, and 1.68 Mcal/kg vs. 35.2%, 12.3%, and 1.59 Mcal/kg, respectively).

The NRC (2001) evaluation of the dietary treatments is shown in Table 3.4. The NE_L and MP balances averaged 1.4 Mcal/d and −158 g/d for the HS diets (HS and HS/MLH), and 0.3 Mcal/d and −80 g/d for RS diets (RS and RS/MLH), respectively. The MP deficiency ranged from −180 g/d for cows fed the HS diet without RP-MLH to −53 g/d for those fed the RS diet with RP-MLH. All 4 dietary treatments provided adequate RDP (on average, ~4% above the requirements), whereas dietary RUP balance varied from 5 to 14% below the requirements and displayed a pattern like that observed for the MP balance. Deficiencies of digestible His, Met, and Lys were observed for the experimental diets without RP-MLH supplementation (HS and RS) and the balances of digestible His, Met, and Lys became positive with supplementing RP-MLH.

Intake and Milk Yield and Composition

Dry matter intake, milk yield and composition, feed efficiency, BW, BCS, and concentrations of PUN and blood hemoglobin are shown in Table 3.5. There were no significant interactions between dietary SL and RP-MLH supplementation for production and blood parameters. However, a trend ($P = 0.08$) for a SL × MLH effect on milk true protein percentage was observed.

Cows fed the HS diets had lower DMI (24.7 vs. 25.4 kg/d) and greater milk yield (40.1 vs. 37.9 kg/d) than those offered the RS counterparts. Consequently, feed efficiency, expressed as milk yield/DMI, improved with feeding the HS vs. RS diets (1.64 vs. 1.50 kg/kg). Yield of 4%

FCM averaged 37.9 kg/d and was not affected by dietary starch concentration. However, ECM yield showed a trend ($P = 0.09$) to increase for the HS diets relative to the RS diets. Feed efficiencies, expressed as 4% FCM yield/DMI (1.56 vs. 1.49 kg/kg) or ECM yield/DMI (1.68 vs. 1.58 kg/kg), were higher for the HS diets than for the RS diets. Compared with RS cows, HS cows had lower milk fat percentage but higher percentages and yields of milk true protein and lactose. Milk N efficiency was greater for HS cows than for RS cows (29.2 vs. 25.4%). Both MUN (11.0 vs. 12.6 mg/dL) and PUN (11.6 vs. 13.3 mg/dL) concentrations were lower for the HS diets relative to the RS diets. Blood hemoglobin concentration, BCS, BW, and BW gain were not affected by dietary SL.

Supplementation of RP-MLH increased milk true protein percentage (2.84 vs. 2.91%) but decreased milk lactose percentage (4.96 vs. 4.88%). There was a trend ($P = 0.06$) for greater BW gain in association with supplemental RP-MLH. However, supplementation of the MP-deficient diets with RP-MLH had no effects on DMI, feed efficiencies, yields of milk and milk components, and milk fat percentage (Table 3.5).

Plasma AA and His-containing Metabolites

Plasma concentrations of EAA, NEAA and His-containing metabolites (i.e., carnosine and 3-methyl-His) are shown in Table 3.6. Significant interactions between SL and RP-MLH were observed for plasma Arg and Lys concentrations, which increased with RP-MLH in RS cows but were not changed in HS cows. The plasma concentrations of all EAA, except Met and Thr decreased with feeding the HS vs. RS diets. As a result, HS cows had lower plasma concentration of total EAA than RS cows. Moreover, supplemental RP-MLH improved plasma

His and Met concentrations by 32.5% and 69.3%, respectively. The plasma concentration of total EAA was higher for RP-MLH as compared with no RP-MLH supplementation.

As for plasma NEAA, there was a trend ($P = 0.06$) for a SL × RP-MLH interaction on plasma Asn concentration, with an increase in response to RP-MLH in RS cows and no change in HS cows. Additionally, higher dietary SL increased the plasma concentrations of Asn, Gly, Pro and Tau, and decreased that of Tyr. There was a trend ($P = 0.06$) to increase the plasma concentration of Asp with feeding the HS vs. RS diets. Replacing beet pulp and soyhulls with ground corn tended ($P = 0.06$) to increase the plasma concentration of NEAA. The plasma concentration of Tau significantly increased by supplementation of RP-MLH. Further, dietary SL did not influence the plasma concentrations of carnosine and 3-methyl-His; however, supplemental RP-MLH reduced the plasma concentration of 3-methyl-His.

Nutrient Digestibility and Urinary N Excretion

Apparent total-tract digestibility of nutrients and urinary excretion of nitrogenous metabolites are presented in Table 3.7. There were no significant SL × MLH interactions, as well as RP-MLH effects for apparent total-tract digestibility of nutrients and urinary excretion of nitrogenous compounds. In addition, digestibilities of DM (mean = 70.1%), OM (mean = 71.5%), and CP (mean = 68.6%) were not altered by dietary SL. In contrast, digestibilities of NDF (43.2 vs. 58.9%) and ADF (47.6 vs. 62.4%) were lower in cows fed the HS diets than those fed the RS diets.

Higher SL reduced urinary excretion of urea N, expressed as g/d, or as a proportion of total urinary N output or N intake, and tended ($P = 0.06$) to decrease urinary excretion of uric acid. However, dietary SL had no impact on volume (mean = 37.5 L/d), and urinary excretions of

creatinine (mean = 5.12 m*M*), total-N (mean = 316 g/d), allantoin (mean = 594 mmol/d), and purine derivatives (mean = 688 mmol/d).

Gaseous Emissions

Emissions of CO_2 and CH_4 are presented in Table 3.8. No significant dietary SL × RP-MLH interactions, and supplemental RP-MLH effects on these gas emission parameters were observed. Although dietary starch concentration did not change CO_2 emission (mean = 12.0 kg/d), substitution of beet pulp and soyhulls with ground corn reduced daily CH_4 production (545 vs. 434 g/d), yield (21.6 vs. 17.7 g/kg of DMI), and intensity (13.6 vs. 10.7 g/kg of ECM) by 20, 18, and 21%, respectively.

Dietary Energy Utilization

Dietary energy utilization and milk energy efficiency are presented in Table 3.9. We did not find any significant SL × RP-MLH interactions and supplemental RP-MLH effects for variables related to energy partitioning. However, intakes of GE (115 vs. 102 Mcal/d) and DE (86.4 vs. 74.5 Mcal/d) were higher for the HS diets compared with RS diets. Energy losses as enteric CH_4 emission was reduced in dairy cows consuming the HS vs. RS diets (6.13 vs. 7.71 Mcal/d). Additionally, DE expressed as a proportion of GE tended ($P = 0.08$) to be higher in HS cows relative to RS cows. When ME, urinary energy, CH_4 energy, and HP were expressed as a percentage of GE, all of them decreased in cows fed the HS vs. RS diets. A trend ($P = 0.08$) was observed for fecal energy (expressed as % of GE) to decrease with feeding the HS vs. RS diets. Milk energy efficiency, expressed as a proportion of GE intake, was reduced when cows were

fed the HS vs. RS diets (25.3 vs. 27.6%). Moreover, supplementation of the MP-deficient diets with RP-MLH tended ($P = 0.07$) to enhance energy stored in tissues.

DISCUSSION

It has been clearly demonstrated that low-protein or MP-deficient diets can improve efficiency of N utilization, and reduce N excretion to the environment (Castillo et al., 2000; Olmos Colmenero and Broderick, 2006). However, MP-deficient diets often limit production performance of dairy cattle, especially in high-producing cows. Previous work has found that supplementation of MP-deficient diets with ruminally fermentable energy (e.g., starch) or RP-MLH sustained to some extent milk yield in dairy cows (Broderick, 2003; Lee et al., 2012a). Nevertheless, the effects of varying dietary starch concentrations and supplemental RP-MLH in regulating production performance and nutrient utilization have not been examined yet in lactating dairy cows fed MP-deficient diets.

In the present study, based on the NRC (2001) evaluation, all dietary treatments (HS, HS/MLH, RS, and RS/MLH) provided insufficient MP (2-7% deficiency) and RUP (5-14% deficiency), and the HS diets had higher dietary SL (34.4 vs. 12.3%), NE_L concentration (1.68 vs. 1.59 Mcal/kg), and NE_L balance (1.4 vs. 0.3 Mcal/d) relative to the RS diets. Also, the amounts of RP-MLH supplements were determined to meet digestible MLH requirements in dairy cows (Schwab et al., 2005). The lack of interactions between dietary SL and RP-MLH supplementation for most response variables in our study illustrated that elevated supply of fermentable energy via corn starch did not optimize utilization of digestible Met, Lys, and His provided by RP-MLH toward milk protein synthesis.

Intake, Milk Yield, and PUN

We observed that elevated dietary SL achieved by replacing beet pulp and soyhulls with ground corn reduced DMI in lactating dairy cows fed MP-deficient diets. Conversely, Ranathunga et al. (2010) and Boerman et al. (2015) reported that substitution of fibrous byproducts (e.g., soyhulls) with ground corn enhanced DMI, which could be explained by decreased rumen fill. In the present study, the ruminal physical fill likely did not play a major role in limiting DMI due to the low inclusion of soyhulls (10%, DM basis) in the RS diets. Also, the energy requirements of dairy cows could be met by consuming lower amounts of the HS diets with higher NE_L density (NRC, 2001), and according to Allen (2000), propionate is more effective to depress DMI relative to acetate. Although the HS diets reduced DMI by −0.7 kg/d, milk yield improved by 2.2 kg/d with feeding the HS diets compared with the RS diets. In support, previous studies have demonstrated that milk yield increased with feeding more starch accomplished either by decreasing dietary forage-to-concentrate ratio or replacing byproducts with corn grain (Broderick, 2003; Rius et al., 2010b). When feeding high-starch diets, more ruminal propionate was produced to support gluconeogenesis in the liver and mammary synthesis of lactose, the osmotic regulator of milk volume (Oba and Allen, 2003). The observed increases in feed efficiency (i.e., milk yield, 4% FCM yield, and ECM yield per unit of DMI) with feeding the HS vs. RS diets resulted from increased milk yield and decreased DMI.

In the present study, milk fat concentration decreased from 4.01 to 3.69% in response to substitution of beet pulp and soyhulls with ground corn. In comparison, there was a linear decrease in milk fat concentration with incremental substitution of soyhulls and cottonseed hulls with ground corn (Beckman and Weiss, 2005). Reduced milk fat concentration is commonly observed when dietary NDF was replaced with starch (Erdman, 1988; Batajoo and Shaver,

1994). Feeding starch at the expense of non-forage NDF reduced the ruminal acetate-to-propionate ratio, which in turn decreased milk fat synthesis in the mammary gland (Erdman, 1988). In our study, milk fat yield was not different between the HS and RS diets, possibly caused by enhanced milk yield in HS cows. Similarly, Beckman and Weiss (2005) and Ranathunga et al. (2010) did not observe any changes in milk fat yield in response to incremental substitution of non-forage fiber sources (i.e., soyhulls, cottonseed hulls, and dried corn distillers grains with solubles) with starch provided by ground corn. Moreover, elevated dietary SL increased both concentration and yield of milk true protein in the current study. Consistent with our results, Boerman et al. (2015) reported that milk protein concentration and yield increased when dairy cows were fed 30% ground corn in place of 30% soyhulls on a DM basis. It may due to the fact that corn starch is more ruminal digestible than non-forage NDF and can provide more energy to capture more ammonia N for microbial protein synthesis (NRC, 2001; Voelker and Allen, 2003b). Notably, milk true protein concentration (mean = 2.87%) in our study was lower than the average milk protein content (3.11%) in Holstein cows (CDCB, 2018), which could be explained by deficiencies of MP in the diets. In support, milk true protein concentration was 2.94% in dairy cows fed a MP-deficient diet (Lee et al., 2012a). Compared with the RS diets, feeding the HS diets improved milk lactose concentration and yield, which may be attributed to increased ruminal propionate production achieved by ruminal starch degradation (Oba and Allen, 2003). In support, Boerman et al. (2015) reported that milk lactose concentration was greater for the diet with 30% ground corn vs. that with 30% soyhulls.

Milk urea N and PUN have been used as indicators of N use efficiency in dairy cows (Eastridge, 2006). Compared with the RS diets, the HS diets reduced MUN and PUN concentrations by 14% and 15%, respectively. The decline in MUN with feeding a HS diet has

been also observed by Dann et al. (2014), in which wheat middlings, dried corn distillers grains with solubles, and beet pulp were replaced by ground corn. Milk N efficiency was 15% greater in HS cows than in RS cows, suggesting that elevated dietary SL is able to improve efficiency of N utilization in dairy cows. The mechanism by which addition of starch promotes N efficiency is that starch can provide more fermentable energy than fibrous byproducts, and capable of capturing ruminal ammonia N for more microbial protein synthesis (Oba and Allen, 2003).

Regarding supplementation with RP-MLH, we observed no changes in DMI, milk yield and feed efficiencies. Likewise, Lee et al. (2015) and Giallongo et al. (2016) did not report any significant effects of RP-AA on these parameters in dairy cows fed MP-deficient diets (10 and 2% deficiency, respectively); however, an earlier study by Lee et al. (2012a) revealed that RP-MLH improved milk yield when feeding a MP-deficient diet (13% deficiency) to dairy cows. The outcomes suggest that supplementing RP-MLH to the diets with ≤ 10% MP deficiency has limited effect on promoting feed intake and milk yield in dairy cows. Moreover, yields of milk components were not modified by RP-MLH in the present study. In contrast, previous research has showed that supplementation with RP-MLH enhanced yields of milk fat (Giallongo et al., 2016), milk protein (Lee et al., 2012; Giallongo et al., 2016), and milk lactose (Lee et al., 2012). Our diets were less MP deficient than those reported by Lee et al. (2012) and Giallongo et al. (2016), which may result in minimal responses of milk components to supplemental RP-MLH. However, milk true protein concentration increased from 2.84 to 2.91% with RP-MLH, which was similar to previous results (Giallongo et al., 2015; Giallongo et al., 2016). It has been widely established that supplementation of RP-Met, RP-Lys, or RP-His is able to improve milk protein concentration when dairy cows were fed MP-deficient diets (Lee et al., 2012b; Zang et al., 2019).

