Assessing availability and metabolism of amino acids in dairy cattle using stable isotope-based approaches

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ACADEMIC ABSTRACT

Determining the AA availability and metabolism in ruminant is a big challenge due to the rumen fermentation and complicated post absorption utilization. Current techniques used for direct determination of AA absorption and metabolism are laborious and expensive with large variation. The objectives of this project were to investigate AA availability of rumen undegradable protein, develop a stable isotope technique for determination of microbial protein and to evaluate the metabolism of amino acids in mammary glands of dairy cattle using stable isotope-based approaches. In the first experiment, seven heifers (258 ± 28 kg BW) were randomly chosen and assigned to 8 treatment sequences in a 7 x 8, incomplete, Latin square design. Treatments were a basal diet (BD), and 10% (DM basis) of BD replaced by corn silage (CS), grass hay (GH), alfalfa hay (AH), dried distillers grain (DDGS), soybean hulls (SH), wet brewers grain (BG), or corn grain (CG). Individual essential AA availabilities for corn silage, grass hay, alfalfa hay, dried distillers grain, soyhulls, brewers grain and corn grain were 33.4, 29.9, 34.1, 40.6, 28.8, 41.2, and 36.5% of the essential AA in each of the respective ingredients when a loss of 8.27% to splanchnic utilization during first pass was assumed; however, availability varied across individual essential AA. In the second experiment, twelve cows were blocked into 3 groups according to days in milk and randomly assigned to 4 treatments in a repeated 4 x 4 Latin square design with 2 factors to evaluate the essential AA availability from microbial protein and rumen undegradable protein under different rumen fermentation conditions. The 4 treatments were high rumen undegradable protein and high starch (HPHS), low rumen undegradable protein and high starch (LPHS), high rumen undegradable protein and low starch (HPLS) and low rumen undegradable protein and low starch

(LPLS). Microbial protein synthesis calculated from purine derivatives was positively associated with rumen degradable protein, which was consistent with total microbial AA entry derived from the isotope dilution model indicating that the isotope based approach was representative. The individual essential AA availability from microbial protein was determined by isotope technique, whereas the PD method was just total PD absorption reflecting CP absorption. The metabolizable AA estimates from NDS nutritional model was similar to results from isotope dilution models, but with smaller difference among treatments. The microbial protein estimated from White's model showed the same trend among treatments compared to isotope dilution model, which may imply it represents the rumen fermentation better. The average essential AA digestibility for microbial AA was 82%, which varied across individual AA and treatments. In the third experiment, four cows (78 ± 10 DIM) were used to study the effects of jugular infusion of 2 groups of AA on essential AA uptake and metabolism by mammary glands in a 4 x 4 Latin square design. Treatments were jugular infusion of saline (CON), methionine plus lysine plus histidine (MKH), isoleucine plus leucine (IL), or MKH plus IL (MKH+IL). The MKH increased milk protein yield in high producing dairy cows. The IL infusion increased milk and milk lactose yields. The production response was associated with a change in mammary plasma flow together with changes in AA uptake and metabolism in mammary gland. Mammary uptake of essential AA was 135 % of milk protein output. Efflux of EAA from the mammary to blood was 13-61% of influx, which was high for BCAA but low for Met and Lys. Changes in influx and efflux resulted in net uptake difference of infused essential AA that were responsive to varying supplies resulting in maintenance of homeostasis. The proportion of AA catabolized and used for milk protein was affected by EAA infusion, which demonstrated plasticity of mammary gland in AA metabolism. Overall, results suggested essential AA availability from rumen undegraded protein and microbial

protein varied across individual AA and diets and can be affected by rumen fermentation. After absorption, EAA transport into mammary tissue was bi-directional and their metabolism was affected by AA supply and energy. Using a single coefficient to represent all AA digestibility in MCP or feed ingredient and an integrated efficiency of MP-AA converted into milk protein is inaccurate.

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PUBLIC ABSTRACT

Studies in monogastric animals have showed that balancing AA supply with animal requirements can improve the efficiency of N utilization. In order to build a model for AA balanced diet formulation, the composition of feed ingredients, the profile and digestibility of EAA for the rumen undegradable protein and microbial protein, the partition and efficiency of EAA utilization in mammary glands must be determined accurately. However, current AA degradation, digestibility and metabolism data used in nutritional models are from in vitro and in situ studies, which have not been fully validated against in vivo observations. This research used an in vivo stable isotope-based approach to determine amino acid availability for commonly used feed ingredients in dairy industry. The microbial protein AA and rumen undegradable protein AA availability was determined by adapting this isotope technique and introducing another isotope into rumen to label microbes. In addition, by coupling stable isotope tracers with arterio-venous difference technique and compartmental modelling, essential AA metabolism in mammary glands of dairy cows were qualified. Total essential AA availabilities for corn silage, grass hay, alfalfa hay, dried distillers grain, soyhulls, brewers grain and corn grain were similar to values from meta-analysis of mobile bag results, but the availabilities of individual AA were more variable compared to in vitro and in situ results. The model derived microbial AA availability was consistent with the microbial protein calculated from NDS and Felming's model. However, our model predicted a lower proportion of metabolizable AA from microbial protein under diets including low rumen degradable protein, which might imply the NDS nutritional model overestimates microbial protein under low protein diets. The microbial protein estimated from White's model showed the same

trend among treatments compared to isotope dilution model, which may imply it represents the rumen fermentation better. The averaged essential AA digestibility form microbial protein was 82%, which varied across individual AA and treatments. After absorption, mammary uptake of essential AA was 135 % of milk protein output. Cellular efflux represented 13 to 61% of essential AA uptake. The proportion of AA catabolized and used for milk protein was affected by essential AA infusion, which demonstrated the plasticity of mammary glands in AA metabolism. In conclusion, the results from isotope technique quantified the essential AA availability from rumen undegradable protein for various feed ingredients and from microbial protein under different feeding conditions. The essential AA transport and metabolism in mammary glands were regulated by multi factors and essential AA supply.

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CHAPTER 1: Introduction

Nitrogen (N) has played an important role as nutritive component in animal production. However, ruminants are relatively inefficient in using N with averaged 25% dietary N retained in milk (Jonker et al., 2002). Nitrogen waste is excreted through urine and feces, contributing to environment pollution (Külling et al., 2001). Reports show that around 1.3 million tons N is excreted by dairy cows per year, accounting for 12.3% of total N waste from animal production (Jonker et al., 2002, Caraviello et al., 2006, EPA, 2014, USDA-ERS, 2019). Compared to fecal N, urinary N excretion has more variation, which implied an opportunity of manipulation (Dijkstra et al., 2013). Huhtanen and Hristov (2009) also reported that the dietary N used for milk varied widely from 14 to 45% .Therefore, ruminant nutrition research has been focusing on understanding the process of N utilization and studying practices that can improve the efficiency of N utilization for productive purposes (Schwab and Broderick, 2017). However, N utilization in ruminant involved in complex processes, including degradation in rumen, digestion and absorption in intestine, and metabolism in different tissues, which makes it hard to manipulate N utilization.

Efforts have been made to improve nutritional models to represent processes of N utilization to estimate the N requirement and supply, which however fail to improve the N efficiency beyond 25% (Hristov et al., 2011). The potential reasons are the shortcomings of the current metabolizable protein (MP) approach, which requires the estimates of microbial protein (MCP), rumen undegraded protein(RUP) and endogenous protein (ECP) and their digestibility (Arriola Apelo et al., 2014). Current methods for assessing protein flows and digestibility in small intestine all have inherent disadvantages (Hristov et al., 2019). The uncertainty was then introduced into models since nutritional models were built and evaluated based on those results. Therefore, the development of more accurate and practical methods to evaluate N flow and

digestibility is required. Lapierre et al. (2012) also suggested a unique coefficient of AA utilization after absorption was not accurate, and better knowledge of AA partition and efficiency of utilization in mammary gland can give us more accurate and precise AA requirement for milk synthesis.

It is a big challenge to solve above mentioned problems. Firstly, determining the AA availability in ruminant is a big challenge due to the rumen fermentation. The nutritive values of feed protein is greatly influenced by the extent to which it is degraded in the rumen and the digestibility of RUP in the small intestine (Paz et al., 2014). Techniques used for direct determination of MCP synthesis are laborious and expensive with large variation, and indirect approaches have been shown not accurate enough to be widely adopted (Hristov et al., 2019). A lot of methods, like in situ, in vivo, and in vitro have been used to detect RUP and MCP digestibility in the gut, but results are inconsistent across different methods (Jahani-Azizabadi et al., 2009). The in situ method may overestimate rumen degradation and intestinal digestibility due to the assumption that all solubilized N is used by microbes (Apelo et al., 2014, White et al., 2017). In vitro techniques are also problematic because enzyme digestion can't mimic in vivo process perfectly, such as enzyme specificity, ammonia recycling, digesta flow, and energy supply (Stern et al., 1997, Paz et al., 2014). The in vivo approach can overcome some of the problems of in situ or in vitro methods, but it is technically hard to conduct due to duodenum or ileum cannula insertion and variation caused by digesta flow markers. Secondly, the process from intestinal AA absorption to milk protein secretion involves complex interactions between organs (Lapierre et al., 2012). Techniques for studying mammary AA metabolism, like isotope-based methods, are always laborious and expensive, and the methods based on estimated MP and blood flow cause variable results (Linzell, 1974, Mepham, 1982). Therefore, in the past decade, efforts to improve the

estimates of AA requirement didn't show great progress. Requirement for maintenance and milk protein synthesis were still calculated by a single aggregated coefficient (Lapierre et al., 2014). NRC (2001) model has been used to describe nutrient intake and conversion to products (milk, growth, etc.) and factors affecting efficiency, which, however, are derived with the input and output known, hence, not accurate for predicting purpose (St-Pierre and Thraen, 1999). Therefore, current model predictions particularly for milk protein are not always consistent with experimental observations. For example, nutritional models are not sensitive to the profile of AA, energy status, affinity of mammary uptake of AA, or signaling effects of AA. Doepel et al. (2016) reported that Phe deficiency greatly decreased milk protein yield, while Thr and Tyr deficiency didn't. The potential reason was the increased blood flow responding to Thr shortage. Therefore, more knowledge of regulation of AA uptake and protein synthesis by the mammary gland is required to improve current models available for ration formulation.

Estes et al. (2018) adapted a stable isotope technique to assess the RUP-AA availability from individual feed ingredients used based on method used by Maxin et al. (2013). Errors of determination for AA availability from each ingredient are approximately 10% using this method, which is a large improvement compared to previously used methods (Titgemeyer et al., 1989). We assume that this method can be used to detect the RUP-AA availability for commonly used feed ingredients. The MCP-AA availability may also be detected by adapting this isotope technique and introducing another isotope (^{15}N) into rumen to label microbes. In addition, by coupling stable isotope approach with arterio-venous difference technique and mammary compartmental modelling, we can quantify essential AA uptake and metabolism in mammary glands of dairy cows. Therefore, the objectives of this project were as follows:

1) Review current literatures relative to protein/AA availability and metabolism in dairy cattle.
2) Determine EAA availability for seven feed ingredients commonly used in dairy rations: dried distillers grain, corn grain, brewers grain, soybean hulls, corn silage, alfalfa hay and grass hay.
3) Evaluate a stable isotope-based approach for determination of the availability of EAA from MCP and RUP in response to starch and RDP in lactating dairy cows.
4) Assess EAA uptake and metabolism in mammary glands that may mediate changes in milk protein yield due to EAA infusion.

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CHAPTER 2: Literature Review

2.1. Overview

In current dairy production system, average dietary N captured in milk is 25%, with the remainder being excreted into environment through urine and feces, and 60% of N excretion occurs after absorption. Nitrogen excretion can cause environment problems, like eutrophication of aquatic ecosystems, increased atmospheric particles, decreased stratospheric ozone concentrations, climate change, acid precipitation and drinking water pollution (Wolfe and Patz, 2002b). In addition, protein represents approximately 42% of the feeding cost (St-Pierre, 2012). From a producer perspective, N not retained in milk or tissue represents waste of an expensive dietary nutrient. To reduce the impact on the environment and to increase economic profits of the dairy industry requires improving efficiency of dietary N incorporation into milk protein. A lot of efforts have been made to improve the N efficiency in cattle (Külling et al., 2001, Lapierre et al., 2005, Agle et al., 2008, Bouwman et al., 2013), which however made little progress. Huhtanen and Hristov (2009) reported the mean N efficiency varied from 14 to 45%. The wide variation implies the possibility for manipulation. Dijkstra et al. (2013) also reported a maximum theoretical efficiency of 43%. However, the N efficiency on commercial dairy farm is often far lower from 43%. The main reason for inefficient N utilization is that producers tried to maximize milk yield to improve profit margins by overfeeding protein in the past decades (Doepel et al., 2004, Colmenero and Broderick, 2006a). The simplest strategy to optimize N utilization can be lowering dietary protein supply (Kebreab et al., 2010), which however may causes MP deficiency (NRC, 2001) and decreases milk protein production (Cabrita et al., 2011). Studies in monogastric animals have showed that balancing AA supply with animal requirements can improve the efficiency of N utilization and maintain the production at the same time (Baker, 1996, Nahm, 2002). (Haque et al.,

2012) also indicated feeding cows diets with "ideal" EAA profile proposed by (Rulquin et al., 2007) increased milk protein yield and N efficiency at both 13.6 and 15.2% protein supply. Building a model for balancing AA requires a supply of nutrients that matches requirements exactly. This challenges dairy scientists to accurately define the AA output and then to determine the composition of feed ingredients, the profile and digestibility of AA for RUP and MCP, the partition and efficiency of AA utilization by mammary glands. Although current models have been improved for balancing diets for limiting AA like Met and Lys (NRC, 2001) and His (Lapierre et al., 2008b, Lee et al., 2012a, Giallongo et al., 2017), limitations still exist and current knowledge doesn't support models based on quantified EAA supply and requirement.

In the future, it will remain a big challenge to assure higher feed efficiency while maintain profitability. To achieve improvement of N efficiency, better knowledge is required for better description of nutrient availability and more accurate and precise estimates of animals' nutrient requirements. This literature review will summarize the N efficiency in dairy cattle, the progress on protein and AA nutrition, methods used to determine AA availability and requirement, the factors that affect AA uptake and metabolism in mammary glands.

2.2. Significance of Improving Nitrogen Efficiency in Dairy Cattle

Nitrogen is essential nutrient subcomponent of a diet for animal growth and productivity. Animal production is responsible for a large part of nitrous oxide and ammonia emissions, i.e. 40% in Europe (Morard, 1999) and 70-85% in United State with 12.3% from dairy cattle (EPA, 2014). Excessive nitrate and nitrite can cause eutrophication and hypoxia of waterways, increased nitrates pollution in precipitation, soil, and water (Wolfe and Patz, 2002a, Leytem, 2014). Compared to monogastric animals, ruminants are relatively inefficient in feed N utilization. Hristov et al. (2004) indicated that the gross N efficiency was 24.7 ± 3.99% in a meta-analysis by using data from 846

experimental diets with similar CP. Jonker et al. (2002) also indicated that milk yield accounted for 25% of the variation in N efficiency according to survey from 463 farms in Maryland, Virginia, West Virginia, and Delaware. According to a national survey carried out on 103 large dairies, the average CP in rations was 17.8 ± 0.1% CP and average DMI was 22.1 kg/d (Caraviello et al., 2006). If we assumed 9 million dairy cattle in the United States (USDA-ERS, 2019), the calculated N waste from dairy cows is 1.3 million tons per year. Improvement of protein efficiency by 10% units (i.e. 35%) for dairy production would reduce N output by 0.17 million tons annually (assumed 9 million cows consuming 22.1 kg DM with 17.8% CP), which will be important for global competitiveness of US dairy being environmentally sustainable and economically efficient. Nitrogen efficiency has been shown to be higher under experimental conditions than under commercial conditions (Powell et al., 2010), suggesting that it is possible to improve N efficiency in feeding practices. In experimental settings, mean N efficiency was 30% and ranged between 18 and 42% (Hristov et al., 2004, Huhtanen and Hristov, 2009, Phuong et al., 2013). Low N efficiency in dairy cows is partly due to excessive protein intake (Castillot et al., 2000, Broderick, 2003, Ipharraguerre et al., 2005). Broderick (2003) found that increasing feed CP by 1.7% caused 3% reduction in N efficiency. Colmenero and Broderick (2006b) also reported a reduction of 5.4% in N efficiency by increasing 3% CP in the diet. Therefore, reducing N intake is the simplest strategy to reduce N excretion (Dijkstra et al., 2011, Hristov et al., 2011). However, according to VandeHaar and St-Pierre (2006), underfeeding protein has greater risk than overfeeding protein, so farmers had no incentive to improve protein efficiency in the past. Thus, it is a big challenge in N management to reduce environmental impact without impairing animal performance. The overall N efficiency in lactating sows or pigs for meat production are 43 and 57%, which was achieved through AA balancing (Millet et al., 2018). Therefore, it is assumed that N efficiency can

also be improved in ruminants without hurting production through AA balancing. Thus it is important to study N metabolism and develop accurate and precise techniques to evaluate N utilization in ruminants.

2.3. Protein and AA Nutrition in Dairy Cattle

Protein is one of most important nutrients for dairy cattle. However, over time people realized that crude protein (CP) intake does not provide enough information to meet animal requirement since CP is greatly changed in the rumen, which has been fully described in papers published in the Journal of Dairy Science (JDS) in the past 60 years. As a result, protein nutrition of dairy cows has moved from dietary CP to the ammonia and AA needs of ruminal fermentation for MCP synthesis and the metabolism protein (MP) or MP-AA for the cow. Compared to monogastric animals, the dairy cattle have a rumen where feed can be greatly changed, which makes the resources of MP more complex. The MP-AA sources in dairy cattle consists of AA absorbed from digested true protein in small intestine, which includes MCP, RUP and endogenous protein (ECP) (NRC, 2001).

Ruminally Synthesized MCP. The first step of ruminal protein degradation involves attachment of microbes to feed particles, followed by microbial proteases activity (Brock et al., 1982). Ruminal MCP includes a mixture of bacteria, protozoa, and fungi and flows to the lower gastrointestinal tract with the ruminal digesta. For most diets, more than 50% feed protein are degraded and used for MCP synthesis in the rumen, which thus accounts for more than half of the protein that passes to the small intestine if all of degraded protein is captured in MCP (Fleming et al., 2019b). Santos et al. (1998) suggested that inadequate rumen degradable protein (RDP) supply was associated with decreased MCP production. Clearly, sufficient RDP are required to support microbial formation in the rumen. The requirement of RDP is calculated as 1.18 x MCP, and the

requirement of RUP is calculated as the difference between MP requirement and MCP supply. Thus the MCP estimates have important effect on both supply and requirement functions in current nutritional models. Therefore, it is important to quantify MCP and understand factors influencing its synthesis and availability in dairy cattle. There are many factors such as type of protein, ruminal dilution rate, ruminal PH and substrate and nutrient interaction that can affect the microbial activity in the rumen and thus the MCP synthesis. Protein solubility is a key factor determining their degradability. For example, prolamins and glutelins are less soluble and thus degraded more slowly compared to globulins (Romagnolo et al., 1994). Russell et al. (1992) suggested that peptides and free AA from protein degradation could stimulate MCP synthesis in the rumen. Additionally, the intake of fermentable carbohydrates affected the disappearance of ammonia and free AA used for MCP synthesis. The fat, especially unsaturated fat intake can also affect the microbial activity due to toxic production. The flows of MCP-AA to small intestine were estimated by using an average AA profile of bacteria from previous results. According to 62 literature reports, the MCP is considered to have average 82.5% (SE = 28.3) AA-N, which is slightly higher than model value 80% AA-N, 10% RNA-N (SE = 8.3) and 5.2% (SE = 9.4) DNA-N (Storm et al., 1983). Although amino acid composition of MCP has been reported to be constant and well balanced with respect to requirements of absorbed AA (Weller, 1957, Purser and Buechler, 1966, Bergen et al., 1968, Ørskow et al., 1986), analysis of AA composition of 441 bacterial samples from 35 experiments reported significantly different AA composition (Clark et al., 1992b). In addition, the profile of MCP varies between bacteria and protozoa, and can be affected by diets. The studies on MCP digestibility is limited and they are all apparent digestibility. NRC (2001) assumed the intestinal digestibility of MCP is 80% without considering the individual AA digestibility, which is difficult and expensive to detect. Storm and Ørskov (1983) obtained an average MCP digestibility of 85%

with values ranged from 68-88% in sheep by infusing freeze dried rumen bacteria and then regressing bacterial AA input on ileum passage. Tas et al. (1981) reported microbial AA digestibility to be 87% by using the same method in sheep fed conventional diet. These experimental values are different from model values. If diets are to be formulated so that RUP-AA complement MCP-AA, it is important to know their contribution to the MP-AA supply (Schwab and Broderick, 2017). Therefore, determining the AA composition of microbial subpopulations and their intestinal digestibility is critically important to understand the nutritive value of MCP and develop nutritional models for AA balanced diets.