Plasma Concentration of AA

Supplementation of RP-MLH increased the plasma concentrations of Arg and Lys in RS cows but not in HS cows, which can be explained by reduced mammary AA uptake, lower AA requirements, or both in RS cows. Substitution of beet pulp and soyhulls with ground corn reduced all circulating EAA except Met and Thr, possibly because greater dietary energy supply led to increased extraction of circulating EAA by the mammary gland for milk protein synthesis (Rulquin et al., 2004). Rius et al. (2010a) demonstrated that abomasal infusion of starch decreased arterial concentrations of Ile, His, Leu, Lys, and Phe in lactating dairy cows. The plasma concentrations of His and Met increased with supplemental RP-MLH, indicating that supplemental RP-MLH were able to provide digestible MLH effectively. Both Lee et al. (2012a) and Giallongo et al. (2016) demonstrated that supplementation with RP-MLH increased the plasma concentrations of Met, Lys, and His.

We detected that increased dietary SL improved the plasma concentrations of Gly, Pro, and Tau but decreased those of Asn and Tyr. There was a trend for increased plasma Asp in association with elevated dietary starch concentration. Hurtaud et al. (1998) demonstrated that incremental amounts of abomasally infused glucose increased plasma Gly concentration in a linear fashion without effects on other NEAA (Hurtaud et al., 1998). Although we observed increased plasma Tau concentration for supplemental RP-MLH, others did not report any change in this AA in response to RP-MLH (Lee et al., 2012a; Giallongo et al., 2016). A trend for increased plasma concentration of total NEAA with feeding the HS diets may be related to reduced mammary extraction of NEAA in HS cows (Bequette et al., 2000). According to Bequette et al. (2000), the capacity of the mammary gland to extract His increased by 43-fold but to extract other AA decreased by 2- to 3-fold in dairy cows fed a His-deficient diet. Reduced

plasma concentration of total AA concentration with the HS diets was mainly caused by decreased plasma concentration of total EAA. Moreover, our results indicated that supplementation of the MP-deficient diets with RP-MLH had limited effect on the metabolism of NEAA but improved the availability of total AA in the blood due to the increases in His, Met and Tau.

We did not observe any RP-MLH effect on the plasma concentration of carnosine, similar to the results of Lee et al. (2012a). Conversely, plasma carnosine concentration increased linearly with incremental amounts of RP-His (Zang et al., 2019). Digestible His from RP-His in our study may be mainly used for milk protein synthesis rather than converted to carnosine due to the higher milk true protein yield compared with that in Zang et al. (2019). Blood hemoglobin, as well as intramuscular carnosine (β-alanyl-l-His) and anserine (β-alanyl-N-methylHis) have been considered endogenous His pools, which can be degraded to supply His during deficiency (Lapierre et al., 2008). However, we did not observe any changes in blood hemoglobin when feeding more RP-His to dairy cows.

Nutrient Digestibility and Urine Excretion of Nitrogenous Metabolites

Dietary SL did not influence the apparent total-tract digestibilities of DM, OM, and CP. Consistent with our results, Voelker and Allen (2003a) reported that replacement of high-moisture corn with 24% beet pulp had no effects on apparent total-tract digestibilities of DM and OM. However, the apparent total-tract NDF and ADF digestibilities were promoted by 36 and 31% when dairy cows were fed the RS vs. HS diets, which may be related to the high fiber digestibility of beet pulp and soyhulls. Our NDF digestibility results corroborate those reported by Boerman et al. (2015), in which NDF digestibility was higher for the diet containing 30%

soyhulls vs. 30% ground corn. Compared with RS cows, the apparent total-tract digestibilities of OM and CP were similar and that of NDF reduced by 27% in HS cows, indicating that the starch digestibility was likely increased with feeding the HS diets.

Reduced urinary urea N excretion (g/d, % of total urinary N, and % of N intake) with feeding the HS vs. RS diets in our study probably contributed to higher capture of ruminal ammonia N by starch and less ammonia N for urea formulation in the liver (Oba and Allen, 2003), which was also supported by lower MUN and PUN concentrations and greater milk N efficiency in HS cows. We did not find any difference in urinary excretion of purine derivative between the HS and RS diets, suggesting that microbial protein synthesis may not be altered by dietary SL. According to González-Ronquillo et al. (2004), urinary excretion of purine derivatives can be effective to reflect changes in rumen microbial protein synthesis. The discrepancy may be related to the inaccurately estimated urine volume derived from the spot sampling technique. There was a trend for reduced urinary uric acid excretion in association with elevated dietary SL, and the mechanism behind it is still not fully understood.

Gaseous Production and Energy Utilization

As discussed above, we observed feeding the RS diets reduced yields of milk and milk protein and elevated urinary urea N excretion. Another issue related to replacing ground corn with fibrous byproducts is to increase CH_4 emissions and decrease energy utilization in dairy cows. The positive relationships between CH_4 emissions and CH_4 energy output, and dietary NDF have been established by Nielsen et al. (2013). In our study, CH_4 production (g/d), CH_4 yield (g/kg of DMI), and CH_4 intensity (g/kg of ECM) increased in dairy cows receiving the RS vs. HS diets. Similar to our results, Aguerre et al. (2011) reported that linear increases in these

same enteric CH_4 variables when lactating dairy cows were fed diets with increasing forage-to-concentrate ratio and starch concentration ranging from 20 to 29% on a DM basis. Pirondini et al. (2015) reported a trend for increased CH_4 production with replacement of ground corn with 6.5% soyhulls (dietary starch content, 28 vs. 23.8%).

Despite increased GE and DE intakes in HS vs. RS cows, there were no treatment differences in ME intake, indicating that the HS diets may lead to more energy in urine and CH_4, which were not supported by our results. The increase in CH_4 emission resulted in more energy losses as CH_4 when dairy cows receiving the RS vs. HS diets, which is further confirmed the need to reduce CH_4 production from dairy cows when feeding diets containing fibrous byproducts. According to Coppock (1985), dietary energy can be lost via feces, urine, CH_4, and heat, of which fecal energy and HP individually account for 30 to 35%. Increased fecal energy, urinary energy, and HP, expressed as a proportion of GE, with feeding the RS diets resulted from similar values of these variables and lower GE in RS cows. Compared with HS cows, milk energy efficiency was improved in RS cows, even milk energy output tended to be lower for RS cows. It was attributed to the big difference in GE between HS and RS groups (115 vs. 102 Mcal/d). Our study demonstrated that RP-MLH had no significant impact on modify energy partitioning of lactating dairy cows.

CONCLUSIONS

Increased dietary SL achieved by replacing fibrous byproducts with ground corn improved milk and milk true protein yields of dairy cows fed MP-deficient diets. Compared with the RS diets, improved production for the HS diets may be caused by enhanced mammary uptake of EAA, which may support by the decreases in plasma EAA. Substituting beet pulp and

soyhulls with ground corn in the MP-deficient diets promoted milk N efficiency and mitigated urinary urea N excretion. Furthermore, feeding fibrous byproducts in place of ground corn increased CH_4 emissions and energy losses as CH_4 in dairy cows. Milk energy output tended to decrease in RS cows relative to HS cows. Our results showed that supplementation of MP-deficient diets with RP-MLH had limited effects on lactation performance and nutrient utilization. In spite of these benefits of feeding the HS diets observed in our study, there has been considerable interest in including fibrous byproducts in dairy rations. Therefore, comparative effects of corn grain and a combination of RP-fat and byproducts on production performance and nutrient utilization need further investigations.

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Table 3.1. Nutrient composition of dietary ingredients (mean ± SD) used in the experimental diets (% of DM, unless otherwise noted)

Item	Corn silage	Haylage[1]	Ground corn	Beet pulp	Soyhulls	Soybean meal	Canola meal	DDGS[2]	Urea
No. of samples	4	2	2	2	2	3	3	3	3
DM, % of fresh matter	29.4 ± 3.37	28.5 ± 0.00	85.6 ± 0.37	86.7 ± 0.54	89.4 ± 1.63	85.9 ± 0.64	85.5 ± 1.40	83.5 ± 0.93	98.9 ± 0.45
CP	8.18 ± 0.22	16.9 ± 1.27	8.20 ± 0.14	8.55 ± 0.07	11.8 ± 2.19	53.8 ± 0.30	41.9 ± 2.65	31.7 ± 0.35	283 ± 1.82
Soluble protein, % CP	66.5 ± 1.29	47.5 ± 2.12	26.0 ± 4.24	14.5 ± 0.71	29.5 ± 4.95	21.7 ± 3.79	20.0 ± 1.00	17.7 ± 12.5	NA[3]
aNDF	43.5 ± 3.00	53.8 ± 0.57	8.25 ± 0.07	35.5 ± 4.95	61.0 ± 5.87	9.40 ± 0.95	29.0 ± 0.71	34.9 ± 2.63	NA
ADF	24.9 ± 1.90	36.3 ± 2.76	2.80 ± 0.28	22.6 ± 0.64	44.8 ± 3.96	7.77 ± 0.15	20.1 ± 0.26	15.7 ± 0.56	NA
ADL	2.85 ± 0.17	6.05 ± 1.06	0.95 ± 0.35	5.30 ± 0.42	2.15 ± 0.07	0.97 ± 0.23	8.07 ± 0.81	4.40 ± 0.61	NA
NFC[4]	41.2 ± 2.67	15.7 ± 2.19	77.7 ± 0.49	41.2 ± 4.88	19.2 ± 2.19	27.2 ± 1.42	17.1 ± 1.65	11.8 ± 4.00	NA
Starch	33.3 ± 0.95	0.75 ± 0.07	74.5 ± 2.05	0.20 ± 0.00	1.85 ± 1.20	0.30 ± 0.00	0.57 ± 0.31	1.40 ± 0.17	NA
Ether extract	3.33 ± 0.19	4.45 ± 0.49	3.75 ± 0.07	1.20 ± 0.00	2.45 ± 1.34	1.57 ± 0.29	5.00 ± 1.65	14.1 ± 1.71	NA
NE_L, Mcal/kg of DM	1.62 ± 0.05	1.30 ± 0.06	2.06 ± 0.02	1.31 ± 0.05	1.52 ± 0.09	1.83 ± 0.05	1.63 ± 0.08	2.12 ± 0.06	NA
Ash	3.81 ± 0.18	9.20 ± 0.21	2.18 ± 0.53	13.6 ± 0.06	5.68 ± 0.18	7.99 ± 0.88	7.01 ± 0.20	7.45 ± 0.60	NA
Ca	0.15 ± 0.01	0.66 ± 0.03	0.01 ±0.00	1.43 ± 0.06	0.52 ± 0.01	0.28 ± 0.02	0.61 ± 0.02	0.03 ± 0.03	NA
P	0.30 ± 0.02	0.40 ± 0.01	0.33 ± 0.01	0.11 ± 0.01	0.15 ± 0.06	0.82 ± 0.03	1.10 ± 0.05	1.11 ± 0.09	NA
Mg	0.15 ± 0.03	0.26 ± 0.01	0.10 ± 0.00	0.26 ± 0.00	0.23 ± 0.01	0.25 ± 0.02	0.52 ± 0.03	0.31 ± 0.03	NA
K	0.95 ± 0.11	2.72 ± 0.02	0.39 ± 0.01	0.36 ± 0.01	1.48 ± 0.00	2.11 ± 0.11	1.08 ± 0.05	1.17 ± 0.11	NA
Na	0.01 ± 0.00	0.06 ± 0.01	0.01 ± 0.00	0.03 ± 0.00	0.01 ± 0.01	0.00 ± 0.00	0.06 ± 0.04	0.09 ± 0.06	NA
S	0.10 ± 0.01	0.27 ± 0.01	0.10 ± 0.01	0.25 ± 0.03	0.14 ± 0.02	0.44 ± 0.03	0.80 ± 0.08	0.44 ± 0.06	NA
Fe, mg/kg of DM	335 ± 197	315 ± 126	35.0 ± 4.24	2,210 ± 198	491 ± 36.1	85.0 ± 2.65	128 ± 1.53	120 ± 43.5	NA
Zn, mg/kg of DM	24.8 ± 3.86	31.5 ± 2.12	18.0 ± 1.41	32.0 ± 0.00	56.0 ± 0.00	41.7 ± 2.31	54.0 ± 4.36	63.7 ± 7.51	NA
Cu, mg/kg of DM	6.00 ± 1.41	10.5 ± 0.71	2.00 ± 1.41	9.50 ± 2.12	8.00 ± 1.41	12.7 ± 0.58	5.33 ± 0.58	8.00 ± 3.61	NA
Mn, mg/kg of DM	14.3 ± 2.06	47.5 ± 3.54	4.00 ± 0.00	102 ± 24.0	18.0 ± 1.41	30.3 ± 0.58	58.3 ± 4.04	19.3 ± 11.0	NA
Mo, mg/kg of DM	1.73 ± 0.26	3.85 ± 0.07	0.75 ± 0.21	1.20 ± 0.14	0.95 ± 0.21	4.60 ± 0.46	1.37 ± 0.15	1.53 ± 0.32	NA

[1]Haylage = mixed mostly grass-legume haylage.
[2]DDGS = corn dried distillers grains with solubles.
[3]NA = not analyzed
[4]NFC = 100 – (CP% + aNDF% + ether extract% + ash%)

Table 3.2. Amino acid composition of dietary ingredients used in the experimental diets (n = 1 composited sample per feedstuff)

Item	Corn silage	Haylage[1]	Ground corn	Beet pulp	Soyhulls	Soybean meal	Canola meal	DDGS[2]
EAA, % CP								
Arg	1.93	3.25	4.79	2.58	5.03	7.40	6.25	4.69
His	1.40	1.67	2.87	3.44	2.80	2.63	2.87	2.78
Ile	4.55	5.31	3.56	4.87	4.29	4.87	4.49	4.25
Leu	11.6	9.15	11.8	7.59	7.08	7.82	7.50	11.5
Lys	2.63	4.82	3.28	4.01	7.08	6.52	6.22	3.44
Met	1.75	1.77	1.78	1.86	1.12	1.32	2.20	1.90
Phe	4.55	5.91	4.92	4.87	4.29	5.29	4.44	5.34
Thr	3.50	4.63	3.69	5.30	3.82	3.91	4.63	4.03
Trp	0.53	1.08	0.82	0.72	0.75	1.38	1.31	0.81
Val	5.95	6.79	4.65	7.31	4.85	4.87	5.47	5.16
NEAA, % CP								
Ala	12.4	9.45	7.39	5.59	4.47	4.35	4.66	6.99
Asp	5.60	9.06	6.70	8.74	9.69	11.3	7.34	6.63
Cys	1.58	1.08	2.33	1.43	1.96	1.42	2.79	2.23
Gly	5.08	5.91	3.97	5.16	7.83	4.27	5.44	4.32
Glu	12.3	9.15	17.8	10.5	12.0	18.4	18.4	16.6
Orn	0.53	0.89	0.00	0.14	0.09	0.06	0.03	0.11
Pro	8.23	6.30	8.62	5.73	5.87	5.37	6.92	8.75
Ser	3.50	3.84	4.65	4.73	5.13	4.55	4.13	4.72
Try	2.28	2.95	2.74	4.30	4.10	3.79	3.04	3.84
Tau	3.50	1.28	3.01	4.44	2.61	0.26	0.36	0.33

[1]Haylage = mixed-mostly grass-legume haylage.
[2]DDGS = dried corn distillers grains with solubles.