Rumen Undegradable Protein. Rumen undegraded protein is the part of feed protein that is not degraded in the rumen, which is considered to be 100% true protein and its digestibility varies from 50 to 100% according to RUP fraction of individual feedstuff. It has long been clear that the value of a protein is largely determined by the extent to which it is degraded in the rumen and methods used to quantify RDP and RUP in feedstuffs (Hristov et al., 2019). Ideally, ruminal protein degradation should be determined in lactating cows at normal DMI by using in vivo technique, which however is impractical for routine use due to difficulty of omasal sampling. Three N fractions are used in NRC (2001), A, B and C quantified with in situ procedure to estimate RUP and RDP. Fraction A includes rapidly solubilized protein and non-protein nitrogen and small particle that can escape incubation bags. Fraction C is the part that can't be degraded in rumen. Fraction B is separated into two parts by using degradation rate (kd) and passage rate (kp). The RUP is the undegraded fraction B and fraction C. Although in situ results were well correlated with in vivo protein degradation, there are concerns about small particle loss and microbial contamination. In addition, in situ assay cannot accurately evaluate protein degradation of certain

feeds (e.g. canola) (Broderick et al., 2015). Therefore, better in vivo assay should be developed to extend the protein degradation of current feed library and evaluate in situ values.

The RUP flows are digested and absorbed in small intestine. The digestibility of RUP was usually obtained through in vitro or mobile bag method. The most commonly used procedure to estimate RUP digestibility is the 3-step procedure (TSP) by Calsamiglia and Stern (1995), which includes ruminal incubation, then digestion with pepsin and pancreatin. However, the in vitro RUP digestibility was not validated with in vivo data. The RUP digestibility is not a constant. The NRC (2001) model recognizes that intestinal digestibility of RUP varies across and within feedstuffs but fails to represent differences in digestibility of individual AA of RUP, which is largely due to the difficulty in obtaining these estimates in ruminant animals. Developing AA based models requires better understanding of RUP-AA digestibility and factors that may affect in the future.

Endogenous Protein. Endogenous protein also contributes to protein flows to the small intestine. The ECP includes mucoprotein in saliva, sloughed epithelial cells and enzymatic secretions. It is hard to measure the content and digestibility of ECP because it is difficult to separate ECP from MCP and RUP. One possible approach is to feed animals RUP free diet. The ECP is assumed as the difference between the total N intake from the diet and the microbial N and total non-ammonia N flows into duodenum (Hannah et al., 1991, Lintzenich et al., 1995). By using this method, they reported an average 2.2 g/kg ECP from rumen and 17.2 g N/d from abomasum. Brandt et al. (1980) concluded 9-12% NAN passage to the small intestine by constantly infusing ^{15}N enriched urea into rumen. In 2002, stable isotope dilution techniques were developed and widely used (Ouellet et al., 2002). It estimated that the endogenous N contribution could represent 14 to 30% of the duodenal flow and 18 to 31% of the fecal flow in dairy cows, depending on the dilution methods used (Ouellet et al., 2002, Lapierre et al., 2008a, Ouellet et al., 2010). The NRC (2001) assumed true

protein proportion and digestibility of ECP to be 40% and 80%, thus 32% ECP contributes to MP. However, in CNCPS (Van Amburgh et al., 2009), total feed MP is the sum of each feed MP, which is problematic for not including ECP supply given that the ECP reabsorbed in the cow can account for 15% of total protein supply (O'Connor et al., 1993, Council, 2001, NRC, 2001).

Amino Acid Nutrition. Microbial protein plus typical amounts of RUP will generally meet MP requirement for low producing cows, but is often inadequate to meet the need of some EAA for high producing cows (VandeHaar and St-Pierre, 2006). The NRC (2001) thus includes requirements for RUP, RDP, and Lys and Met. Although MP is still a widely used concept in many nutritional models, researchers realize MP-AA instead of MP is better nutrition indicator. Therefore, the next step would be to define the MP supply in terms of the requirements of AA used at the cell level for protein synthesis (Pacheco et al., 2006). Amino acids are the essential building blocks for protein synthesis and precursors for gluconeogenesis or oxidized as energy. Some AA, like Lys, Met, His, Leu, Ile, Arg, Trp, Thr, Phe and Val, are considered essential because they can't meet the animal requirement by de novo synthesis. Deficiency of essential AA (EAA) may compromise animal production. As we know, MP is not necessarily AA balanced. One EAA deficiency can also adversely affect other AA utilization, and decrease total N efficiency (Mitchell and Block, 1946). Therefore, balancing AA to match animal needs is a good way to improve N efficiency with both economic and environmental benefits (Schwab et al., 2014). This has led to define the most limiting AA under different feeding conditions. In the past decades, Lys and Met have been recognized as most limiting AA in MP in dairy cattle. However, limited data are available to identify limiting AA after Met and Lys. Studies in lactating dairy cows showed positive milk and milk protein response to Met and Lys (Schwab et al., 1976, Noftsger and St-Pierre, 2003b, St-Pierre and Sylvester, 2005b, Appuhamy et al., 2011a, Chen et al., 2011b, Lee et

al., 2012b). However, Sinclair et al. (2014b) reported that no benefit in milk yield and only small increase in milk protein was observed when cows were fed diets less than 15% CP with supplementation of Met and Lys. One potential reason is poor representation of post-absorptive AA metabolism by using an inaccurate efficiency of MP converting into milk. The inconsistent results and inherent bias may slow the industry adoption of reducing dietary CP while balancing for EAA. Other possible challenges are the high cost of synthetic AA, lack of high quality and consistent rumen protected AA, and inaccurate estimates of AA supply and animal requirements (Schwab and Broderick, 2017). It is apparent that progress in balancing diet for AA has been slow because of the complex rumen fermentation and its effect on AA availability and the lack of comprehensive knowledge of post-absorptive AA metabolism of dairy cattle.

2.4. Protein and AA Availability

Currently, the general consensus among researchers is that an integrative model of AA flow and metabolism in major sites of the dairy cow (small intestine, portal-drained viscera (PDV), liver, muscle, and the mammary gland) should be developed to accurately predict milk protein yield (Hanigan et al., 1998a, Cant et al., 2003, Fleming et al., 2019a) . The first step in developing such a model will be accurately estimating EAA flow and their digestibility in small intestine, and the losses of EAA across the PDV. Accurate estimates of these variables should allow better matching of MP-AA supply and requirements. Several systems have been developed to predict MCP and RUP availability, e.g. NRC, CNCPS, and they are based on estimates from in vivo, in vitro and in situ techniques.

NRC. In the NRC (2001) nutritional model, feed CP, along with estimates of degradability in the rumen and digestibility in the intestine, were used to calculate digestible RUP and MCP. Together with ECP, digestible RUP and MCP were used to estimate MP supply. In NRC (2001), a 3-pool

protein system is used to describe protein partition. Pool A is assumed to be totally degradable, pool B is partially degradable, and pool C is undegradable in the rumen. Along with their fractional degradation rate (kd) and passage rate (kp), 3 fractions were used to calculate the RDP. The RUP then is calculated as difference of CP and RDP. Although the model gives reasonable estimates of RDP and RUP, weaknesses still exist. Huhtanen and Hristov (2009) suggested that part of protein in pool A may pass as RUP. White et al. (2017a) evaluated the model performance on estimating post ruminal non-ammonia nitrogen and found bias are caused by poorly specified feed fractions or kd. The kp equations in the NRC (2001) were also inaccurate compared with experimental results (Krizsan et al., 2010).

According to Pacheco et al. (2012), NRC (2001) greatly underestimated MCP under low DMI, non-corn-based diets and grass-based diet, while overestimated MCP when DMI was high (>22 kg/d). In NRC, MCP is predicted from RDP supply (85 g of MCP/100 g of RDP) or energy supply (130 g of MCP/kg of total digested nutrient (TDN)), which implies that a single nutrient affects MCP synthesis. However, a single limiting nutrient model may fail to represent biological process of microbial activity because both energy and protein are utilized simultaneously to optimize microbial growth (Hackmann and Firkins, 2015). For example, Russell (1986) demonstrated that energy spilling of rumen microbes happened when carbohydrate exceeds or other nutrients limits, which can depress MCP synthesis efficiency. Van Kessel and Russell (1996) also found that rumen bacteria spilled more energy when ammonia-N was limiting compared to when amino-N was limiting. In addition, unsaturated fatty acids have been associated with reduced microbial activity and depressed milk fat synthesis (Allen, 2000, Baumgard et al., 2001). Therefore, predicting MCP yield from a single nutrient lacks the flexibility to accommodate other

factors that affect organic matter digestion in the rumen, like carbohydrate and fat (Russell et al., 1992).

The NRC (2001) assumes an integrated digestibility of 80% for MCP without considering the individual AA digestibility of particular bacteria, which can be problematic because the amino acid composition of MCP varies across microbial species (Clark et al., 1992b). Storm and Ørskov (1983) obtained an average MCP digestibility of 85% with values ranged from 68-88% in sheep by infusing freeze dried rumen bacteria and then regressing bacterial AA input on ileum passage. Tas et al. (1981) reported microbial AA digestibility to be 87% by using the same method in sheep fed conventional diet. The intestinal digestibility of RUP is calculated from digestion coefficients for each feed ingredient protein (Apelo et al., 2014a). In the previous NRC (1998), the RUP digestibility of all feeds was assumed to be 80%. In current NRC, RUP digestibility was estimated by summarizing 48 mobile bag studies and 6 three-step procedure studies. For those feeds with limited or no data, the sheep digestibility values from the French PDI system were used (Jarrige, 1989). Because of this approach, the current NRC feed library better reflects the variability in the digestibility of RUP across feedstuffs than the previous versions. However, Bateman et al. (2001) evaluated NRC model and found it underpredicted individual EAA flows to the duodenum. After they increased the digestibility of grass hay, grass silage, and corn silage to 88%, and increased the digestibility of the MCP fraction from 80 to 85%, the predicted digestible EAA flow fitted observed net portal EAA better except for Met, which was still underpredicted. In addition, using the mean values of RUP digestibility to predict AA availability is not ideal due to the wide variation within feedstuffs (Taghizadeh et al., 2005, Estes et al., 2018).

The NRC model cannot estimate the digestibility of individual AA in RUP (RUP-AA) or MCP (MCP-AA), which are simply assumed to be equal to the digestibility of total RUP and MCP.

However, predicted MP-AA were not consistent with observed results (Hvelplund and Hesselholt, 1987, White et al., 2017b). The estimates of observed AA by NRC (2001) likely do not represent all the variations observed by Pacheco and Lapierre (2004). However, the RUP and MCP digestibility values from NRC are still widely used due to the lack of a standardized and commercially accepted technique for estimating RUP-AA and MCP-AA digestibility. In order to improve current nutritional models, the digestibility of individual AA in the RUP and MCP should be accurately predicted, and a more practical in vivo method should be developed to evaluate current library values.

CNCPS. The current CNCPS model balances amino acids supply by using a factorial approach based on the amino acid content of the predicted MP supply, which is the sum of each feed MP. In the CNCPS, more complex equations are used to calculate protein degradation in the rumen. The CP is divided into 5 pools, from which A1 is the ammonia, A2 is soluble true protein, B1 is insoluble protein, B2 is fiber bound protein and C is indigestible protein. The RDP is calculated from A1, A2, B1, B2 and fractional kd and kp and RUP is the difference of CP and RDP. The soluble pools (A1 and A2) are assigned to flow with the liquid passage rate in CNCPS model because soluble fraction of feed N is reported to contribute 5 to 15% of the total AA flow to the small intestine (Hristov et al., 2001, Choi et al., 2002, Reynal et al., 2007).

Mechanistic equations are used in CNCPS to predict MCP from fermentable carbohydrate and non-starch carbohydrate intake, rates of fermentation, the availability of amino N, and PH, which represents the ruminal biology better compared to NRC (2001). In the CNCPS, ruminal microorganisms are categorized as bacteria that ferment fiber carbohydrate (FC) and non-fiber carbohydrate (NFC) (Russell et al., 1992). Generally, FC bacteria use cellulose and hemicellulose as energy source and ammonia as N source for MCP synthesis. The NFC bacteria utilize starch,

pectin, and sugars as energy source and can also use AA as N sources. The bacterial growth is determined by degradable carbohydrate in the rumen and their digestion rates, which is adjusted according to the N balance and physically effective NDF supply (Fox et al., 2004).

The absorption of MCP and RUP is calculated by multiplying each fraction by its respective digestibility (Fox et al., 2004). In CNCPS, the total tract digestibility of bacterial true protein is assumed to be 100%, which is inconsistent with literature value (Sniffen et al., 1992). Storm and Ørskov (1983) indicated the true digestibility of ruminal bacterial RNA and DNA were 89 and 80%, respectively, which is similar to previous values 87 and 81% by Smith and McAllan (1971). Better descriptors must be developed for nutrient digestibility in the future.

Another limitation of CNCPS model might be not accounting for ECP supply (O'Connor et al., 1993, NRC, 2001). The AA composition of ECP and their digestibility are available from estimates by Shabi et al. (2000), Ouellet et al. (2002), and Marini et al. (2008). Incorporating these data into current CNCPS model would result in predicting the ECP supply and its utilization by microbes in rumen and re-absorption by the cow.

In both NRC and CNCPS, a lot of efforts have been invested to develop complex rumen sub-models to better predict the flow of proteins and the associated digestible AA flows. Although both models estimate duodenal flows of proteins or digested AA with good accuracy, variance still exist (Bateman et al., 2001, Pacheco et al., 2006). Both NRC and CNCPS models were similar in sensitivity to variation in protein fractions and their degradation rates and digestibility when predicting MP supply, which is not surprising considering that they are based on common principles (Lanzas, 2006). For example, both models use the first limiting nutrient to estimate microbial growth and use degradation and passage rates to predict the available nutrients at digestion sites. Lanzas (2006) indicated that MP estimates were sensitive to the degradation rates

of the B protein fraction in the NRC and the B2 fraction in the CNCPS and AA digestibility in small intestine. However, both the degradation rates and the intestinal digestibility are from in vitro and in situ estimates, which are not accurate (Sniffen et al., 2006, Hatew et al., 2015, Estes, 2017). A better approach to integrate protein degradation rates and digestibility are necessary. In addition, more data of protein fractions and their digestion rates are needed to decrease the uncertainty of model inputs.

In Vitro. In vitro procedures have been widely used to determine nutrient degradation and digestibility because of the low cost and high efficiency. The in vitro gas production technique is a common approach used to estimate N degradation in the rumen via linear regression of gas production and ammonia N production. Although it has been improved by Karlsson et al. (2009), variation in estimates of protein degradation still can be caused by variation in rumen fluid and ammonia background. Therefore, employment of in vitro techniques for N degradation in research is very limited. Tilley and Terry (1963) developed the two-step in vitro method to determine protein digestibility by utilizing hydrochloric acid (HCl) and pepsin to mimic abomasal digestion. Calsamiglia and Stern (1995) added pancreatin to simulate intestinal digestion and modified two-step method to a three-step procedure (TSP), which includes ruminal incubation, pepsin digestion, and pancreatin digestion. After ruminal incubation, the residues are digested in centrifuge tubes with pepsin first, and then further digested with pancreatin, and undigested protein is precipitated with TCA. The TSP was however not used to estimate RUP-AA digestibility at the beginning. Gargallo et al. (2006) modified the TSP to determine the RUP-AA digestibility. With the modifications, polyester bags was used to replace centrifuge tubes to digest the rumen residues, which allows for collection and analysis of a final undigested residue, for example AA content, thus intestinal RUP-AA digestibility can be calculated as proportion of AA that disappear from

the bags. In 2009, Boucher et al. (2009a) modified it to a two-step procedure using ruminal incubation and a cecetomized rooster to determine amino acid digestibility. In 2013, Ross et al. (2013) suggested using a glass Erlenmeyer flask to replace centrifuge tubes considering the use of centrifuge tubes by Calsamiglia and Stern (1995) was unfavorable for rumen bacterial growth and the use of bags by Gargallo et al. (2006) can create a microbial barrier and cause a high loss of soluble components. This new Cornell method or Multi Step Protein Evaluation was developed to minimize sample loss and correct enzyme activity and concentration to better simulate digestion (Ross et al., 2013). The new in vitro method still uses porcine pepsin for abomasal digestion, but levels were decreased 40% given its higher activity (Kassell and Lang, 1971). An enzyme "cocktail" including lipase, amylase, trypsin, chymotrypsin and pancreatin was used to better simulate intestine digestion. In the last step of this assay, filtration under vacuum was used to replace the use of TCA, which greatly improved the recovery efficiency. Ross et al. (2013) tested various ingredients for undigested feed nitrogen using this new method with either the enzyme cocktail or just pancreatin. Only 3 out of 20 feed ingredients were found to be different in undigested feed nitrogen level, whereas similar ingredients such as distiller's grains yielded different nitrogen digestibility. When compared this new method with modified TSP by Boucher et al. (2009b), Ross et al. (2013) found the modified TSP resulted in much higher rumen degradation which was likely due to loss of small particles through the porous bags during incubation. Although in vitro approaches may be useful for comparative purposes, there are large discrepancies when in vitro values are compared to in vivo results (Sniffen et al., 2006, Hatew et al., 2015, Estes, 2017).

In Vivo. In vivo techniques were commonly used for evaluating nutrient degradation and digestion in early times, which have generally been considered as the standard to which other techniques are

compared. In vivo methods used to determine protein or AA degradation and digestibility includes the use of cannulated animals with the collection of feed, feces and digesta from different sites of digest tract. Digested protein can also be calculated simply as the protein consumed minus the protein observed in feces. The methodology certainly represents the most physiological process. However, several disadvantages are concerned. The biggest limitation is that in vivo methods for quantification of digesta flow obtains the degradability or digestibility of the overall diet not individual feed ingredients (Hvelplund et al., 1995, Firkins et al., 1998). One possible approach is feeding animals with a basal diet (iso-fermentable OM) with adequate N for MCP synthesis and adding the test feed on the top. The increase in protein flow in duodenum is assumed to attribute to test ingredient (Nocek, 1988, Titgemeyer et al., 1989, Flachowsky and Lebzien, 2006). However, increased supply of preformed AA may affect MCP synthesis. Another limitation is the contribution of digesta with ECP. NRC (2001) used an average value of 1.9 g N/kg DMI to represent endogenous part of duodenal N flow based on previous animal studies using protein free diets (Ørskow et al., 1986) or low protein diets (Hannah et al., 1991, Lintzenich et al., 1995). Similarly, the French system adapted a value of 1.7 g N/kg DMI (Jarrige, 1989). Another more accurate way to estimate ECP flows is tracer-based. Ouellet et al. (2002) found the non-urea endogenous secretions contributed 13% of the duodenal N flow by using ^{15}N-leucine infusion technique, which was equal to 2.3 g of N/kg of DMI. The endogenous urea-N incorporated into rumen microbes averaged 2.1 g of N/kg of DMI, which was approximately equal to the free endogenous secretion. In total, these 2 fractions contributed to 15% of the total duodenal N flow. In another study, Ouellet et al. (2005) found the endogenous derived N flow averaged 18% of total duodenal N flow when silage was fed, and 20% when hay was fed by using the same method, which was approximate 5.9 g of N/kg of DMI. Other variations existing in vivo method are markers

and animals. Ideal markers should be indigestible, stable, label different digesta fractions evenly. Commonly used markers are internal markers like indigestible ADF, indigestible NDF (iNDF) and acid insoluble ash, and external markers like Yb, oxides and salts of Cr and Co. For now, we haven't found an ideal marker. Therefore, all assumptions regarding markers should be accounted when they are used and double- or triple-marker methods are recommended. Animal difference is another source of variation. MacRae et al. (1975) indicated 50 to 95% of the variation in digesta flow measurements were caused by animal difference. Another considerable variation is caused by the microorganism in large intestine, where amino acids can be degraded or synthesized. Fuller and Tomé (2005) indicated ammonia can be absorbed from large intestine and transported to the liver, which represented the part of digested nitrogen unusable by the animal. Lapierre et al. (2006) also mentioned that analyzing feces for protein digestibility can result in misleading results with the concern of protein digestion and synthesis by microorganisms in the large intestine and endogenous protein losses. To solve this problem, animals with proximal duodenal and terminal ileal cannulas were used to measure digestion before the large intestine. However, there are still flaws with this method. For example, it is challenging to obtain a representative sample though a cannula or sampling point (Fuller and Tomé, 2005). One possible solution of this problem is using re-entrant cannula, where all digesta is collected, sampled and then returned to the intestine. The only concern is large feed particles may block the cannulas (Fuller and Tomé, 2005). Therefore, to determine the true digestibility in vivo, dietary and endogenous protein as well as ammonia loss from the rumen and hindgut must be considered.