Table 3.3. Ingredient and nutritional composition (% of DM, unless otherwise noted) of the experimental diets

	Diet	
Item	High starch	Reduced starch
Ingredient, % of DM		
Corn silage	35.7	35.7
Haylage	14.7	14.7
Ground corn	30.0	-
Beet pulp	-	20.0
Soyhulls	-	10.0
Soybean meal	8.71	8.71
BergaFat F100[1]	3.00	3.00
Canola meal	2.76	2.76
Mineral and vitamins mix[2]	2.50	2.50
Sodium bicarbonate	1.00	1.00
DDGS	0.92	0.92
Urea	0.70	0.70
Nutrient composition		
DM, % of fresh matter	46.8	46.8
CP	16.0	16.4
aNDF	27.9	38.6
Forage NDF	23.4	23.4
ADF	16.4	24.6
ADL	2.54	3.53
NFC[3]	47.0	35.2
Starch	34.4	12.3
Ether extract	6.40	5.70
NE_L, Mcal/kg DM	1.68	1.59
Ca	0.60	1.00
P	0.40	0.40

[1]BergaFat F100 is a rumen-stable fat containing 80% palmitic acid (Berg+Schmidt America, LLC, Libertyville, IL).
[2]Mineral and vitamin mix contained (as-fed basis): 269 mg/kg of monensin sodium, 13.8% Ca, 1% P, 11% Na, , 5.50% Mg, 16 mg/kg of Co, 180 mg/kg of Cu, 8.4 mg/kg of Se, 1,280 mg/kg of Zn, 24.0 kIU/kg of vitamin A, 6.64 kIU/kg of vitamin D 3, and 0.29 kIU/kg of vitamin E.
[3]NFC = 100 – (CP% + aNDF% + ether extract% + ash%).

Table 3.4. NRC (2001) evaluation of experimental diets with high versus reduced starch levels supplemented with or without rumen-protected Met, Lys, and His (RP-MLH[1])

	Treatment[3]			
Item	HS	HS/MLH	RS	RS/MLH
NE_L[2], Mcal/d				
Requirement	40.0	40.0	40.0	40.0
Supply	41.4	41.5	40.1	40.4
Balance	1.3	1.4	0.1	0.4
MP[2], g/d				
Requirement	2743	2747	2773	2780
Supply	2564	2612	2666	2727
Balance	-180	-135	-107	-53
RDP[2], g/d				
Requirement	2539	2548	2479	2497
Supply	2623	2633	2615	2634
Balance	84	85	136	137
RUP[2], g/d				
Requirement	1544	1541	1675	1669
Supply	1321	1366	1537	1590
Balance	-223	-175	-137	-79
dHis[2], g/d				
Requirement[4]	60	60	61	61
Supply from the diet	55	55	58	59
Supply from RP-His	0	7	0	7
Balance	-5	2	-3	5
dMet[2], g/d				
Requirement[4]	60	60	61	61
Supply from the diet	48	48	49	49
Supply from RP-Met	0	15	0	15
Balance	-12	3	-12	3
dLys[2], g/d				
Requirement[4]	181	181	183	181
Supply	172	173	177	178
Supply from RP-Lys	0	16	0	16
Balance	-9	8	-7	13

[1]RP-MLH = 25 g/d of RP-Met (Smartamine® M; Adisseo USA Inc., Alpharetta, GA), 76 g/d of RP-Lys (AjiPro®-L; Ajinomoto Heartland, Inc., Eddyville, IA), and 110 g/d of RP-His (a prototype supplement; Ajinomoto Co. Inc., Kawasaki-shi, Japan).
[2]All values were estimated using the NRC (2001) model with actual DMI and nutrient composition of dietary ingredients during the experiment and milk yield and components before the experiment.
[3]HS = high starch diet, HS/MLH = high starch diet supplemented with RP-MLH, RS = reduced starch diet, and RS/MLH = reduced starch diet supplemented with RP-MLH.
[4]Requiements of dHis, dMet, and dLys were calculated as 2.2, 2.2, and 6.6% of MP requirements, respectively.

Table 3.5. Dry matter intake, milk yield and composition, plasma urea N (PUN), blood hemoglobin (Hb), BCS, and BW in lactating dairy cows fed diets with high versus reduced starch levels with or without rumen-protected Met, Lys, and His (RP-MLH[1])

	Treatment[2]					*P*-value[3]		
Item	HS	HS/MLH	RS	RS/MLH	SEM	SL	MLH	SL × MLH
DMI, kg/d	24.6	24.7	25.3	25.5	0.67	0.02	0.59	0.88
Milk yield, kg/d	40.2	40.0	37.4	38.3	1.16	<0.001	0.49	0.34
Milk yield/DMI, kg/kg	1.64	1.64	1.49	1.51	0.03	<0.001	0.75	0.58
4% FCM,[4] kg/d	37.8	38.4	37.8	37.4	1.08	0.37	0.88	0.44
4% FCM/DMI, kg/kg	1.55	1.57	1.51	1.47	0.03	<0.01	0.76	0.18
ECM,[5] kg/d	40.7	41.3	39.9	39.9	1.12	0.09	0.62	0.62
ECM/DMI, kg/kg	1.67	1.69	1.59	1.57	0.03	<0.001	0.97	0.27
Milk fat, %	3.64	3.74	4.05	3.97	0.11	<0.001	0.82	0.14
Milk fat, kg/d	1.45	1.49	1.51	1.49	0.05	0.35	0.84	0.33
Milk true protein, %	2.87	2.91	2.80	2.91	0.06	0.02	<0.001	0.08
Milk true protein, kg/d	1.15	1.17	1.05	1.09	0.03	<0.001	0.13	0.43
Milk lactose, %	4.98	4.92	4.93	4.83	0.06	0.05	0.02	0.60
Milk lactose, kg/d	2.02	1.98	1.86	1.82	0.06	<0.001	0.20	0.95
Milk N, % of N intake	29.5	28.8	25.5	25.3	0.52	<0.001	0.20	0.39
MUN, mg/dL	10.8	11.2	12.5	12.6	0.69	<0.01	0.54	0.82
PUN, mg/dL	11.3	11.9	13.2	13.4	0.54	<0.001	0.38	0.64
Blood Hb, g/dL	10.1	10.2	10.2	10.3	0.15	0.15	0.40	0.51
BCS	2.90	2.87	2.83	2.91	0.11	0.74	0.63	0.29
BW, kg	703	704	703	702	12.2	0.64	0.96	0.78
BW change, kg/d	0.09	0.39	0.05	0.32	0.15	0.71	0.06	0.91

[1]RP-MLH = 25 g/d of RP-Met (Smartamine® M; Adisseo USA Inc., Alpharetta, GA), 76 g/d of RP-Lys (AjiPro®-L; Ajinomoto Heartland, Inc., Eddyville, IA), and 110 g/d of RP-His (a prototype supplement; Ajinomoto Co. Inc., Kawasaki-shi, Japan).
[2]HS = high starch diet, HS/MLH = high starch diet supplemented with RP-MLH, RS = reduced starch diet, and RS/MLH = reduced starch diet supplemented with RP-MLH.
[3]SL = main effect of dietary starch level, MLH = main effect of RP-MLH supplementation, and SL × MLH = interaction between dietary starch level and RP-MLH.

[4]4% FCM = (0.4 × kg of milk) + (15 × kg of milk fat); Gaines and Davidson (1923).
[5]ECM = (0.327 × kg of milk) + (12.95 × kg of milk fat) + (7.65 × kg of milk protein); Tyrrell and Reid (1965).

Table 3.6. Concentrations of plasma AA and His-containing metabolites in lactating dairy cows fed diets with high versus reduced starch levels with or without rumen-protected Met, Lys, and His (RP-MLH[1])

	Treatment[2]					*P*-value[3]		
Item	HS	HS/MLH	RS	RS/MLH	SEM	SL	MLH	SL × MLH
EAA, μ*M*								
Arg	70.4	72.7	81.8	95.0	3.27	<0.001	<0.01	0.04
His	37.1	50.9	43.0	55.2	2.01	<0.01	<0.001	0.67
Ile	108	111	139	147	4.68	<0.001	0.18	0.52
Leu	117	118	132	137	5.68	<0.001	0.48	0.75
Lys	66.5	70.7	77.2	95.3	3.68	<0.001	0.001	0.03
Met	23.2	37.5	21.1	37.5	1.55	0.40	<0.001	0.37
Phe	43.5	43.5	48.1	51.0	1.42	<0.001	0.28	0.28
Thr	97.5	95.8	96.5	104	3.93	0.24	0.33	0.14
Trp	45.7	43.7	47.9	48.8	1.27	<0.01	0.67	0.23
Val	202	205	250	262	9.09	<0.001	0.22	0.46
NEAA, μ*M*								
Ala	298	291	275	299	15.1	0.41	0.38	0.13
Asn	44.7	44.7	44.9	51.2	1.91	0.05	0.06	0.06
Asp	3.16	3.18	2.86	3.07	0.13	0.06	0.29	0.37
Gln	206	208	210	220	6.87	0.21	0.32	0.51
Glu	39.6	40.3	38.7	39.0	1.72	0.27	0.63	0.85
Gly	299	285	242	245	9.90	<0.001	0.46	0.29
Pro	92.5	90.0	81.2	88.2	4.44	0.03	0.44	0.11
Ser	77.3	74.3	71.2	74.5	2.56	0.14	0.95	0.11
Tau	39.0	45.6	30.6	40.1	1.86	<0.001	<0.001	0.33
Tyr	46.2	45.2	49.3	52.2	2.57	<0.01	0.59	0.30
Sum of AA								
∑ EAA, μ*M*	810	848	936	1,037	28.9	<0.001	<0.01	0.18
∑ NEAA, μ*M*	1,292	1,274	1,191	1,272	35.9	0.06	0.25	0.07
Total AA,[4] μ*M*	2,102	2,122	2,128	2,309	59.3	0.03	0.04	0.09
His-containing metabolites, μ*M*								
Carnosine	18.2	18.7	17.4	18.0	0.66	0.19	0.37	0.97
3-MHis[6]	1.60	1.41	1.58	1.30	0.11	0.55	0.04	0.71

[1]Rumen-protected MLH = 25 g/d of RP-Met (Smartamine® M; Adisseo USA Inc., Alpharetta, GA), 76 g/d of RP-Lys (AjiPro®-L; Ajinomoto Heartland, Inc., Eddyville, IA), and 110 g/d of RP-His (a prototype supplement; Ajinomoto Co. Inc., Kawasaki-shi, Japan).
[2]HS = high starch diet, HS/MLH = high starch diet supplemented with RP-MLH, RS = reduced starch diet, and RS/MLH = reduced starch diet supplemented with RP-MLH.
[3]SL = main effect of dietary starch level, MLH = main effect of RP-MLH supplementation, and SL × MLH = interaction between dietary starch level and RP-MLH.
[5]Total AA = EAA + NEAA.
[6]3-MHis = 3-methylHis.

Table 3.7. Apparent total-tract digestibility of nutrients and urinary excretion of nitrogenous compounds in lactating dairy cows fed diets with high versus reduced starch levels with or without rumen-protected Met, Lys, and His (RP-MLH[1])

	Treatment[2]					*P*-value[3]		
Item	HS	HS/MLH	RS	RS/MLH	SEM	SL	MLH	SL × MLH
Apparent total-tract digestibility								
DM	70.3	70.0	70.4	69.8	0.36	0.89	0.18	0.70
OM	71.6	71.0	71.8	71.6	0.42	0.33	0.37	0.59
CP	68.3	69.1	68.6	68.4	0.60	0.69	0.49	0.28
aNDF	43.7	42.7	59.1	58.6	0.75	<0.001	0.27	0.74
ADF	48.1	47.0	63.0	61.8	0.94	<0.001	0.12	0.93
Urinary Excretion								
Creatinine (m*M*)	5.65	5.11	4.71	5.01	0.46	0.16	0.74	0.25
Volume (L/d)	36.3	37.2	39.0	37.6	2.25	0.36	0.90	0.48
Urea-N (g/d)	149	168	196	195	9.88	<0.001	0.34	0.29
Total-N (g/d)	297	317	323	325	14.0	0.17	0.37	0.44
Urea-N, % of total N	50.7	54.8	63.2	59.6	2.36	<0.001	0.89	0.06
Urea-N, % N intake	24.1	24.8	30.3	28.6	1.61	<0.01	0.73	0.39
Total-N, % N intake	47.8	49.0	48.3	47.6	1.99	0.81	0.92	0.61
Uric acid, mmol/d	93.5	91.9	102	102	5.38	0.06	0.85	0.90
Allantoin, mmol/d	575	620	596	584	36.5	0.80	0.56	0.32
Purine derivatives, mmol/d	665	704	699	684	39.4	0.83	0.71	0.39

[1]Rumen-protected MLH = 25 g/d of RP-Met (Smartamine® M; Adisseo USA Inc., Alpharetta, GA), 76 g/d of RP-Lys (AjiPro®-L; Ajinomoto Heartland, Inc., Eddyville, IA) and 110 g/d of RP-His (a prototype supplement; Ajinomoto Co. Inc., Kawasaki-shi, Japan).
[2]HS = high starch diet, HS/MLH = high starch diet supplemented with RP-MLH, RS = reduced starch diet, and RS/MLH = reduced starch diet supplemented with RP-MLH.
[3]SL = main effect of dietary starch level; MLH = main effect of RP-MLH supplementation; and SL × MLH = interaction between dietary starch level and RP-MLH.