In Situ. In situ techniques to some extent combine advantages of in vivo and in vitro methods by allowing feed stuffs to occur in vivo systems while allowing recovery of the test feed by using mobile bags, which thus is the most widely used technique for determining protein degradation

and digestibility. The principle of in situ method is allowing feed stuffs to digest in vivo but also allowing collection of the test feed by using nylon bags. Bags with feed ingredient are incubated in the rumen of cannulated animals for different time period. Then bags are collected and washed, and the residues weight is recorded. The pore size of nylon bags is critical for this method, which should be large enough to allow bacteria to get in but small enough to retain the undegraded feed. In the end, nutrient degradation at different time points is used to derive the general degradability profile. Madsen and Hvelplund (1994) tested the rumen degradation of 5 feed ingredients in 23 labs across 7 countries by using the in situ approach and found inconsistent results, which was likely caused by the variation existing in feed preparation and processing and nylon bag materials. Generally, the variation associated to estimation of degradability and digestibility of concentrate feeds are more caused by particle size and pore size (Madsen and Hvelplund, 1994), whereas forages are more affected by microbial contamination and incubation time (Klopfenstein et al., 2001).

The mobile nylon bag technique can also be used to test postruminal digestion of RUP or MCP. After incubated in the rumen and abomasum, nylon bags with feed residues are placed into the duodenum and then recovered from the ileum or feces (Hvelplund et al., 1992). The degraded AA or protein were calculated as the proportion of protein or AA that disappeared from bags (Hvelplund and Weisbjerg, 2000). One disadvantage of recovering bags from feces is the effect of microbial fermentation in hindgut, thus retrieval of bags from the ileum is preferred for estimating digestibility in the small intestine (Stern et al., 1997).

The most apparent disadvantage of in situ method is that cannulated animals are needed to place and recover the bags in different areas of digestive tract. Although this technique seems to yield reliable results by using biological system in animals, there are still many sources of

variation, like dimensions and pore sizes of the bags, feed sources, length of incubation time, bacteria contamination as well as the wash method of the bags (Mohamed and Chaudhry, 2008). These factors caused the low repeatability of in situ technique. To minimize the variation, Nocek (1988) recommended guidelines to standardize the in situ digestion procedure. For example, they suggested 40 to 60 um bag porosity; the particle size should vary according to feed type (2 mm for protein and energy supplements, 5 mm for whole cereal grain, fibrous byproducts and forage); the sample size to bag surface area should be 10-20 mg/cm^2; and standard ingredients should be used with test feed. The incubation time should cover the retention time of the test feed in digestion sites.

Overall, a lot of limitations still exist in current in-vitro and in-situ methods for protein degradation in the rumen and AA digestibility in small intestine. The poor accuracy and precision of in vitro and in situ degradation rates may cause an overprediction of the ranges in RDP-RUP flows. Better laboratory methods and a better approach to integrate protein degradation rates and digestibility are necessary. A better in-vivo method should be developed since it is the "standard" used to evaluate other techniques. The in vivo method of plasma AA concentration responses after an abomasal pulse dose seems promising. This method has been used to assess rumen-protected Met and Lys (Graulet et al., 2005, Whitehouse et al., 2016), which is however difficult to be applied to all AA in individual feed ingredient. Estes et al. (2018) and Huang et al. (2019) adapted a stable isotope approach used by Maxin et al. (2013) to assess the AA availability from individual feed ingredients. This method makes use of a 2 h or longer constant infusion of a ^{13}C labelled AA mixture derived from enriched algae to assess the plasma absorption rate of each AA. Because infusions and sampling are via the jugular vein, measurements can be made with minimal animal preparation. Errors of determination for AA availability from each ingredient are approximately

10% using this method, which is a large improvement than previously used methods (Titgemeyer et al., 1989).

2.5. Amino Acid Requirement

The RUP and MCP are digested to AA and peptides in the small intestine and mostly absorbed there. After absorption, AA enter into general circulation and can be used by all tissues for maintenance, reproduction, growth and productive purposes. However, once AA are absorbed, the estimates of milk protein excretion from calculated inputs are determined based on a constant efficiency of conversion. Although this simplification of AA utilization for lactation was necessary as a starting point due to the limited knowledge of nutrient metabolism, Lapierre et al. (2007) indicated current nutritional models often underestimate MP allowable milk in dairy cows fed low protein diet but overestimate in animals fed high protein diet due to utilization of an inaccurate efficiency (67%) of MP for both maintenance and lactation functions. The use of a constant efficiency of MP has been challenged by previous study (Hanigan et al., 1998a), which was reported to vary from 0.70 to 0 as MP supply increased (Metcalf et al., 2008). Likewise, Apelo et al. (2014a) and Daniel et al. (2016) also suggested that the efficiency of MP decreases with increasing MP supply. Current nutritional models (e.g. NRC, CNCPS) used a constant MP efficiency thus variation was introduced. Therefore, to match MP-AA supply with requirements for different functions requires a more accurate and precise prediction of MP efficiency and determination of AA requirements. Due to the limitation in current nutritional models, more complex models that include factors affecting MP efficiency, such as MP supply, AA profile, energy supply and potential of cows should be built.

NRC. In current NRC model, only MP requirement is presented, which is the sum of maintenance requirement, growth requirement, reproduction requirement, and lactation requirement without

consideration of the individual AA. Maintenance is assumed to not change with the level of production. Requirements for growth, reproduction, and production are functions of protein excretion in products and efficiencies from MP to products. The NRC (2001) only has pregnancy requirement for animals more than 190 days pregnant, and animals that are more than 279 days pregnant have the same requirement as animals that are 279 days pregnant. Therefore, the MP requirement for pregnancy was an equation of days of pregnancy and calf birth weight. Metabolism protein used for lactation is directly determined by milk protein yield. Growth requirement for heifers or steers is usually calculated from retained energy, daily weight gains and equivalent shrunk BW. Metabolism protein is considered to be used with 65% efficiency for milk protein synthesis. The model assumes fixed efficiency for milk protein synthesis above requirements. However, studies showed that observed efficiency for milk protein synthesis are smaller than predicted values and decreased as MP supply increased (Hristov et al., 2004, Doiron et al., 2009, Lapierre et al., 2010). Although the energy and individual EAA are considered as the nutrients most likely to limit milk production, NRC subcommittee believed that current knowledge is too limited to build a factorial model that can accurately estimate AA requirements for dairy cattle. However, some EAA like Met and Lys requirement were determined based on dose-response relationships between changes of EAA content in MP and animal responses to it. This is a more direct way to define the ideal content of EAA in MP considering the uncertainty of AA flow to small intestine and to functional sites. The 5-step dose response approach described by Rulquin et al. (1993) was used in NRC (2001): (1) predicting digestible Lys and Met in MP and production response (36 experiments); (2) determining the fixed concentration of Lys and Met in MP intermediate to the lowest and highest values. For example, Lys concentration in MP varies from 4.33% to 9.83%, so 6.67% was chosen as fixed concentration of Lys; (3) calculating reference

production value for each experiment that corresponded to fixed Lys or Met level; (4) calculating the production response corresponded to reference production value; (5) regressing the production response to the predicted concentration of Lys or Met. This method indicated that 7.2% Lys and 2.4% Met in MP were required for maximal milk protein yield, which means the optimum ratio of Lys and Met in MP is 3:1 in this model. One potential disadvantage of this technique is that the AA concentration in MP were predicted from model. In addition, the model only evaluated diets for percent not grams of EAA in MP, and didn't adjust for AA or energy supply change (Schwab et al., 2014). There are still a lot of limitations existing in our current post absorption AA metabolism model, exploration of which will improve our future knowledge.

CNCPS. Different from NRC (2001), CNCPS has adapted a factorial approach to estimate AA requirements, which included 3 steps: (1) quantify the true protein (TP) excretion or accretion; (2) determine their AA profile; and (3) determine the efficiency of utilization of MCP- AA to AA in TP of excretion and accretions. The TP used for maintenance are the sum of scurf protein, urinary protein, and metabolic fecal protein (MFP); The TP excretion for lactation are milk protein content; and the TP accretion for optimum growth of replacement heifers and young cows depends on rate, composition, and efficiency of daily gain. The AA composition of proteins are usually obtained after a 21-h or 24-h acid hydrolysis. However, Pacheco et al. (2006) indicated 24-h hydrolysis could underestimate some AA. Lapierre et al. (2016) proposed correction factors by combining the ratios of the maximal value, theoretical value and extrapolated value relative to 24-h measurement. The NorFor system also corrects the weight of each AA for hydration after hydrolysis (Nielsen and Volden, 2011). The EAA composition of proteins for each fraction was determined by this method. For integumental proteins, AA composition was combined from AA composition of the head, hide, feet and tail (Williams, 1978). For the endogenous urinary

excretion, the AA composition was assumed the same with the whole empty body AA composition (Williams, 1978, Van Amburgh et al., 2015). For the MFP, the AA composition were determined from ruminal and abomasal isolates and the endogenous flow at the ileum in pigs (Ørskow et al., 1986, Jansman et al., 2002). For the milk protein, AA composition was defined by determining AA profiles of different proteins in milk (Farrell Jr et al., 2004). The efficiency of utilization of MP is assumed to be 0.64 in CNCPS, which is a single combined efficiency based on AA metabolism research by Lapierre et al. (2007). However, the large range (0.35 to 1.01) suggests the efficiency is variable. Lapierre et al. (2016) indicated that MP/NEL supply or MP supply/DMI were linearly related to efficiency, and proposed efficiency of 1.06 (±0.04) - 0.0078 (±0.0007) × MP/NEL supply (g/Mcal), which however still needs to be evaluated. Metcalf et al. (2008) also questioned the use of a static efficiency and suggested the range of 0.77 to 0.50 as MP supply increased. They further optimized the data fit and narrowed the efficiency range to 0.62 - 0.64, which is lower than our current model value but is consistent with the value (62.2%) by Doepel et al. (2004). In addition, the static efficiency for individual AA can cause variations. Recent studies in lactating cattle challenged current static efficiencies for either MP or individual AA considering the functional roles of certain AA (Doepel et al., 2004, Pacheco et al., 2006, Metcalf et al., 2008). For example, His may play a role in regulating the blood flow. However, individual AA efficiency is hard to detect due to limited data on AA balance beyond Met and Lys.

Limiting EAA. Although some AA can be synthesized de novo by animals, ten EAA cannot be synthesized in body or their synthesis cannot meet the animal requirement, including Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Trp, and Val. In addition, EAA are necessary for some nonessential AA (NEAA) synthesis. Therefore, the EAA must be supplied in the diets. If a diet is deficient in one EAA, protein synthesis cannot be beyond the rate at which that AA is available, which is called a

limiting amino acid. Therefore, MP efficiency to some extent is determined by its EAA profile. In the 1970s, researchers came up with the question whether some AA are more limiting than others. Lysine and Met are considered the most limiting AA for cows fed corn-based rations or when high forage is fed (Schwab et al., 1976, NRC, 2001, Noftsger and St-Pierre, 2003a, St-Pierre and Sylvester, 2005a, Appuhamy et al., 2011b, Chen et al., 2011a, Lee et al., 2012a, Giallongo et al., 2016a, Zhou et al., 2016). However, some studies have shown that His is likely the most limiting AA in lactating dairy cows fed high grass silage/haylage based diets (Vanhatalo et al., 1999, Kim et al., 2000, Korhonen et al., 2000, Huhtanen et al., 2002). Therefore, His has also been considered a limiting AA after Lys and Met in animals fed low CP diets based on corn silage or alfalfa haylage (Lee et al., 2012a, Giallongo et al., 2016c). These findings indicate the most limiting AA is different under different feeding conditions. Therefore, more knowledge of limiting AA are required to optimize AA profile in MP (NRC, 2001). In addition, the effect of some EAA like Lys and Met appears to extend beyond the production and health benefits. For example, maternal Met supplementation can affect gene expression (Peñagaricano et al., 2013) and increase maturation of gluconeogenesis and fatty acid oxidation in the liver of the newborn calves (Jacometo et al., 2016). According to Mitchell and Block (1946), the most limiting AA limits protein synthesis in the body and addition of other AA has no effect. Based on this idea, the well-known "barrel theory" was built, which believes in that the most limiting AA determines milk protein yield maxima (Schwab et al., 2014). Traditionally, Met, Lys, and possibly His are considered to be most limiting and their requirement are incorporated into nutrition models assuming fixed efficiency for maintenance, milk protein, and growth independent of MP or energy supply (Doepel et al., 2016). However, animal responses to Met, Lys and His are inconsistent. For example, in low CP diets (< 15% CP), rumen protected Met and Lys supplementation had no effect on fat corrected milk production in a

meta-analysis across 16 experiments (Sinclair et al., 2014a). In a recent study, addition of Met, Lys, and His to MP deficient diet over a 9-week period decreased milk and ECM yields (Giallongo et al., 2016b), which was likely due to low arterial plasma Leu concentration. Leucine and Ile were known as mTOR stimulators, but their effects on milk protein yield are not well documented, particularly in long term animal studies (Doelman et al., 2015, Nichols et al., 2017). Some studies didn't observe increased milk protein yield with infused BCAA, which were likely due to the deficiency of other AA or increased non-mammary use of EAA (Appuhamy et al., 2011b, Kassube et al., 2017, Curtis et al., 2018). The removal of BCAA or Leu from EAA infusate have shown negative effects on milk protein yield in some studies (Rulquin and Pisulewski, 2006, Doelman et al., 2015, Tian et al., 2017), but no effect in another study (Weekes et al., 2006). Although the manipulation of AA content may not increase production on N sufficient diets, it can significantly increase the N efficiency by reducing the supply of AA that exceeds the need. For example, the swine and poultry industries have improved the N efficiency by reducing the CP in the diet and supplementing with the limiting AA. However, it is unrealistic to detect all 10 EAA and their combination of each feed ingredient under all feeding conditions. Therefore, more studies of the potential interaction of EAA are required to better predict responses to additive of these EAA and better predict their requirements

2.6. Factors that Affect AA Availability

There are a lot of factors that can affect the profile and quantity of amino acids in the small intestine for absorption, and further plasma amino acids used for milk synthesis. For example, management, feed quality including its amino acid balances, DMI, protein degradation in rumen and post-ruminal digestion can greatly influent AA availability in blood. King et al. (1990) found that dietary protein source greatly affected the profile and quantity of amino acids presented in the

small intestine of lactating dairy cows, and Lys and Thr were more degradable in the rumen compared to other EAA. Foldager et al. (1980) also found plasma BCAA were influenced by dietary CP, stage of lactation, and production. To optimize the AA quantity and composition in blood, the factors that can affect AA availability should be well studied.

Feed Sources. Metabolizable protein mainly comes from MCP synthesized in the rumen and RUP from the feed. Today the milk production potential of dairy cows is much higher than before thus MCP alone can't meet the protein requirements. The RUP is usually formulated into diet to meet the animal requirements. Santos et al. (1998) indicated the production responses to increased RUP supply were inconsistent. This is likely associated with a poor AA profile of RUP, which fails to increase the supply of AA that is limiting in MCP. Therefore, the ideal AA content of RUP should complement the AA profile of MCP. This is further supported by Korhonen et al. (2002), who indicated that the dietary changes had no effect on AA profiles of individual microbial fractions. Piepenbrink and Schingoethe (1998) studied AA composition of RUP, and intestinal digestibility of blood meal, canola meal, corn gluten meal and menhaden fish meal, and found blood meal, canola meal, and fish meal had lowest Ile, and corn gluten meal had lowest Lys. Although canola meal had an estimated AA profile in the intestinal tract that was closest to the AA profile in milk protein, but the RUP content is low. Blood meal and corn gluten meal are deficient in several AA and shouldn't be fed as the only protein source. Boucher et al. (2009a) studied the intestinal digestibility of AA in RUP of fish meal and distillers dried grains and found rumen incubation changed the AA profile and digestibility of distillers grain but not feather meal, which implied different feed sources varied in their AA availability. However, simply supplying an excess of RUP has no benefits. Voigt and Piatkowski (1987) suggested increased dietary RUP had a negative effect on MCP. This was also confirmed in a meta-analysis by Ipharraguerre et al. (2005), who

found a 7% reduction in MCP with increased RUP intake. Therefore, the RUP-AA composition and digestibility has to be well studied to formulate diets to maximize ruminal microbial protein synthesis with perfectly complemented RUP. Selection of feeds that have balanced AA profiles with high intestinal digestibility should improve the MP-AA content and profile.

Nutrients Interaction. A lot of studies have concentrated on diet characteristics that may affect nutrient availability (Petitclerc et al., 2000), which are however complex in ruminants as different combinations of feed can greatly change nutrient output from the rumen (Clark et al., 1992a, Brito et al., 2007) and thus modify the type and amount of AA that are absorbed into the bloodstream (Reynolds et al., 1994). The most well studied nutrient interaction which can affect MCP production and thus the AA availability in dairy cows is the carbohydrate and N interaction in the rumen. The carbohydrate is an important ATP source for microbial growth (Nocek and Russell, 1988). To achieve optimal growth, the rate of ATP production from carbohydrate fermentation should equal its utilization for protein synthesis (Hespell and Bryant, 1979), which implied the interaction of the carbohydrate and protein in the rumen. Herrera-Saldana et al. (1990) also indicated that MCP synthesis was maximized when starch and protein sources with similar degradation rates were fed. The CNCPS defined carbohydrate fractions into sugars, starch, and ruminally available or unavailable fiber (Sniffen et al., 1992), with first three types can be used by ruminal microorganisms. According to Russell et al. (1992), fiber-degrading bacteria preferred to use ammonia as N source in the rumen, which implied the provision of ruminally degradable fiber may increase ruminal ammonia utilization (Firkins, 1997). Feng et al. (1993) also found increasing fiber in the diet can significantly reduce ammonia concentrations in the rumen. Hristov and Ropp (2003) used ^{15}N to label MCP and found estimated proportion of milk protein N originating from ruminal bacterial N was 50%, and more estimated milk protein N originated from MCP when cows

were fed ruminally fermentable fiber diet compared to ruminally fermentable nonstructural carbohydrates diet.

The interaction of post absorption AA and glucose was also studied. When gluconeogenic energy is limiting, the AA will be used for gluconeogenesis. It was assumed that supplying rapidly available energy precursors should save more AA for milk protein synthesis (Rius et al., 2010). However, inconsistent production responses to supplemental glucose were observed, which largely depended on the nutritional status and animals (Cant et al., 2002, Nichols et al., 2016).

Another nutrient that may affect the AA availability is fat. However, the interaction between protein and fat has not been extensively studied in dairy cows. Saturated long-chain fatty acids are often supplemented into dairy rations as a source of nonfermentable energy to maximize the milk production. In contrast, unsaturated fatty acids have been associated with decreased DMI, reduced microbial activity, and depressed milk fat synthesis (Allen, 2000, Baumgard et al., 2001). Rabiee et al. (2012) reported supplemental fat affected DMI and nutrient digestibility and thus cow performance. Harvatine and Allen (2006) indicated fat supplementation reduced nutrient digestibility, which however depended highly on other factors. In contrast to previous studies, Nichols et al. (2018) observed a positive effect of fat supplement on apparent protein digestibility, which might be associated with the lower starch contents in fat-supplemented diets. Under this condition, N loss through NH_3-N production was expected to increase, thus apparent N digestibility increased.

Feed Processing. Feed processing (particle size, heat treatment et. al) may greatly change protein degradability and digestibility. The Maillard reaction between sugar-aldehyde groups and free amino groups during heat processing can increase RUP (Chalupa, 1975, Clark et al., 1987). However, excessive heating decreases the RUP digestibility in the small intestine (Satter, 1986).

Huhtanen (2005) reported that heat-treated rapeseed expeller had lower protein digestibility than solvent-extracted rapeseed (0.82 vs. 0.92). When soybean meal, raw soybean, and soybean extruded at 132 or 149 °C were compared, raw soybean had highest RDP and soybean extruded at 149°C had lowest RDP (Stern et al., 1985), whereas profiles of duodenal EAA were not altered by heat treatment according to Kung et al. (1984) and Hudson et al. (1970).