Table 3.8. Emissions of carbon dioxide (CO2) and methane (CH4)[1] in lactating dairy cows fed diets with high versus reduced starch levels with or without rumen-protected Met, Lys, and His (RP-MLH[2])

	Treatment[3]					*P*-value[4]		
Item	HS	HS/MLH	RS	RS/MLH	SEM	SL	MLH	SL × MLH
CO_2, kg/d	12.1	11.9	11.8	12.0	0.32	0.79	0.99	0.46
CH_4, g/d	434	434	545	545	20.8	<0.001	0.98	0.99
CH_4, g/kg of DMI	17.6	17.7	21.7	21.5	0.77	<0.001	0.95	0.76
CH_4, g/kg of ECM	10.7	10.6	13.5	13.7	0.47	<0.001	0.88	0.65

[1]Gases were measured using GreenFeed (C-Lock Technology Inc., Rapid City, SD). Data were derived from 8 individual measurements over 4-d period.

[2]Rumen-protected MLH = 25 g/d of RP-Met (Smartamine® M; Adisseo USA Inc., Alpharetta, GA), 76 g/d of RP-Lys (AjiPro®-L; Ajinomoto Heartland, Inc., Eddyville, IA), and 110 g/d of RP-His (a prototype supplement; Ajinomoto Co. Inc., Kawasaki-shi, Japan).

[3]HS = high starch diet, HS/MLH = high starch diet supplemented with RP-MLH, RS = reduced starch diet, and RS/MLH = reduced starch diet supplemented with RP-MLH.

[4]SL = main effect of dietary starch level, MLH = main effect of RP-MLH supplementation, and SL × MLH = interaction between dietary starch level and RP-MLH.

Table 3.9. Dietary energy estimations and milk energy efficiencies in lactating dairy cows fed diets with high versus reduced starch levels with or without rumen-protected Met, Lys, and His (RP-MLH[1])

	Treatment[2]					*P*-value[3]		
Item	HS	HS/MLH	RS	RS/MLH	SEM	SL	MLH	SL × MLH
GE, Mcal/d	114	116	101	102	3.46	<0.001	0.42	0.97
DE,[4] Mcal/d	86.4	86.4	75.0	73.9	2.15	<0.001	0.74	0.75
ME,[5] Mcal/d	58.8	59.4	57.5	58.5	1.39	0.26	0.41	0.86
Components, Mcal/d								
Fecal energy	27.8	28.8	25.9	28.0	1.60	0.24	0.17	0.62
Urinary energy[6]	3.82	3.86	3.74	3.80	0.09	0.26	0.43	0.87
CH_4 energy[7]	6.13	6.13	7.71	7.70	0.30	<0.001	0.97	0.99
Heat production[8]	30.6	30.2	30.1	30.4	0.79	0.76	1.00	0.48
Milk energy[9]	27.8	28.2	27.3	27.2	0.80	0.08	0.80	0.59
Tissue energy[10]	0.34	0.99	0.16	0.93	0.40	0.76	0.07	0.88
DE, % of GE	75.2	74.2	73.8	72.0	1.09	0.08	0.17	0.71
ME, % of GE	53.8	53.2	58.7	58.8	1.18	<0.001	0.77	0.66
Fecal energy, % of GE	24.8	25.8	26.2	28.0	1.09	0.08	0.17	0.71
Urinary energy, % of GE	3.50	3.46	3.82	3.83	0.08	<0.001	0.77	0.66
CH_4 energy, % of GE	5.18	5.12	7.28	7.30	0.29	<0.001	0.91	0.83
Heat, % of GE	28.0	26.9	30.4	30.7	0.83	<0.001	0.50	0.21
Milk energy, % of GE	25.3	25.3	27.9	27.3	0.55	<0.001	0.48	0.45
Tissue energy, % of GE	0.46	0.97	0.35	0.87	0.38	0.77	0.17	0.99

[1]Rumen-protected MLH = 25 g/d of RP-Met (Smartamine® M; Adisseo USA Inc., Alpharetta, GA), 76 g/d of RP-Lys (AjiPro®-L; Ajinomoto Heartland, Inc., Eddyville, IA) and 110 g/d of RP-His (a prototype supplement; Ajinomoto Co. Inc., Kawasaki-shi, Japan).
[2]HS = high starch diet, HS/MLH = high starch diet supplemented with RP-MLH, RS = reduced starch diet, and RS/MLH = reduced starch diet supplemented with RP-MLH.

[3]SL = main effect of dietary starch level, MLH = main effect of RP-MLH supplementation, and SL × MLH = interaction between dietary starch level and RP-MLH.
[4]DE = GE − FE (NRC, 2001).
[5]ME = Heat production + milk energy + tissue energy (NRC, 2001).
[6]Urinary energy = ME × 0.065 (Ferris et al., 1999).
[7]CH_4 energy = CH_4 × 13.18 kcal/g (Judy et al., 2019).
[8]Heat production (MJ/d) = 0.0184 × QCO_2 (L/d) + 7.50 (Bayat et al., 2019).
[9]Milk energy = [(0.0929 × milk fat%) + (0.0563 × milk true protein%) + (0.0395 × milk lactose%)] × milk yield (kg/d) (NRC, 2001).
[10]Tissue energy = (body fat% × 9.4 + body protein% × 5.55) × BW change (NRC, 2001).

CHAPTER IV: DIETARY ENERGY SOURCE AND RUMEN-PROTECTED AMINO ACIDS: EFFECT ON MILK YIELD AND NUTRIENT UTILIZATION IN LACTATING DAIRY COWS

ABSTRACT

Our previous study demonstrated that reduced dietary starch level limited milk and milk protein yield in dairy cows receiving MP-deficient diets. Supplementation of reduced-starch diets with fat could be a feeding strategy to improve milk production. Thus, we aimed to investigate the interactions between energy sources (starch vs. fat) and rumen-protected (RP) Met, Lys, and His (MLH) on milk production and utilization of energy and N in dairy cows fed MP-deficient diets. Sixteen multiparous Holstein cows were used in a replicated 4 × 4 Latin square with a 2 × 2 factorial arrangement of treatments. Each period lasted 21 d with 14 d for diet adaptation and 7 d for data and sample collection. Treatments included high starch (HS), HS + RPMLH, reduced starch + RP-fat (RSF), and RSF + RPMLH. The basal diets consisted (dry matter basis) of 50% forage and 50% concentrate. The HS diet contained 26% ground corn, while the RSF diet had 16% ground corn replaced with 15% soyhulls and 1.5% RP-fat (i.e., palmitic acid-enriched supplement). Dietary net energy for lactation, starch, and crude protein averaged 1.53 Mcal/kg, 32.6% and 15.9% for HS diets, and 1.59 Mcal/kg, 21.7% and 16.8% for RSF diets, respectively. Smartamine® M, AjiPro®-L, and an Ajinomoto prototype RP-His supplement were supplemented to meet digestible MLH requirements. Data were analyzed using the MIXED procedure of SAS. Dietary treatments had no effects on DMI and milk yield. However, feeding RSF diets increased feed efficiency (1.57 vs. 1.54 kg/kg) and milk fat yield (1.65 vs. 1.50 kg/d) compared with HS diets. Milk and plasma urea N increased and milk N efficiency (30.6 vs. 29.1%) decreased for RSF versus HS diets. Supplemental RP-MLH tended to improve milk true protein content. Additionally, RSF diets increased the plasma concentrations of Arg, Ile, Thr, and Ala but reduced that of Leu relative to HS diets. Plasma Met and His increased with RP-MLH. Digestibilites of crude protein, neutral and acid detergent fiber increased in cows fed RSF vs. HS

diets. Urinary urea N and total N increased in RSF vs. HS cows. Treatments did not change CH_4 production, CH_4 yield, and CH_4 intensity. Consequently, cows fed HS and RSF diets had similar CH_4 energy losses. Likewise, CO_2 emissions and heat production were not affected by treatments. Overall, substitution of ground corn with soyhulls and RP-fat improved feed efficiency and milk fat yield but reduced N utilization without changing energy balance in dairy cows.

Key words: ground corn, metabolizable protein, soyhulls, starch

INTRODUCTION

The high cost of protein feeds and N pollution from high CP diets have encouraged the dairy industry to feed MP-deficient rations to lactating dairy cows. Olmos Colmenero and Broderick (2006) and Broderick et al. (2009) reported that MP-deficient diets improved milk N efficiency and reduced urinary excretion of urea N and total N, but inconsistent responses of milk and milk protein yields were observed. These inconsistencies may have been caused by deficiencies of EAA, particularly Met, Lys, and His (**MLH**; Bequette et al., 2000), limited dietary energy supply (Broderick, 2003), or a combination of both. Lee et al. (2012) demonstrated that supplementation of rumen-protected (**RP**)-MLH to a MP-deficient diet increased yields of milk and milk protein in dairy cows. Giallongo et al. (2016) also observed an increase in milk protein yield in dairy cows fed MP-deficient diets supplemented with RP-MLH. Furthermore, we demonstrated that cows fed high starch diets produced 6% more milk and 8.4% more milk protein than those offered reduced starch diets formulated by substituting ground corn with a beet pulp-soyhulls mix (Zang et al., unpublished).

Taken together, supplementation of MP-deficient diets with fermentable energy (e.g., ground corn) and RP-MLH may have the potential to improve yields of milk and milk components and nutrient utilization in dairy cows. However, there is a growing interest in feeding reduced starch diets by replacing ground corn with fibrous byproducts (Fredin et al., 2015a,b), but decreased dietary energy supply must be overcome to avoid reductions in yields of milk and milk components. Specifically, supplementing MP-deficient, reduced starch diets with RP-fat may be a viable approach to enhance production performance and efficiency of nutrient use in lactating dairy cows.

It is important to note that reduced starch diets decrease the risks of ruminal acidosis and displaced abomasum among other metabolic disorders (Allen et al., 2009; Allen and Piantoni, 2013). According to van Knegsel et al. (2007) and Boerman et al. (2015), more nutrients were partitioned toward milk production and less to body reserves in dairy cows fed reduced starch diets supplemented with RP-fat compared with those offered high starch diets. West (2003) stated that less metabolic heat production is expected with feeding RP-fat due to its minimal digestion in the rumen relative to starch sources, which can spare energy to produce milk and milk fat. In fact, milk yield and milk fat concentration and yield increased in cows fed supplemental fat sources as reported in a meta-analysis and meta-regression by Rabiee et al. (2012). In contrast, decreased milk protein concentration was associated with fat supplementation to dairy cows (Rabiee et al., 2012). Decreased glucose availability, development of insulin resistance, increased efficiency of milk production, or reduced plasma somatotropin may be involved in the underlining mechanisms linked to decreased milk protein concentration when fat sources are fed to dairy cows (Wu and Huber, 1994). Alternatively, there is evidence of less EAA supply to the mammary gland with feeding supplemental fat (Erickson et al., 1992; Cant et al., 1993). However, there is scare information on how these diets would affect CH_4 emissions and energy utilization.

Therefore, supplementation of reduced starch diets with RP-fat and RP-MLH may simultaneously increase energy and EAA supply to the mammary gland ultimately improving yields of milk and milk components. We hypothesized that a reduced starch, low-protein diet supplemented with RP-MLH and RP-fat would improve milk production and nutrient utilization in dairy cows. Our objectives were to explore the effects of energy source (**ES**; corn starch vs.

RP-fat) and RP-MLH on lactation performance, CH_4 emission, efficiency of N and energy utilization in high-producing dairy cows fed MP-deficient diets.

MATERIALS AND METHODS

All experimental procedures were approved by the Institutional Animal Care and Use Committee (protocol no. 190202) of the University of New Hampshire (Durham). The experiment was conducted at the University of New Hampshire Fairchild Dairy Teaching and Research Center (Durham) from March 11 to June 9, 2019.

Animals, Experimental Design, and Treatments

Sixteen multiparous Holstein cows averaging (mean ± SD) 112 ± 28 DIM, 46 ± 5 kg/d of milk, and 726 ± 97 kg of BW were selected at the beginning of the study. Cows were housed in a tie-stall barn equipped with water bowels for free access to water and feed tubs for individual feeding. Cows were milked twice a day at 0530 and 1630 h, and milk yield was recorded automatically at each milking throughout the experiment. Cows were weighed (Northeast Scale Co. In., Hooksett, NH) immediately after the afternoon milking during 3 consecutive days before the beginning of the study and at the end of each experimental period. Body condition score was recorded by 3 trained investigators before the beginning of the study and on the last day of each period following the procedures outlined by Wildman et al. (1982).

Cows were blocked by DIM and milk yield into 4 squares and randomly assigned to treatment sequences in a replicated 4 × 4 Latin square design with a 2 × 2 factorial arrangement of treatments. Squares were balanced for potential first-order carryover effects in subsequent periods as each treatment immediately preceded and followed every other exactly once in every

square (Williams, 1949; Kim and Stein, 2009). Each experimental period consisted of 14 d for diet adaptation and 7 d for data and sample collection. Dietary treatments were: (1) high-starch diet (**HS**); (2) HS + RP-MLH (**HS/MLH**); (3) reduced-starch diet + RP-fat (**RSF**); and (4) RSF + RP-MLH (**RSF/MLH**). The basal diets were formulated to meet the nutrient requirements, except MP, of a lactating dairy cow averaging 700 kg BW, 120 DIM, 46.4 kg of milk/d, 3.5% of milk fat, 3.1% of milk true protein, 4.98% of milk lactose, and 26 kg/d of DMI using the NRC (2001) evaluation software (v.1.1.9), and contained (DM basis) 40% corn silage, 5% mixed mostly grass-legume haylage, 5% grass hay, and 50% concentrate. The basal HS diet contained 26% ground corn, and the basal RSF diet was achieved by replacing 16% ground corn with 15% soyhulls and 1.5% RP-fat. The RP-fat was BergaFat F100 (Berg+Schmidt America, LLC, Libertyville, IL) that contained at least 80% palmitic acid based on the manufacturer's specification. The RP-MLH supplements were top-dressed on top of the TMR to meet the requirements of digestible MLH (Schwab et al., 2005). The amounts of RP-Met (Smartamine® M; Adisseo USA Inc., Alpharetta, GA), RP-Lys (AjiPro®-L; Ajinomoto Heartland Inc., Eddyville, IA), and RP-His (Ajinomoto prototype supplement; Ajinomoto Co. Inc., Kawasaki-shi, Japan) supplemented were 12, 9, and 15 g/d, respectively. The RP-Met, RP-Lys, and RP-His supplements contained 75% DL-Met with 80% bioavailability (Chirgwin et al., 2015), 40% Lys with 54% bioavailability (Giallongo et al., 2016), and 40% His with 49% bioavailability (T. Takagi, associate director at Ajinomoto Heartland, Inc., personal communication), respectively. These RP-AA supplements (Smartamine® M, AjiPro®-L, and Ajinomoto prototype supplement) were expected to provide 7.2, 1.94, and 2.94 g/d of digestible Met, Lys, and His, respectively.