The effect of ration particle size on dry matter digestibility is controversial. Some studies reported that increasing ration particle size increased dry matter digestibility (Kononoff and Heinrichs, 2003a, Yang and Beauchemin, 2005), whereas others observed decreased dry matter digestibility as particle size increased (Kononoff and Heinrichs, 2003b, Maulfair et al., 2011). Several studies also found ration particle size had no effect on dry matter digestibility (Yang and Beauchemin, 2006, 2007). Clearly this effect is variable based on other factors, i.e. interactions between forage type, forage-to-concentrate ratio, and starch fermentability with forage particle size. The effect of particle size on digestibility of individual nutrient is more consistent. The protein in small particles is likely to be degraded more rapidly in the rumen because of the larger surface area of small particles. Tice et al. (1993) observed decreased protein degradation in the rumen and decreased milk yield as roasted soybean particle size increased. Dhiman et al. (1997) also reported half and quarter size roasted soybean had higher protein availability than whole size and coarsely ground soybean, which was reflected in high milk yield. The potential reason was the intestinal availability of protein increased with finer particles.

2.7. The AA Uptake and Metabolism in Mammary Gland and Factors May Affect this Process

After absorption, part of MP-EAA is captured by mammary glands and used for milk protein synthesis. Milk protein synthesis is a complex process, which can be described as involving

uptake of AA by the mammary glands from the blood supplies and utilization of those AA for milk protein synthesis. Amino acid uptake depends on AA supply and the transport affinity of the mammary glands for AA. The arterial influx is calculated from mammary blood flow (MBF) rate and arterial AA concentration. Currently, our understanding of AA uptake and metabolism in mammary glands is limited (Bequette et al., 1998). For example, our current protein requirement models for dairy cows are unable to predict milk protein response to changes in dietary protein or AA availability, which is partly due to the incomplete understanding of the metabolic transformations of post-absorptive AA (Armentano, 1994, Hanigan et al., 2001). Currently, a fixed factor (0.64 to 0.80) is used to convert MP-AA into milk protein, which is however found to decrease as supply increase (Doepel et al., 2004, Van Duinkerken et al., 2011, Daniel et al., 2016). Therefore, factors that can regulate AA uptake and metabolism by mammary glands should be studied to better predict milk protein synthesis and required AA for this process. Thus, the mechanism that can regulate the availability of AA to the mammary glands and the utilization of AA for milk protein synthesis in udder will need to be represented better so that milk protein response to dietary nutrient change can be predicted more accurately.

The arteriovenous difference technique is usually used to detect EAA uptake by mammary glands and thus characterize the variation (Linzell, 1974). Mammary net uptake of individual EAA is determined by arterial influx and cellular transport activity. Arterial influx is regulated by absorption from the gut, blood flow, and AA uptake in non-mammary tissues (Hanigan et al., 1998a). Mammary transport activity of individual EAA depends on the substrate concentration in extracellular space and the affinity and competition for AA transport system (Baumrucker, 1985). There are several mechanisms that the udder uses to maintain milk protein yield when facing varying supply. Mammary net uptakes of most AA linearly respond to arterial concentrations

(Hanigan et al., 1992). Apelo et al. (2014) reported that Ile, Thr, Met, and Leu uptake is proportional to extracellularly supplies in pig mammary tissue explants. Hurley et al. (2000) and Jackson et al. (2000) also observed that intracellular Val and Lys increased as extracellular AA supply increased. The other finding is that AA transporters are not influenced by dietary AA (Manjarin et al., 2012, Manjarin et al., 2014, Shennan and Boyd, 2014, Huber et al., 2016, Osorio et al., 2016), which implies reduced arterial AA concentrations decreases intracellular mammary AA concentrations. If the intracellular concentration of one AA becomes low, efflux will decrease resulting in a net uptake increase. Efflux of EAA happened immediately after uptake by mammary glands (i.e. 55, 53, and 69% for Leu, Met, and Val in lactating goats) as the net EAA uptake is demonstrated to be a consequence of bidirectional transport across the plasma membranes of mammary epithelial cells (Bequette et al., 2000, Hanigan et al., 2009). Therefore, marginal efficiency of MP-AA converted into milk protein increases when arterial AA supplies are reduced (Whitelaw et al., 1986, Doepel et al., 2004). For example, the ratio of EAA uptake to output in milk protein decreased when their supplies are reduced (Lapierre et al., 2012, Doepel et al., 2016, Guo et al., 2017b). Inversely, excessive uptake of EAA may be used for the de novo synthesis of NEAA for which uptake was found to be less than or equal to milk outputs (Guinard and Rulquin, 1994).

The pattern and availability of AA to mammary glands may also be affected by stage of lactation, which is likely due to change of mammary blood flow (MBF), tissue protein turnover, hormone sensitivity and secretory cells. For example, EAA infusion to cows in early lactation increased milk protein content to larger extend compared to the same cows in midlactation, which was the consequence of higher efficiency of converting the infused EAA into milk protein in early lactation (Crompton et al., 1996). Daily milk yield decreased after peak lactation is due primarily

to decreased cell numbers and not a change in cell activity (Capuco et al., 2001). The number of secretory cells in mammary glands is affected by cell proliferation, differentiation, and apoptosis (Cant et al., 2018). Nichols et al. (2017) indicated that differentiation of secretory cells is sensitive to EAA supplies, which is related to ER biogenesis. Some studies found that energy restrict can also decrease secretory cell number (Nørgaard et al., 2008, Dessauge et al., 2011). Hormone is another factor that affects milk protein synthesis in different lactation stages. Manjarin et al. (2014) reported increased gene expression of several members of the SLC families for AA transporters in the udder at the onset of lactation, which is likely due to increased GH (Sciascia et al., 2015).

Local blood flow can significantly affect the AA uptake by the tissues. For example, Hanigan et al. (1998b) indicated that increased blood flow would increase total EAA supply and removal of them by liver and mammary glands. Blood flow through the mammary appears to be regulated locally. If we assumed these local factors are produced in response to changes in mammary intracellular metabolism. Thus, these local control mechanisms may govern the partition of nutrients between the mammary gland and other tissues. Several studies have investigated local effects of nutrients, like AA, on MBF, but results were inconsistent. Doepel and Lapierre (2010) found abomasal infusion of EAA mixture reduced MBF by 10%, whereas infusion of NEAA tended to increase MBF by 7% when cows were fed MP deficient diet. The effect of individual AA was also investigated. Guinard and Rulquin (1994) and Guinard and Rulquin (1995) found increased Lys had no effect on MBP, whereas 16g/d duodenal infusion of Met greatly reduced MBF. Bequette et al. (2000) observed 33% increases in MBF when the goats were His deficient. Similarly, shortages of His, Lys, and Thr have also been found to cause substantial changes in MBF (Bequette et al., 2000, Rius et al., 2010, Doepel et al., 2016, Guo et al., 2017a, Curtis et al., 2018). In contrast, Cant et al. (2001) found no effect of His on MBF. The potential reason to cause

this inconsistence is not clear yet. Guo et al. (2017a) found the MBP decrease with jugular Lys infusion was related to linear decrease in concentrations of NO_3 + NO_2 in mammary venous outflow, which may also be applied to other EAA. However, this effect seems only happening in mammary glands as blood flow in the portal vein was reported to respond to energy supply but not protein supply (Reynolds, 1995, Doepel et al., 2004). Whether the change in MBF was caused by NO_3 + NO_2 concentration or other mechanism is to be elucidated. However, it is demonstrated that mammary glands are able to maintain an adequate supply of nutrients to the udder for maintenance of milk production even under deficient conditions.

After uptake by mammary glands, 90% of AA on a net basis are used for milk protein synthesis with the remaining AA used for catabolism or transamination according to Cant et al. (2018). The net uptake of BCAA, Arg, and Lys largely exceeds milk protein needs while the Phe, Met and His uptake is close to output in milk (Lapierre et al., 2012b). The excessive EAA are utilized for oxidation and transamination to NEAA, which thus produces an inefficiency of EAA use. Infusion studies also supported that BCAA and Arg supply did not increase milk protein production (Korhonen et al., 2002, Appuhamy et al., 2011b, Kassube et al., 2017, Curtis et al., 2018). These findings however are contradictory to the effect of BCAA on mTOR regulation (Arriola Apelo et al., 2014c, Hallett and Manning, 2016, Saxton et al., 2016a, Wolfson et al., 2016). A possible reason for lack of BCAA effect on milk protein synthesis is the interaction with other factors, e.g. milk protein gene transcription, protein abundance of translation apparatus machinery. Overall, a better understanding of mechanisms of EAA uptake and metabolism in mammary glands and factors affecting this process can help build nutrition models to better identify EAA requirement and supply (Cant et al., 2018).

2.7. Conclusion

It is widely accepted now that balancing for EAA and reducing dietary CP can improve N efficiency. However, current nutrition models cannot always predict production accurately from AA supplement, which is likely due to the bias existing in estimates of nutrient flows at different sites and inflexible post-absorptive metabolism model of nutrition models. The study of N utilization in ruminants is challenging due to complex microbial activity in the rumen, subsequent digestion in small intestine and utilization in body tissue. Therefore, it is important to study these processes using more accurate and precise methods to improve the efficiency of N utilization. Current feeding systems used by the dairy industry are mostly empirical and not sensitive to dietary AA other than Met and Lys. This is assumed to contribute to errors of prediction for milk and milk components and low nutrient efficiency (~25% for N) (NRC, 2001). Addressing these limitations requires a more mechanistic understanding of AA availability and utilization in lactating animals.

Dietary protein degradability in the rumen and digestibility in small intestine are critical in determining the AA availability. The in situ, in vitro, in vivo or model approaches used to evaluate AA availability all have inherent advantages and disadvantages. Techniques used to assess protein degradation in the rumen are laborious, expensive and inaccurate. For example, in situ degradability is largely affected by animal species, diet composition, feed intake, sample processing, particle size, bag pore size, incubation time, sample washing and microbial contamination. In vitro techniques can only be used for comparative purposes due to large discrepancy with in vivo data. In vivo methods include the use of cannulated animals and markers, which cannot be applied to test individual feedstuff (Hvelplund et al., 1995, Firkins et al., 1998). Approaches for studying postruminal digestion of AA have the same disadvantages mentioned above. These limitations are related to inaccuracy of estimates of AA supply and requirement by

nutritional models. In addition, current AA degradation and digestibility database is incomplete and contains inadequate experimental replication for commonly used feeds in the field. More importantly, these values were mainly from in vitro and in situ studies, which have been found different from in vivo values. In addition, many unknown factors affect AA availability, which requires more studies.