Dietary ingredients were mixed and offered as TMR twice daily at 0600 and 1700 h using a Super Data Ranger mixer (American Calan Inc., Northwood, NH). Orts were collected and

weighed once daily before the afternoon feeding. Feed offered was adjusted daily to achieve 5 to 10% orts. Feed intake was recorded throughout the experiment.

Feed Sampling and Analyses

Samples of corn silage, mixed mostly grass-legume haylage, grass hay, TMR, and orts were collected thrice weekly and composited by week. The composite samples were dried (55°C, 48 h) in a forced-air oven (VWR Scientific, Radnor, PA) for determination of DM to adjust the TMR on an as-fed basis and to calculate DMI. Samples of forages, concentrates (i.e., ground corn, soyhulls, AminoMax, soybean meal, and urea), TMR, and orts were collected thrice during the sampling week of each period and composited by week. Weekly ingredients were lyophilized for 48 h (Labconco Inc., Kansas City, MO), ground using a Wiley mill (A. H. Thomas Co., Swedesboro, NJ) to pass through a 1-mm screen, and stored in air-tight glass jars until nutritional analysis.

Lyophilized and ground samples of dietary ingredients were shipped to Dairy One Cooperative Inc. (Ithaca, NY) and analyzed for DM, CP, soluble protein, aNDF, ADF, ADL, starch, ether extract, ash, and individual minerals (Ca, P, Mg, K, Na, S, Fe, Zn, Cu, Mn, and Mo) following the procedures used by Dairy One Cooperative Inc. (https://dairyone.com/download/forage-forage-lab-analytical-procedures; Accessed Mar. 19, 2020). Moreover, TMR and orts were analyzed for CP, aNDF, ADF, and ash at Dairy One Cooperative Inc. laboratory. Samples of dietary ingredients were further ground (Wiley mill; A. H. Thomas Co.) to pass through a 0.5 mm screen and used for determination of AA by cation exchange chromatography (cIEC-HPLC) coupled with post-column ninhydrin derivatization using norleucine as the internal standard (method 982.30; AOAC International, 2016) at the

University of Missouri Agricultural Experiment Station Chemical Laboratory (Columbia, MO). Tryptophan was determined after alkaline hydrolysis and sulfur AA were analyzed after performic acid oxidation (method 988.15; AOAC International, 2016).

Milk and Blood Sampling and Analyses

Milk samples were collected using automatic samplers during 4 consecutive milkings starting in the afternoon milking of d 15 of each period. Milk samples were transferred into tubes preserved with 2-bromo-2-nitropropane-1,3 diol (Broad Spectrum Microtabs II; Advanced Instruments Inc., Norwood, MA) and stored at 4°C until analysis. Milk samples were shipped to Dairy One Cooperative Inc. laboratory and analyzed for concentrations of fat, true protein, lactose, TS, and MUN by mid-infrared reflectance spectroscopy in a Milkoscane (Foss Inc., Hillerød, Denmark), and SCC using flow cytometry in a Fossomatic (Foss Inc.).

Blood samples were collected into vacutainer 15% EDTA tubes (Monoject, Mansfield, MA) via the coccygeal vein or artery approximately 4 h after the morning feeding on d 16 and 17 of each period. Tubes were immediately placed in a chill bucket with beads (Chemglass Life Sciences, Vineland, NJ) and transported to the laboratory for centrifugation (2,155 × g, 20 min, 4ºC) using an Eppendorf centrifuge (model 5810; Eppendorf, Hamburg, Germany). Plasma samples were composited by cow and period, and the composite samples were used to determine the concentrations of AA, carnosine, and urea N (**PUN**) at Ajinomoto Co. Inc. using a High-Speed AA analyzer L-8900 (Hitachi High-Technologies Co., Tokyo, Japan) following the procedures described by the manufacturer (https://www.hitachi-hightech.com/us/library/literature/brochure-l-8900-amino-acid-analyzer.html; Accessed March

19, 2020). Codified plasma samples were sent to Ajinomoto Co. Inc. to preserve treatments identity.

Fecal and Urinary Sampling and Analyses

Fecal grab samples were taken directly from the rectum or during voluntary defecation at 8 time points (0600 h and 1500 h on d 18; 0300 h, 0900 h, and 1800 h on d 19; and 0000 h and 1200 h and 2100 h on d 20) during the sampling week of each period. Fecal samples (~200 g/sampling) were collected into 100-mL specimen containers and transferred into 4-L storage bags to obtain composited samples by cow per period. Next, samples were dried in a forced-air oven (VWR Scientific) at 55°C for approximately 72 h and ground (Wiley mill; A. H. Thomas Co.) to pass through a 1-mm screen. Fecal samples were analyzed for DM, CP, aNDF, ADF, and ash at Dairy One Cooperative Inc. laboratory as reported above. In addition, triplicate samples (~0.5 g) of feces, TMR, and orts were weighed into Ankom F57 bags (25 µm pore size; Ankom Technology, Macedon, NY), placed in a larger laundry nylon bag, and inserted in the rumen of 1 ruminally cannulated late-lactation Holstein cow for 12 d. The cannulated cow was fed a corn silage- and grass silage-based diet with a forage-to-concentrate ratio of 50:50. After removal from the rumen, bags were rinsed with tap water and analyzed in-house for NDF using an Ankom^{2000} fiber analyzer (Ankom Technology). Indigestible NDF (**iNDF**) was used as the internal marker to estimate fecal output of DM and apparent total-tract digestibility of nutrients (Cochran et al., 1986; Huhtanen et al., 1994).

Spot urine samples were collected concurrently with fecal samples into 100-mL specimen containers through stimulation of the pudendal nerve by massaging the area below the vulva or during voluntary urination in the sampling week of each period. After each sampling, 1 mL of

urine was pipetted into 50-mL centrifuge tubes containing 32 mL of 0.072 *N* H_2SO_4 to obtain composited urine samples by cow per period. Urine samples were stored at −20°C before analyses of nitrogenous metabolites. After thawing at room temperature, samples were analyzed for concentrations of creatinine (assay kit no. 500701, Cayman Chemical Co., Ann Arbor, MI) and uric acid (catalog no. DIUA-250, QuantiChrom Uric Acid Assay Kit, BioAssay Systems Inc., Hayward, CA) using a chromate microplate reader set at a wavelength of 492 nm and 540 nm, respectively (Awareness Technology Inc., Palm City, FL), allantoin (Chen et al., 1992), urea N (Stanbio Urea Nitrogen Kit 580; Stanbio Laboratory Inc.), and total N (micro-Kjeldahl analysis, AOAC, 1990; Dairy One Cooperative Inc.). Allantoin and urea N were determined at wavelengths of 522 and 520 nm, respectively, in a UV/visible spectrophotometer (Beckman Coulter Inc., Pasadena, CA). Daily urine volume was estimated from urinary creatinine concentration assuming a constant creatinine excretion rate of 29 mg/kg of BW (Valadares et al., 1999). Urinary excretion of urea N, total N, allantoin, uric acid, and purine derivatives (allantoin plus uric acid) were calculated by multiplying the concentration of each of these metabolites by the urinary volume.

Measurements of Gaseous Fluxes

Emissions of CO_2 and enteric CH_4, and consumption of O_2 were measured at 8 timepoints (0200 h and 1400 h on d 15; 0500 h and 1700 h on d 16; 0800 h and 2000 h on d 17; and 1100 h and 2300 h on d 18) to account for diurnal variation in gaseous fluxes using the GreenFeed system (C-Lock Inc., Rapid City, SD) during the sampling week of each period (Harper et al., 2017). The GreenFeed unit was placed in front of each cow for approximately 5 min to sample breath gases and then moved to the alley for 2 min to sample background gases. The unit was

moved from cow to cow in a sequential manner. About 2 weeks prior to the beginning of the study, cows were trained to have access to the GreenFeed unit by using a bait feed (Hi-Line 16% Dairy/Beef Pellet, Poulin Grain Inc., Newport, VT). The bait feed was a pelleted product, and contained 16% CP, 6.2% crude fiber, 31% NFC, 19% starch, and 3.9% ether extract. Approximately 25 g of pellets were dropped from the dispenser of the GreenFeed unit every 15 sec, resulting in a total consumption of ~0.5 kg of pellets per sampling (20 drops in total). One cow refused to consistently access the unit, and her data were not included in the statistical analyses. Data were averaged by cow per period. A complete description of the gaseous sampling protocol and calculations used herein has been reported elsewhere (Dorich et al., 2015).

Calculations and Statistical Analyses

Yields of milk components were calculated using milk yield and concentrations of milk components at each milking, summed for daily total, and averaged by period. Energy losses as CH_4 was calculated by multiplying CH_4 emissions (g/d) by a constant of 0.0132 Mcal/g (Judy et al., 2019). Heat production was calculated using the equation reported by Bayat et al. (2019): heat production (Mcal/d) = [0.0184 × CO2 (L/d) + 7.50] ÷ 4.184.

Data were analyzed using the MIXED procedure of SAS (version 9.4; SAS Institute Inc., Cary, NC) according to a replicated 4 × 4 Latin square design with a 2 × 2 factorial arrangement of treatments. The following model was used:

$$Y_{ijklm} = \mu + S_i + C_{j(i)} + P_k + ES_l + MLH_m + ES_l \times MLH_m + e_{ijklm}$$

where, Y_{ijklm} = dependent variable, μ = overall mean, S_i = fixed effect of square (i = 1 to 4), $C_{j(i)}$ = random effect of cow nested within square, P_k = fixed effect of period (k = 1 to 4), ES_l = fixed effect of ES (l = starch or fat), MLH_m = fixed effect of supplemental RP-MLH (m = yes or no),

$ES_l \times MLH_m$ = interaction between dietary ES and supplemental RP-MLH, and e_{ijklm} = residual error. Normality of the residuals was checked with normal probability and box plots and homogeneity of variances with plots of residual versus predicted values. Data were considered as outliers and removed from analysis when Studentized residuals were >3.0 or < −3.0. All results were expressed as least squares means and standard errors. Significance was declared at $P \leq 0.05$ and trends at $0.05 < P \leq 0.10$. The PDIFF option of SAS was used for mean comparisons when interaction between dietary ES and RP-MLH was significant.

RESULTS

The chemical composition and AA profile (% of CP) of the individual ingredients are presented in Table 4.1 and 4.2, respectively. The ingredient and nutritional composition of the basal diets are listed in Table 4.3. The basal diets consisted of 50% forage (i.e., corn silage, mixed mostly grass-legume haylage and grass hay) and 50% concentrate on a DM basis. The HS basal diet contained (DM basis) 26% ground corn and 7% soyhulls. Ground corn was partly replaced with soyhulls and RP-fat to obtain the RSF basal diet, which contained 10% ground corn, 22% soyhulls, and 1.5% RP-fat (palmitic acid-enriched supplement). The basal diets were originally formulated to be isonitrogenous and isoenergetic; however, the RSF basal diet had greater CP level than the basal HS diet (16.8 vs. 15.9%). Dietary NE_L levels were similar between the HS and RSF basal diets (1.53 vs. 1.59 Mcal/kg). Substituting ground corn with soyhulls and RP-fat increased the dietary concentrations of aNDF and ADF and decreased those of NFC and starch; however, the content of forage NDF was not altered. The RSF diet had higher level of ether extract compared with the HS diet (5.9 vs. 4%).

The NRC (2001) evaluation of the dietary treatments is presented in Table 4.4. The balances for NE_L and MP averaged 0.85 Mcal/d and −84 g/d for the HS diets, and 2.4 Mcal/d and −27 g/d for the RSF diets, respectively. The balances of RDP and RUP were 162 and -34 g/d for the RSF diets and 95 and −104 g/d for the HS diets, respectively. Deficiencies of digestible His, Met, and Lys were observed for both experimental diets without RP-MLH supplementation. Supplemental RP-MLH alleviated these shortages of Met, Lys, and His to some degree.

Intake and Milk Yield and Composition

Dry matter intake, milk yield and components, PUN, BCS, and BW are shown in Table 4.5. There was a significant interaction of ES and RP-MLH supplementation effect on milk fat concentration. Specifically, milk fat concentration tended ($P = 0.08$) to decrease in HS cows but did not change in RSF cows when feeding RP-MLH. Milk yield showed a trend ($P = 0.07$) for an interaction between dietary ES and RP-MLH.

Substitution of ground corn with soyhulls and RP-fat did not significantly change DMI (mean = 29.0 kg/d) and milk yield (mean = 45 kg/d) but improved feed efficiency expressed as milk yield/DMI (1.57 vs. 1.54 kg/kg). Moreover, the RSF diets had greater yields of 4% FCM ($P < 0.001$; 42.9 vs. 40.1 kg/d) and ECM ($P < 0.001$; 47.0 vs. 44.8 kg/d) compared with the HS diets, respectively. Consequently, feed efficiencies, expressed as 4% FCM yield/DMI ($P < 0.001$; 1.48 vs. 1.38 kg/kg) or ECM yield/DMI ($P < 0.001$; 1.62 vs. 1.53 kg/kg), increased with feeding the RSF vs. HS diets, respectively. Compared with HS cows, RSF cows had greater yields of milk fat ($P < 0.001$; 1.50 vs. 1.65 kg/d) and TS ($P < 0.001$; 5.55 vs. 5.72 kg/d) and milk TS concentration ($P < 0.001$; 12.5 vs. 12.6%), but lower contents of milk true protein ($P < 0.001$; 3.17 vs. 3.10%) and milk lactose ($P < 0.01$; 4.99 vs. 4.94%), respectively. Milk N efficiency ($P <$

0.001; 29.1 vs. 30.1%) decreased for the RSF diets relative to the HS diets, respectively. However, milk SCC (mean = 47,900 cells/mL) was not impacted by dietary ES. Both MUN ($P <$ 0.001; 14.9 vs. 12.1 mg/dL) and PUN ($P < 0.001$; 15.6 vs. 12.3 mg/dL) concentrations increased in RSF cows compared with HS cows, respectively. Body condition score, BW, and BW change were not modified by dietary ES ($P \geq 0.15$).

Supplementation with RP-MLH (HS/MLH and RSF/MLH) tended ($P = 0.06$) to increase milk true protein concentration (3.15 vs. 3.12%, respectively) compared with the diets without RP-MLH (HS and RSF). There was a trend ($P = 0.09$) for greater BW gain in association with supplemental RP-MLH. However, the present study indicated that supplementing RP-MLH to the basal diets had no effects on DMI, feed efficiencies, yields of milk and milk components, BCS, and BW.

Plasma AA and Carnosine

The plasma concentrations of AA and carnosine are shown in Table 4.6. No significant interactions between dietary ES and RP-MLH were observed for plasma AA and carnosine concentrations. Regarding EAA, substituting ground corn with soyhulls and RP-fat increased ($P \leq 0.05$) the plasma concentrations of Arg, Ile and Thr, but decreased ($P < 0.01$) plasma Leu concentration. The plasma concentrations of His and Phe tended ($P \leq 0.09$) to decrease, while those of plasma Lys and Val tended ($P \leq 0.09$) to increase with feeding the RSF vs. HS diets. As for NEAA, the RSF diets enhanced ($P = 0.02$) the plasma concentration of Ala and tended ($P \leq$ 0.08) to reduce plasma Ser and Tau compared with the HS diets. Plasma carnosine concentration was lower for cows receiving the RSF vs. HS diets (17.7 vs. 18.9 μ*M*, respectively).

Moreover, supplementation with RP-MLH elevated ($P \leq 0.04$) plasma His and Met concentrations and tended ($P = 0.08$) to reduce plasma Thr concentration. Likewise, a trend ($P = 0.07$) was observed for lower plasma Gly concentration with supplemental RP-MLH. Plasma concentration of Tau was enhanced ($P = 0.001$) by supplemental RP-MLH (34.9 vs. 38.9 μ*M*). Neither dietary ES nor RP-MLH supplementation were able to modify the plasma concentrations of EAA, NEAA, and total AA.

Nutrient Digestibility and Urinary N Excretion

The apparent total-tract digestibility of nutrients and urinary excretion of nitrogenous metabolites are presented in Table 4.7. There were no significant dietary ES × RP-MLH supplementation interactions, as well as RP-MLH effects on digestibility and urinary excretion variables. In addition, the apparent total-tract digestibilities of DM (mean = 66.8%) and OM (mean = 67.8%) were not changed by dietary ES. However, the total-tract digestibilities of CP (67.0 vs. 68.1%), aNDF (42.7 vs. 49.2%), and ADF (49.7 vs. 54.4%) were lower in HS cows than RSF cows, respectively.

In the present study, RSF cows had reduced urinary creatinine concentration ($P < 0.001$; 5.57 vs. 6.92 m*M*) and greater urine volume ($P < 0.001$; 36.3 vs. 29.3 L/d) relative to HS cows. Both urinary urea N ($P < 0.001$; 185 vs. 239 g/d) and total N ($P < 0.001$; 279 vs. 335 g/d) were increased when ground corn was partially replaced with soyhulls and RP-fat. The urinary excretion of urea N, expressed as a proportion of total urinary N (72.0 vs. 66.4%) or N intake (31.0 vs. 25.2%), increased for the RSF diets relative to the HS diets. Similarly, urinary excretion of total N, expressed as a percentage of N intake ($P < 0.001$; 43.4 vs. 38.0%) also increased with feeding the RSF diets compared with the HS diets. Feeding the RSF diets tended ($P = 0.09$) to

elevate the urinary excretion of uric acid. However, dietary ES had no impact on urinary allantoin and purine derivatives.

Gaseous Emissions

Emissions of CO_2 and enteric CH_4, and O_2 consumption are presented in Table 4.8. No significant interactions between dietary ES and RP-MLH supplementation on these gaseous fluxes were observed. Additionally, neither dietary ES nor supplemental RP-MLH affected O_2 consumption (mean = 12.8 kg/d), emissions of CO_2 (mean = 16.2 kg/d) and CH_4 (534 g/d), CH_4 yield (mean = 18.5 g/kg of DMI), and CH_4 intensity (mean = 11.6 g/kg of ECM). Subsequently, dietary treatment had no impact on CH_4 energy (mean = 7.08 Mcal/d) and heat production (mean = 37.8 Mcal/d).

DISCUSSION

Feeding MP-deficient diets to lactating dairy cows has gained considerable attention in recent years in order to reduce feed costs and environmental N pollution (Castillo et al., 2000). However, MP-deficient diets often limit production of dairy cows, which is likely associated with shortages of EAA (Lee et al., 2011; Lee et al., 2012). Therefore, RP-AA supplements have been used to meet EAA requirements for synthesis of milk and milk protein in dairy cows fed MP-deficient diets (Lee et al., 2012; Giallongo et al., 2016). The effects of different dietary energy concentrations on mitigating milk yield losses have also been investigated when feeding MP-deficient diets to dairy cows (Broderick, 2003; Rius et al., 2010). Recently, we demonstrated that feeding a diet with reduced starch concentration decreased milk and milk protein yields in dairy cows receiving MP-deficient diets compared with a high starch diet (Zang et al.

unpublished). Nevertheless, it has been well established that high starch diets increase the risks of ruminal acidosis and displaced abomasum among other metabolic disorders (Allen et al., 2009; Allen and Piantoni, 2013). Thus, a comparison between fermentable energy (e.g., starch) and nonfermentable energy (e.g., RP-fat) for the improvement of milk production and nutrient utilization is needed when feeding MP-deficient diets.

In the present study, based on the NRC (2001) evaluation, all dietary treatments (HS, HS/MLH, RSF, and RSF/MLH) provided sufficient energy (NE_L balance, +0.1 to 2.4 Mcal/d) and insufficient MP (1-3% deficiency) and RUP (2-6% deficiency), and the HS and RSF diets contained similar levels of NE_L (1.53 vs. 1.59 Mcal/kg) but different levels of CP (15.9 vs. 16.8%), starch (32.6 vs. 21.7%), and ether extract (4 vs. 5.9%), respectively. We aimed to formulate isoenergetic and isonitrogenous basal diets; however, the CP difference was caused by an unexpected increase in the CP level of soyhulls. Supplemental RP-MLH alleviated these shortages of digestible Met, Lys, and His to some degree. The lack of interactions between dietary ES and RP-MLH supplementation for all response variables except milk yield (interaction, $P = 0.07$) and milk fat concentration (interaction, $P = 0.04$) in our study illustrated that neither fat nor corn starch optimized utilization of digestible Met, Lys, and His provided by RP-MLH toward milk protein synthesis.

Intake, Milk Yield, and PUN

In our study, there were no differences in DMI and milk yield between the HS (HS and HS/MLH) and RSF (RSF and RSF/MLH) diets, which are consistent with results reported by van Knegsel et al. (2007), which examined two diets with same forage-to-concentrate ratios and forages, but different concentrates (glucogenic vs. lipogenic). An earlier review by Ipharraguerre

and Clark (2003) summarized that corn grain can be replaced by up to 30% soyhulls on a DM basis without negatively affecting production performance of dairy cows. Although the RSF diets had higher dietary aNDF concentration than the HS diets, the same forage NDF level may lead to similar ruminal fill that determines DMI of dairy cows (Allen, 2000). We observed that both milk fat concentration and yield increased with feeding the RSF diets, similar to observations reported by Boerman et al. (2015), in which a high starch diet (a forage-to-concentrate ratio of 40:60) containing 33% corn grain and a high-fiber and high-fat diet (a forage-to-concentrate ratio of 50:50) containing 2.5% palmitic acid-enriched supplement were fed to mid-lactation dairy cows. The observed increase in milk fat production in RSF cows may result from supplemental RP-fat and higher dietary aNDF concentration in the RSF diets, which provided more palmitic acid (Piantoni et al., 2013), and acetate (Cunningham et al., 1993), respectively. Elevated supply of these FA improved milk fat synthesis in the mammary gland (Mathews et al., 2016; Urrutia et al., 2019). Moreover, 4% FCM yield and ECM yield increased by 7 and 5%, respectively with feeding the RSF diets, which can be explained by elevated milk fat yield. The RSF diets also improved feed efficiencies, expressed as 4% FCM yield/DMI and ECM yield/DMI, which resulted from higher yields of 4% FCM and ECM and similar DMI in RSF cows compared with HS cows.

We did not observe an increase in milk true protein yield in RSF cows, even though the RSF diets had higher dietary CP level than the HS diets. According to Oba and Allen (2003a), substitution of corn grain for fibrous byproducts captured more ruminal ammonia N to synthesize more microbial protein for milk protein synthesis in the mammary gland. Thus, more microbial protein may be produced in HS cows to compensate for a reduction in dietary CP level. In the present study, milk lactose concentration increased but milk lactose yield did not

change for the HS diets relative to the RSF diets. Similarly, Fredin et al. (2015a) reported that substitution of ground corn with soyhulls at ~8% of diet DM did not modify milk lactose concentration and yield. Milk urea N has been identified an indicator of efficiency of N utilization (Schepers and Meijer, 1998) and urine N excretion (Kauffman and St-Pierre, 2001). Compared with the HS diets, the RSF diets increased MUN and PUN concentrations by 23 and 27%, respectively, suggesting that RSF cows did not utilize N as effectively as HS cows that probably resulted from higher dietary CP level (Broderick and Clayton, 1997) and lower dietary starch concentration (Oba and Allen, 2003a). Reduced milk N efficiency for replacement of ground corn with soyhulls and RP-fat further confirmed this point.

Supplementation of RP-MLH failed to improve milk protein synthesis in the present study. Conversely, Giallongo et al. (2016) reported that these two variables were enhanced for RP-MLH in dairy cows fed a MP-deficient diet. Milk protein concentration and yield increased significantly with jugular infusion of MLH in dairy cows fed a MP-deficient diet (Yoder et al., 2020). The lower degree of MP deficiency in our study (mean = 1.75% deficiency) compared with that (mean = 3.5% deficiency) from Giallongo et al. (2016) and that (mean = 16% deficiency) from Yoder et al. (2020) may be related to no change in milk protein yield in response to RP-MLH.

Plasma Concentration of AA

Increased plasma concentrations of Arg, Ile, Lys, Thr, and Val with feeding the RSF (RSF and RSF/MLH) vs. HS (HS and HS/MLH) diets were mainly caused by higher dietary CP content (15.9 vs. 16.8%). Unexpectedly, the plasma concentrations of His, Leu, and Phe were lower for RSF cows relative to HS cows. These results could be attributed to higher proportions

of His (2.83 vs. 2.68% of CP), Leu (12.1 vs. 7.59% of CP), and Phe (4.98 vs. 4.85%) in ground corn as compared with soyhulls in our diets, respectively. The plasma concentrations of circulating EAA can be impacted by many factors including dietary supplies of EAA, ruminal protein synthesis, utilization of EAA in portal-drained viscera, hepatic EAA metabolism, and catabolism and anabolism of EAA in the mammary gland (Lapierre et al., 2006). Plasma concentrations of EAA in dairy cows can also be impacted by dietary starch level. For instance, substitution of an energy concentrate composed of 54.2% peas, 38.2% corn starch, 4.4% sugar molasses, 2.0% soy oil, and 1.2% NaCl for corn silage at 17% of diet DM, increased blood flow toward the mammary gland and mammary uptake of EAA, which in turn reduced the plasma concentrations of some EAA including Arg, His, Phe, and Thr (Omphalius et al., 2019). Furthermore, mammary catabolism of group 2 AA (i.e., Lys, Ile, Leu, and Val), hepatic catabolism of His + Met + Phe, and the plasma concentrations of most EAA (i.e., Ile, Lue, Lys, Phe, and Val) decreased with abomasal infusion of glucose (Omphalius et al., 2020). Taken together, the type of energy (corn starch vs. fat) may not play a significant role on AA utilization in tissues of dairy cows.

Additionally, feeding the RSF diets enhanced the plasma concentration of carnosine, which may be associated with higher plasma His concentration. More than 99% of carnosine, which is synthesized by carnosine synthase using His and Ala, is found in skeletal muscles (Maynard et al., 2001; Boldyrev et al., 2013). Therefore, it is conceivable that increased circulating concentrations of carnosine observed herein may be related to its transportation from skeletal muscles to plasma.

As expected, the plasma concentrations of His and Met were improved by 8% and 27% by supplemental RP-MLH. However, we found no change in plasma Lys concentration with RP-

MLH, probably due to the small increase (~2 g/d) in digestible Lys supplied by RP-Lys. For comparison, plasma concentrations of His, Met, and Lys increased in dairy cows consuming MP-deficient diets supplemented with RP-MLH (Lee et al., 2012; Giallongo et al., 2016). Overall, the effect of RP-MLH on plasma AA concentrations are influenced by the amounts and bioavailability of RP-AA products, dietary MP balance, and lactation stage (Lee et al., 2012; Giallongo et al., 2016; Zang et al., 2019).

Digestibility of Nutrients and Urinary Excretion of Nitrogenous Metabolites

Feeding the RSF diets (RSF and RSF/MLH) increased the apparent total-tract digestibility of CP compared with the HS counterparts (HS and HS/MLH). This may have been related to the high quality of soyhulls (17.5% CP, 53.4% aNDF, and 39.2% ADF) in the present study. The wide ranges of CP (9.4 to 19.2%), NDF (53.4 to 73.7%), and ADF (39.6 to 52.8%) of soyhulls have been reported (Ipharraguerre and Clark, 2003). Nevertheless, an earlier review by Ipharraguerre and Clark (2003) demonstrated that feeding soyhulls as a replacement for grain did not often change apparent total-tract digestibility of CP. Moreover, the RSF diets had higher total-tract NDF and ADF digestibilities presumably because soyhulls is a source with highly digestible NDF and ADF (Ipharraguerre and Clark, 2003).

Our results demonstrated that RSF cows produced 24% more urine than HS cows presumably because of higher dietary CP level in the RSF vs. HS diets. According to Holter et al. (1982), increased N intake led to more urinary volume. In support, Olmos Colmenero and Broderick (2006) reported that urine volume showed a linear increase to increasing dietary CP (13.5 to 19.4%). Elevated urinary urea N (g/d, % of total urinary N, and % of N intake) and total N (g/d and % of N intake) for the RSF vs. HS treatments are consistent with enhanced dietary

supply of CP and lower ruminally fermentable energy from starch. Olmos Colmenero and Broderick (2006) demonstrated that urinary excretion of urea N (g/d and % of total urinary N) and total N (g/d and % of N intake) increased linearly in response to incremental amounts of dietary CP fed to lactating dairy cows. Furthermore, fermentable energy from starch can optimize ammonia N utilization in the rumen and ultimately reduce ureagenesis in the liver (Oba and Allen, 2003b). In support, urinary urea N excretion (g/d, % of total urinary N, and % of N intake) decreased by elevated dietary starch level (Zang et al. unpublished).

Gaseous Fluxes

We observed that feeding the RSF diets improved feed efficiency and milk fat and TS yields. However, some concern exists about replacing fibrous byproducts with corn grain in dairy diets because it can increase greenhouse gas emissions and decrease energy utilization in dairy cows (Nielsen et al., 2013). The dairy industry contributes ~4% of global anthropogenic greenhouse gas emissions with CH_4, N_2O, and CO_2 representing 63, 25, and 12% of total emissions from dairy systems, respectively (FAO, 2019).

In the present study, there were no differences in emissions of enteric CH_4 and CO_2 between the HS diets and RSF diets. On the contrary, Hammond et al. (2016) demonstrated that elevated dietary NDF concentration via replacing cracked wheat with barley straw and soyhulls increased CH_4 emissions in dairy cows. The lack of response in enteric CH_4 emissions to increased aNDF concentration herein may have been caused by replacing ground corn with soyhulls rather than a forage source. Heat production and CH_4 energy did not differ between the HS and RSF treatments due to similar production of CH_4 and CO_2

CONCLUSIONS

Substitution of ground corn with soyhulls and RP-fat increased feed efficiency and milk fat and TS yields in dairy cows fed MP-deficient diets. Compared with the HS diets (HS and HS/MLH), enhanced milk fat production for the RSF diets (RSF and RSF/MLH) may have been caused by elevated ruminal fiber fermentation and palmitic acid supply. However, feeding the RSF diets decreased milk N efficiency and increased urinary excretion of urea N and total N. Supplementation of MP-deficient diets with RP-MLH tended to increase milk true protein concentration; however, RP-MLH had limited effects on production performance, apparent total-tract digestibility of nutrients, and urinary excretion of N metabolites in lactating dairy cows. Furthermore, we did not observe any treatment effects on emissions of CO_2 and enteric CH_4, as well as heat production and energy losses as CH_4. Further research is needed to compare MP-adequate diets and MP-deficient diets containing fibrous byproducts and RP-fat on production performance and balance of N and energy in lactating dairy cows.

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Table 4.1. Nutrient composition of dietary ingredients (mean ± SD) used in the experimental diets (% of DM, unless otherwise noted)

Item	Corn silage	Haylage[1]	Grass hay	Ground corn	Soyhulls	AminoMax[2]	Soybean Meal	Urea
No. of samples	4	4	4	4	4	4	4	4
DM, % of fresh matter	29.9 ± 1.17	34.0 ± 0.67	82.1 ± 0.57	81.5 ± 1.90	86.2 ± 1.98	82.8 ± 1.23	85.3 ± 1.61	99.7 ± 0.17
CP	7.93 ± 0.05	16.5 ± 0.78	9.35 ± 0.66	8.80 ± 0.08	17.5 ± 1.27	43.6 ± 0.21	52.6 ± 0.29	290 ± 1.82
aNDF	41.8 ± 1.35	54.4 ± 1.45	67.3 ± 1.61	8.60 ± 0.61	53.4 ± 2.51	24.8 ± 0.50	10.8 ± 0.96	NA[3]
ADF	24.7 ± 1.12	36.7 ± 1.26	42.2 ± 0.94	2.33 ± 0.26	39.2 ± 2.10	17.6 ± 0.75	7.85 ± 0.66	NA
Lignin	4.15 ± 0.74	7.20 ± 0.66	6.28 ± 0.50	1.05 ± 0.37	3.60 ± 1.55	7.38 ± 0.53	2.25 ± 1.57	NA
NFC[4]	42.7 ± 1.44	15.9 ± 0.97	16.8 ± 0.73	77.2 ± 1.20	17.1 ± 2.57	17.4 ± 0.80	27.4 ± 0.79	NA
Starch	34.3 ± 1.00	1.75 ± 0.30	0.50 ± 0.29	70.3 ± 1.83	2.95 ± 1.27	1.43 ± 0.30	1.23 ± 0.17	NA
Ether extract	3.70 ± 0.14	4.75 ± 0.26	1.95 ± 0.24	4.20 ± 0.22	7.05 ± 0.85	6.15 ± 0.54	1.30 ± 0.14	NA
NE_L, Mcal/kg of DM	1.62 ± 0.05	1.28 ± 0.03	1.03 ± 0.03	2.10 ± 0.01	1.72 ± 0.11	1.72 ± 0.04	1.78 ± 0.03	NA
Ash	3.86 ± 0.20	8.49 ± 0.82	4.65 ± 0.46	1.22 ± 0.43	5.09 ± 1.01	8.10 ± 0.73	7.94 ± 0.57	NA
Ca	0.17 ± 0.01	0.82 ± 0.04	0.40 ± 0.04	0.01 ± 0.01	0.51 ± 0.03	0.67 ± 0.01	0.58 ± 0.05	NA
P	0.32 ± 0.01	0.35 ± 0.03	0.23 ± 0.01	0.32 ± 0.01	0.26 ± 0.01	1.00 ± 0.02	0.80 ± 0.02	NA
Mg	0.16 ± 0.01	0.27 ± 0.03	0.19 ± 0.02	0.11 ± 0.01	0.27 ± 0.01	0.52 ± 0.01	0.33 ± 0.01	NA
K	1.10 ± 0.05	2.56 ± 0.25	1.60 ± 0.20	0.40 ± 0.02	1.50 ± 0.10	1.74 ± 0.03	2.44 ± 0.05	NA
Na	0.01 ± 0.00	0.06 ± 0.02	0.09 ± 0.01	0.00 ± 0.00	0.01 ± 0.00	0.10 ± 0.01	0.01 ± 0.01	NA
S	0.11 ± 0.00	0.24 ± 0.02	0.15 ± 0.01	0.10 ± 0.00	0.18 ± 0.01	0.82 ± 0.02	0.43 ± 0.01	NA
Fe, mg/kg of DM	141 ± 20.7	248 ± 76.5	94.3 ± 20.5	40.0 ± 5.23	399 ± 25.6	177 ± 7.62	90.8 ± 6.65	NA
Zn, mg/kg of DM	22.5 ± 1.29	27.5 ± 1.73	25.8 ± 1.26	21.3 ± 0.96	51.8 ± 2.22	58.5 ± 1.73	49.0 ± 0.82	NA
Cu, mg/kg of DM	5.00 ± 2.00	5.25 ± 0.50	5.00 ± 0.82	0.00 ± 0.00	6.25 ± 0.50	8.50 ± 0.58	13.3 ± 0.50	NA
Mn, mg/kg of DM	13.5 ± 1.29	34.8 ± 4.99	49.8 ± 4.65	5.00 ± 0.00	19.3 ± 0.96	55.3 ± 1.26	36.3 ± 1.26	NA
Mo, mg/kg of DM	0.80 ± 0.00	4.85 ± 0.68	2.03 ± 0.13	0.70 ± 0.29	1.70 ± 0.54	3.08 ± 0.25	4.75 ± 0.42	NA

[1]Haylage = mixed mostly grass-legume haylage.
[2]AminoMax = a mix of soybean meal and canola meal.
[3]NA = not analyzed.
[4]NFC = 100 – (CP% + aNDF% + ether extract% + ash%).

Table 4.2. Amino acid composition of dietary ingredients used in the experimental diets (n = 1 composited sample per feedstuff)

Item	Corn silage	Haylage[1]	Grass hay	Ground corn	Soyhulls	AminoMax[2]	Soybean Meal
EAA, % CP							
Arg	1.88	3.71	4.73	4.53	6.13	6.56	7.27
His	1.32	1.77	1.89	2.83	2.68	2.77	2.67
Ile	4.71	5.30	4.89	3.74	4.66	4.67	4.83
Leu	12.1	9.10	8.83	12.1	7.59	7.74	7.91
Lys	2.45	5.21	5.68	3.51	6.96	5.90	6.51
Met	2.07	1.94	1.89	2.04	1.40	1.89	1.42
Phe	4.52	5.83	5.68	4.98	4.85	4.77	5.32
Thr	3.20	4.77	4.89	3.51	3.83	4.31	3.90
Trp	0.56	0.97	0.95	0.68	0.89	1.37	1.46
Val	6.21	6.89	6.47	4.98	4.98	5.41	4.98
NEAA, % CP							
Ala	13.2	8.66	7.10	7.47	4.59	4.67	4.41
Asp	5.27	9.72	10.3	7.02	10.5	9.08	11.3
Cys	1.69	1.15	1.42	2.27	1.79	2.31	1.46
Gly	5.27	5.74	5.68	3.96	6.38	5.13	4.32
Glu	12.8	9.81	11.4	18.5	15.1	18.4	18.2
Orn	0.19	0.62	0.16	0.11	0.06	0.05	0.08
Pro	7.91	5.65	6.15	8.83	5.36	6.17	5.15
Ser	3.01	3.80	4.26	4.53	4.72	4.12	4.30
Try	2.07	3.27	2.52	2.72	3.83	3.43	3.92
Tau	2.07	1.06	2.05	1.25	1.08	0.25	0.19

[1]Haylage = mixed-mostly grass-legume haylage.
[2]AminoMax = a mix of soybean meal and canola meal.

Table 4.3. Ingredient and nutrient composition (% of DM, unless otherwise noted) of the experimental diets

	Diet[1]	
Item	HS	RSF
Ingredient, % of DM		
Corn silage	40.0	40.0
Grass Haylage	5.00	5.00
Grass Hay	5.00	5.00
Ground corn	26.1	10.0
Soyhulls	7.04	22.0
AminoMax	8.00	8.01
Soybean meal	5.81	5.59
Mineral mix[3]	2.00	2.00
BergaFat F100[2]	-	1.50
Urea	0.54	0.46
Sodium bicarbonate	0.50	0.50
Nutrient composition		
DM, % of fresh matter	46.7	47.0
CP	15.9	16.8
aNDF	31.4	38.0
Forage NDF	22.8	22.8
ADF	19.1	24.5
NFC[4]	46.1	36.6
Starch	32.6	21.7
Ether extract	4.00	5.90
NE_L, Mcal/kg DM	1.53	1.59
Ca	0.60	0.60
P	0.40	0.40

[1]HS = high-starch diet, RSF = reduced-starch + RP-fat diet.
[2]BergaFat F100 is a product containing 80% palmitic acid (Berg+Schmidt America, LLC, Libertyville, IL).
[3]Mineral and vitamin mix contained (as-fed basis): 269 mg/kg of monensin sodium, 13.8% Ca, 1% P, 11% Na, , 5.50% Mg, 16 mg/kg of Co, 180 mg/kg of Cu, 8.4 mg/kg of Se, 1,280 mg/kg of Zn, 24.0 kIU/kg of vitamin A, 6.64 kIU/kg of vitamin D 3, and 0.29 kIU/kg of vitamin E.
[4]NFC = 100 – (CP% + aNDF% + ether extract% + ash%).

Table 4.4. NRC (2001) evaluation of the experimental diets with different energy sources (corn starch vs. RP-fat) with or without rumen-protected Met, Lys, and His (RP-MLH[1])

	Treatment[3]			
Item[2]	HS	HS/MLH	RSF	RSF/MLH
NE_L, Mcal/d				
Requirement	43.6	43.6	43.5	43.5
Supply	44.3	44.5	45.9	45.9
Balance	0.7	1.0	2.4	2.4
MP, g/d				
Requirement	3,154	3,160	3,148	3,148
Supply	3,062	3,084	3,121	3,121
Balance	−92	−76	−27	−27
RDP, g/d				
Requirement	2,814	2,830	2,846	2,846
Supply	2,909	2,925	3,007	3,007
Balance	94	95	162	162
RUP, g/d				
Requirement	1,827	1,822	1,817	1,817
Supply	1,714	1,729	1,836	1,836
Balance	−113	−94	−34	−34
dHis,[2] g/d				
Requirement[3]	69	70	69	69
Supply from the diet	66	66	67	67
Supply from RP-His	0	3	0	3
Balance	−3	0	−2	1
dMet,[2] g/d				
Requirement[3]	69	70	69	69
Supply from the diet	58	59	58	58
Supply from RP-Met	0	7	0	7
Balance	−11	−4	−11	−4
dLys,[2] g/d				
Requirement[3]	208	208	208	208
Supply	202	202	209	209
Supply from RP-Lys	0	2	0	2
Balance	−6	−4	1	3

[1]RP-MLH = 12 g/d of RP-Met (Smartamine® M; Adisseo USA Inc., Alpharetta, GA), 9 g/d of RP-Lys (AjiPro®-L; Ajinomoto Heartland, Inc., Eddyville, IA), and 15 g/d of RP-His (a prototype supplement; Ajinomoto Co. Inc., Kawasaki-shi, Japan).
[2]All values were estimated using the NRC (2001) model with actual DMI and nutrient composition of dietary ingredients during the experiment and milk yield and components before the experiment.
[3]HS = high starch diet, HS/MLH = high-starch diet supplemented with RP-MLH, RSF = reduced starch + RP-fat diet, and RSF/MLH = reduced-starch + RP-fat diet supplemented with RP-MLH.
[3]Requiements of dHis, dMet, and dLys were calculated as 2.2, 2.2, and 6.6% of MP requirements, respectively.

Table 4.5. Dry matter intake, milk yield and composition, plasma urea N (PUN), BCS, and BW in lactating dairy cows fed different energy sources (corn starch vs. RP-fat) with or without rumen-protected Met, Lys, and His (RP-MLH[1])

	Treatment[2]					*P*-value[3]		
Item	HS	HS/MLH	RSF	RSF/MLH	SEM	ES	MLH	ES × MLH
DMI, kg/d	29.0	29.2	28.9	28.9	0.66	0.45	0.66	0.62
Milk yield, kg/d	44.6	45.0	45.6	44.8	1.18	0.17	0.52	0.07
Milk yield/DMI, kg/kg	1.54	1.54	1.58	1.56	0.04	0.03	0.39	0.33
4% FCM,[4] kg/d	40.1	40.0	42.7	43.0	1.32	<0.001	0.84	0.70
4% FCM/DMI, kg/kg	1.38	1.37	1.47	1.49	0.03	<0.001	0.88	0.42
ECM,[5] kg/d	45.0	44.6	46.8	47.1	14.0	<0.001	0.96	0.45
ECM/DMI, kg/kg	1.53	1.53	1.61	1.63	0.03	<0.001	0.70	0.55
Milk fat, %	3.40	3.29	3.59	3.66	0.11	<0.001	0.62	0.04
Milk fat, kg/d	1.52	1.47	1.64	1.65	0.06	<0.001	0.48	0.15
Milk true protein, %	3.15	3.19	3.09	3.11	0.03	<0.001	0.06	0.85
Milk true protein, kg/d	1.41	1.42	1.40	1.41	0.04	0.65	0.36	0.82
Milk lactose, %	4.99	4.99	4.95	4.93	0.03	<0.01	0.49	0.26
Milk lactose, kg/d	2.22	2.24	2.26	2.23	0.06	0.53	0.85	0.46
Milk TS, %	12.5	12.4	12.6	12.6	0.12	<0.001	0.96	0.14
Milk TS, kg/d	5.56	5.54	5.72	5.72	0.16	<0.01	0.91	0.85
SCC, ×1,000 cells/mL	47.9	45.6	45.2	53.0	15.7	0.68	0.63	0.35
Milk N, % of N intake	30.4	30.7	28.9	29.2	0.61	<0.001	0.38	0.99
MUN, mg/dL	12.0	12.2	14.7	15.0	0.41	<0.001	0.33	0.90
PUN, mg/dL	12.4	12.1	15.4	15.7	0.46	<0.001	1.00	0.29
BCS	3.13	3.14	3.08	3.11	0.09	0.15	0.39	0.70
BW, kg	776	779	773	775	12.3	0.16	0.20	0.73
BW change, kg/d	0.69	0.96	0.52	0.76	0.15	0.21	0.09	0.91

[1]RP-MLH = 12 g/d of RP-Met (Smartamine® M; Adisseo USA Inc., Alpharetta, GA), 9 g/d of RP-Lys (AjiPro®-L; Ajinomoto Heartland, Inc., Eddyville, IA), and 15 g/d of RP-His (a prototype supplement; Ajinomoto Co. Inc., Kawasaki-shi, Japan).
[2]HS = high starch diet, HS/MLH = high-starch diet supplemented with RP-MLH, RSF = reduced starch + RP-fat diet, and RSF/MLH = reduced-starch + RP-fat diet supplemented with RP-MLH.

[3]ES = main effect of energy source (corn starch vs. RP-fat), MLH = main effect of RP-MLH supplementation, and ES × MLH = interaction between energy source and RP-MLH.
[4]4% FCM = (0.4 × kg of milk) + (15 × kg of milk fat); Gaines and Davidson (1923).
[5]ECM = (0.327 × kg of milk) + (12.95 × kg of milk fat) + (7.65 × kg of milk protein); Tyrrell and Reid (1965).

Table 4.6. Concentrations of plasma AA and carnosine in lactating dairy cows fed different energy sources (corn starch vs. RP-fat) with or without rumen-protected Met, Lys, and His (RP-MLH[1])

	Treatment[2]					*P*-value[3]		
Item	HS	HS/MLH	RSF	RSF/MLH	SEM	ES	MLH	ES × MLH
EAA, μ*M*								
Arg	76.0	73.1	81.6	83.4	3.33	<0.01	0.86	0.41
His	56.0	57.5	49.8	56.4	3.02	0.06	0.04	0.18
Ile	122	114	134	130	5.56	<0.001	0.11	0.62
Leu	145	142	134	132	7.74	<0.01	0.56	0.99
Lys	80.8	78.8	83.7	88.0	4.17	0.08	0.74	0.35
Met	25.6	31.7	25.2	32.6	1.16	0.78	<0.001	0.55
Phe	49.2	49.2	46.5	47.9	1.71	0.09	0.54	0.54
Thr	101	90.6	104	102	4.28	0.05	0.08	0.27
Trp	49.9	48.1	48.8	49.2	1.55	0.99	0.57	0.37
Val	248	244	261	252	10.9	0.09	0.29	0.71
NEAA, μ*M*								
Ala	262	261	279	283	13.3	0.02	0.90	0.79
Asn	48.3	43.6	48.5	48.6	1.80	0.15	0.19	0.19
Asp	3.44	3.32	3.23	3.30	0.17	0.41	0.85	0.51
Gln	290	283	275	274	9.11	0.12	0.59	0.68
Glu	37.4	38.9	38.9	40.2	1.82	0.26	0.28	0.93
Gly	315	302	339	312	19.1	0.14	0.07	0.54
Pro	90.5	90.6	87.7	87.1	3.85	0.32	0.94	0.90
Ser	79.5	75.1	74.0	72.6	2.20	0.07	0.19	0.50
Tau	36.6	39.1	33.1	38.6	1.56	0.08	0.001	0.19
Tyr	51.8	48.9	50.8	51.8	2.78	0.56	0.54	0.20
Sum and ratio of AA								
∑ EAA, μ*M*	953	930	975	974	35.3	0.13	0.58	0.61
∑ NEAA, μ*M*	1,212	1,166	1,230	1,210	35.9	0.24	0.22	0.62
Total AA,[4] μ*M*	2,178	2,092	2,201	2,184	50.5	0.19	0.24	0.42
Lys:Met	3.17	2.54	3.45	2.72	0.13	<0.01	<0.001	0.59
Carnosine, μ*M*	19.3	18.5	17.6	17.8	0.87	0.01	0.55	0.33

[1]RP-MLH = 12 g/d of RP-Met (Smartamine® M; Adisseo USA Inc., Alpharetta, GA), 9 g/d of RP-Lys (AjiPro®-L; Ajinomoto Heartland, Inc., Eddyville, IA), and 15 g/d of RP-His (a prototype supplement; Ajinomoto Co. Inc., Kawasaki-shi, Japan).
[2]HS = high starch diet, HS/MLH = high-starch diet supplemented with RP-MLH, RSF = reduced starch + RP-fat diet, and RSF/MLH = reduced-starch + RP-fat diet supplemented with RP-MLH.
[3]ES = main effect of energy source (corn starch vs. RP-fat), MLH = main effect of RP-MLH supplementation, and ES × MLH = interaction between energy source and RP-MLH.
[4]Total AA = EAA + NEAA.

Table 4.7. Apparent total-tract digestibility of nutrients and urinary excretion of nitrogenous compounds in lactating dairy cows fed different energy sources (corn starch vs. RP-fat) with or without rumen-protected Met, Lys, and His (RP-MLH[1])

	Treatment[2]					*P*-value[3]		
Item	HS	HS/MLH	RSF	RSF/MLH	SEM	ES	MLH	ES × MLH
Apparent total-tract digestibility								
DM	66.6	66.8	66.9	67.0	0.52	0.60	0.77	0.94
OM	67.7	67.7	67.7	67.9	0.52	0.78	0.86	0.88
CP	66.7	67.2	68.0	68.2	0.65	0.03	0.56	0.81
aNDF	43.2	42.1	48.9	49.5	1.22	<0.001	0.76	0.29
ADF	49.5	49.8	54.5	54.3	1.22	<0.001	0.92	0.82
Urinary excretion								
Creatinine, m*M*	6.96	6.88	5.57	5.56	0.21	<0.001	0.77	0.82
Volume, L/d	29.1	29.4	35.9	36.6	1.15	<0.001	0.58	0.84
Urea-N, g/d	188	181	238	240	6.21	<0.001	0.58	0.25
Total-N, g/d	284	273	336	334	7.25	<0.001	0.18	0.37
Urea-N, % Total-N	66.6	66.1	71.8	72.1	1.03	<0.001	0.93	0.67
Urea-N, % N intake	25.6	24.7	30.8	31.1	0.85	<0.001	0.57	0.23
Total-N, % N intake	38.7	37.2	43.6	43.2	0.99	<0.001	0.18	0.43
Uric acid, mmol/d	132	131	134	138	5.64	0.09	0.50	0.28
Allantoin, mmol/d	342	334	345	343	9.97	0.34	0.46	0.65
PD, mmol/d	472	465	479	481	13.8	0.14	0.70	0.53

[1]RP-MLH = 12 g/d of RP-Met (Smartamine® M; Adisseo USA Inc., Alpharetta, GA), 9 g/d of RP-Lys (AjiPro®-L; Ajinomoto Heartland, Inc., Eddyville, IA), and 15 g/d of RP-His (a prototype supplement; Ajinomoto Co. Inc., Kawasaki-shi, Japan).
[2]HS = high starch diet, HS/MLH = high-starch diet supplemented with RP-MLH, RSF = reduced starch + RP-fat diet, and RSF/MLH = reduced-starch + RP-fat diet supplemented with RP-MLH.
[3]ES = main effect of energy source (corn starch vs. RP-fat), MLH = main effect of RP-MLH supplementation, and ES × MLH = interaction between energy source and RP-MLH.

Table 4.8. Consumption of oxygen (O_2), emissions of carbon dioxide (CO_2) and methane (CH_4)[1], CH_4 energy, and heat production in lactating dairy cows fed different energy sources (corn starch vs. RP-fat) with or without rumen-protected Met, Lys, and His (RP-MLH[2])

	Treatment[3]					*P*-value[4]		
Item	HS	HS/MLH	RSF	RS/MLH	SEM	ES	MLH	ES × MLH
O_2, kg/d	12.6	12.6	12.8	13.0	0.25	0.14	0.72	0.71
CO_2, kg/d	16.2	16.3	16.0	16.2	0.33	0.30	0.52	0.73
CH_4, g/d	530	530	536	539	24.5	0.63	0.93	0.92
CH_4, g/kg of DMI	18.3	18.3	18.5	18.7	0.72	0.55	0.85	0.87
CH_4, g/kg of ECM	11.9	11.8	11.4	11.4	0.47	0.25	0.95	0.89
CH_4 energy,[5] Mcal/d	7.03	7.02	7.10	7.15	0.32	0.62	0.93	0.91
Heat production,[6] Mcal/d	37.9	38.1	37.3	37.7	0.74	0.28	0.56	0.75

[1]Gases were measured using GreenFeed (C-Lock Technology Inc., Rapid City, SD). Data were derived from 8 individual measurements over 4-d period.

[2]RP-MLH = 12 g/d of RP-Met (Smartamine® M; Adisseo USA Inc., Alpharetta, GA), 9 g/d of RP-Lys (AjiPro®-L; Ajinomoto Heartland, Inc., Eddyville, IA), and 15 g/d of RP-His (a prototype supplement; Ajinomoto Co. Inc., Kawasaki-shi, Japan).

[3]HS = high starch diet, HS/MLH = high-starch diet supplemented with RP-MLH, RSF = reduced starch + RP-fat diet, and RSF/MLH = reduced-starch + RP-fat diet supplemented with RP-MLH.

[4]ES = main effect of energy source (corn starch vs. RP-fat), MLH = main effect of RP-MLH supplementation, and ES × MLH = interaction between energy source and RP-MLH.

[5]CH_4 energy = CH_4 × 13.18 kcal/g (Judy et al, 2019).

[6]Heat production (MJ/d) = 0.0184 × QCO_2 (L/d) + 7.50 (Bayat et al., 2019).

APPENDIX I: THE IACUC APPROVAL FOR CHAPTER II

University *of* New Hampshire

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

10-Mar-2017

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

IACUC #: 170202
Project: Utilization of Endogenous Histidine Reserves in Dairy Cows Fed a Metabolizable Protein-Deficient Diet
Approval Date: 23-Feb-2017

The Institutional Animal Care and Use Committee (IACUC) reviewed and approved the protocol submitted for this study under Category D on Page 5 of the Application for Review of Vertebrate Animal Use in Research or Instruction - *Animal use activities that involve accompanying pain or distress to the animals for which appropriate anesthetic, analgesic, tranquilizing drugs or other methods for relieving pain or distress are used.*

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

APPENDIX II: THE IACUC APPROVAL FOR CHAPTER III

University *of* New Hampshire

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

10-Apr-2018

Brito, Andre Fonseca De
ANFS
Keener Dairy Research
Durham, NH 03824

IACUC #: 180305
Project: Feeding Rumen-Protected Amino Acids to Lactating Dairy Cows Given Diets with Different Energy Density
Approval Date: 22-Mar-2018

The Institutional Animal Care and Use Committee (IACUC) reviewed and approved the protocol submitted for this study under pain or distress category C - *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,

Jessica A. Bolker, Ph.D.
Chair

cc: File

APPENDIX III: THE IACUC APPROVAL FOR CHAPTER IV

University *of* New Hampshire

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

19-Feb-2019

Brito, Andre Fonseca De
Agriculture, Nutrition, & Food Systems
Keener Dairy Research Building
Durham, NH 03824

IACUC #: 190202
Project: Interactions between Rumen-Protected Amino Acids and Energy Source on Milk Production in Dairy Cows.
Approval Date: 18-Feb-2019

The Institutional Animal Care and Use Committee (IACUC) reviewed and approved the protocol submitted for this study under Category C in Section V 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,

Rebecca Rowe, Ph.D.
Chair

cc: File