The post-absorptive AA utilization in different tissue especially mammary glands is critical in determining AA requirements. Manipulating AA supply to the mammary glands should lead to further improvements in post absorptive AA efficiency, which however cannot be achieved if nutrition models do not accurately represent mammary utilization for each AA. The inability of nutrition models to predict production accurately is partly due to the empirical description of post-absorptive AA metabolism, which used a fixed efficiency for converting MCP-AA to milk. The potential reason for variable efficiency of MP-AA utilization in mammary is that mammary AA metabolism is regulated by a lot of factors. Amino acid transport is highly multivariate and bidirectional. This flexibility demonstrates how udder maintains homeostasis of intracellular AA when supplies change. A lot of studies have been conducted on AA transport and metabolism in udders, which however only studied net uptake in unidirectional way. The limited research was due to the technical difficulty or high cost. Research with mammary epithelial cells of cows were low cost and repeatable, which however are not necessarily representative of in vivo activity. The A-V difference technique based on amino acid exchange from the blood seems useful for assessment of amino acid metabolism in the mammary gland, it however cannot estimate the rates of protein accretion, synthesis and degradation if used alone. Therefore, more comprehensive knowledge of AA transport and better techniques are necessary to provide a framework for nutrition models.

Overall, bias existing in techniques used for determination of AA availability and utilization and lack of knowledge of biologic mechanisms hamper efforts to achieve a perfect match of AA supply and requirements. Developing more accurate and precious and practical methods to evaluate AA availability and utilization and further incorporating these findings into nutrition models should greatly enhance our ability to optimize AA supply and improve N efficiency.

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CHAPTER 3: Assessing Availability of Amino Acids from Various Feedstuffs in Dairy Cattle Using a Stable Isotope-based Approach

3.1. Abstract

Improving N efficiency can be achieved in dairy cows by more precisely supplying essential amino acids (EAA) relative to animal needs, which requires accurate estimates of the availability of individual EAA from feedstuffs. The objective of this study was to determine EAA availability for 7 feed ingredients. Seven heifers (258 ± 28 kg BW) were randomly chosen and assigned to 8 treatment sequences in a 7 x 8, incomplete, Latin square design. Treatments were a basal diet (BD), and 10% (DM basis) of BD replaced by corn silage (CS), grass hay (GH), alfalfa hay (AH), dried distillers grain (DDGS), soybean hulls (SH), wet brewers grain (BG), or corn grain (CG). Total plasma AA entry rates were estimated for each EAA within each diet by fitting a 4-pool, dynamic model to observed plasma, ^{13}C AA enrichment resulting from a 2-h, constant infusion of a ^{13}C algal AA mixture. Individual EAA availability from each test ingredient was determined by regression of entry rates for that AA on crude protein (CP) intake for each ingredient. The derived plasma total EAA entry rates for corn silage, grass hay, alfalfa hay, dried distillers grain, soyhulls, brewers grain and corn grain were 30.6 ± 3.4, 27.4 ± 3.2, 31.3 ± 3.4, 37.2 ± 3.2, 26.4 ± 3.2, 37.8 ± 3.2, and 33.5 ± 3.2% of EAA from each ingredient, respectively. Using the previous estimate of 8.27% EAA utilization by splanchnic tissues during first pass, total RUP EAA absorbed from the gut lumen was 33.4, 29.9, 34.1, 40.6, 28.8, 41.2, and 36.5% of the EAA in each ingredient respectively.

Key words: Amino acid, availability, isotope, dairy cattle

3.2. Introduction

Improving N efficiency of dairy production has received a lot of attention due to feed costs and environment concerns (Külling et al., 2001, Agle et al., 2008, Bouwman et al., 2013) . During the past decades, producers generally maximized milk yield to improve profit margins by overfeeding protein, which is the main cause of inefficient N utilization (Doepel et al., 2004, Colmenero and Broderick, 2006). The simplest strategy to optimize N utilization is to lower dietary protein (Kebreab et al., 2010), but this increases the risk of metabolizable protein (MP) deficiency resulting in decreased milk protein yield (NRC, 2001, Cabrita et al., 2011). Studies in monogastric animals have showed that balancing AA supply with animal requirements can improve the efficiency of N utilization (Baker, 1996, Nahm, 2002). In ruminants, optimizing N efficiency may be achieved by providing RUP with an AA profile that complements microbial AA flow (Ipharraguerre et al., 2005, Haque et al., 2012). However, previous work has demonstrated that AA flow from the rumen was biased when predicted with the NRC (2001) system. This model overestimates RUP flow from the rumen and underestimates microbial protein (MCP) flow (Bateman, 2005, Roman-Garcia et al., 2016, White et al., 2017a). Such bias hampers efforts to achieve a perfect match of supply and requirements.

Bateman (2005) reported that many unknown factors affect RUP content including DMI, protein solubility, heat denaturation, etc. Although some studies showed that the EAA composition of intact feed protein and of RUP did not differ (Boucher et al., 2009b, Edmunds et al., 2013), this may only be true for feedstuffs with low ruminal degradability (Boucher et al., 2009a). Paz et al. (2014a) found that the AA profile of RUP was altered during a 16 h ruminal incubation, and that the extent of change varied by AA and feedstuff. Furthermore, previous studies showed the AA digestibility of RUP varied across feedstuffs and individual AA (White et al., 2017b). Therefore,

using a common digestibility across EAA based on the CP digestibility may lead to errors in predicting EAA availability (Castro et al., 2007).

Soybean meal, dried distillers grain, corn grain, brewers grain, soybean hulls, corn silage, alfalfa hay and grass hay are widely used dietary ingredients in North America dairy rations. In 2016, the total usage of corn grain and soybean meal in the U.S represented 66.3% and 14.7% of concentrate feeds fed to livestock and poultry (USDA, 2017). The exact proportions of overall feed usage represented by each are not available for dairy. According to AFIA (2017), total consumption of corn grain, soybean meal, dried distillers grain and soybean hulls by dairy cows in 2016 was 16.24, 2.84, 2.68 and 1.53 million tons, which represented approximately 68.1%, 11.9%, 11.2% and 6.4% of concentrates fed to dairy cows. However, these estimates may be biased as most of the other byproducts were excluded from the diet simulations. Corn silage and alfalfa hay also represent a large fraction of forages consumed by dairy cattle. For example, total usage of corn silage by US dairy cattle in 2016 was 45.5 million tons (AFIA, 2017). There are some studies that have been conducted to investigate the digestibility of individual AA of RUP for various feed ingredients in the past decades. For example, in situ studies showed the AA digestibility of RUP from soybean meal varied from 92.7 % (Arg) to 95.3% (Thr), and the values for dried distillers grain varied from 87.4% (His) to 95% (Leu) (White et al., 2017b); Van Straalen et al. (1997) indicated the AA digestibility of RUP from soybean hulls varied from 61 % (Tyr) to 85% (Arg). Taghizadeh et al. (2005) found the AA digestibility of RUP AA varied from 60% (Arg) to 85% (Met) for corn silage, from 59% (Met) to 87% (Lys) for alfalfa, and from 76% (Thr) to 92% (Met) for corn grain. But as White et al. (2017b) stated, the current AA degradation and digestibility database is incomplete and contains inadequate experimental replication for commonly used feeds in the field. More importantly, these values were mainly from in vitro and

in situ studies, which have not been fully validated against in vivo observations, and where examined have been found to differ from the in vivo observations (Titgemeyer et al., 1989, Estes, 2017).

Determining the apparent AA digestibility for the diet or RUP in ruminants is technically difficult due largely to the errors of measurement associated with sample collection and animal variation (NRC, 2001). Although in vitro digestibility may represent true values for some ingredients, they are not applicable to all ingredients (Estes et al., 2018), and thus research on intestinal availability of individual AA from feedstuffs is quite limited (Titgemeyer et al., 1989, Ipharraguerre et al., 2005). Measuring in vivo disappearance from the intestine is technically difficult and invasive requiring surgical insertion of cannulas (Titgemeyer et al., 1989). The in vivo method of plasma AA concentration responses after an abomasal pulse dose is simpler, and has been used to assess rumen-protected Met and Lys (Graulet et al., 2005, Whitehouse et al., 2016). However, it is difficult to apply to all AA in feed ingredients. Estes et al. (2018) adapted a stable isotope approach used by Maxin et al. (2013) to assess the AA availability from individual feed ingredients. This method makes use of a 4 to 8 h constant infusion of a ^{13}C labelled AA mixture derived from enriched algae to assess the plasma entry rate of each AA. Because infusions and sampling are via the jugular vein, measurements can be made with minimal animal preparation. Errors of determination for AA availability from each ingredient are approximately 10% using this method, which is a large improvement over previously used methods (Titgemeyer et al., 1989).

Our hypothesis was that the stable isotope-based approach can be used to determine AA availability across various feedstuffs, with high or low protein content. Additionally, extending the knowledge of AA availability to more commonly used feed ingredients will provide a basis for

improvements to our feeding systems. Therefore, the objective of this study was to determine plasma EAA availability and RUP digestibility for 7 feed ingredients commonly used in dairy rations: dried distillers grain, corn grain, brewers grain, soybean hulls, corn silage, alfalfa hay and grass hay.

3.3. Material and Methods

3.3.1 Animals and Treatments

All animal procedures were conducted at the Virginia Tech Kentland Dairy Farm and approved by the Virginia Tech Animal Care and Use Committee. Seven Holstein heifers (258 ± 28 kg BW) were randomly selected and assigned to 8 treatments in a 7 x 8, incomplete, Latin square design with 8 periods of 10 days each. Treatments were a high protein, basal diet (BD), and 10% (DM basis) of BD replaced by corn silage (CS), grass hay (GH), alfalfa hay (AH), soybean hulls (SH), dried distillers grain (DDGS), wet brewers grain (BG), or corn grain (CG) (Table 1). Alfalfa hay, grass hay and corn silage were from Kentland farm of Virginia Tech (Blacksburg, VA) and grains were purchased from Rockingham Milling Company (Harrisonburg, VA). The BD contained a mix of corn silage, dried grass hay, soybean meal, and vitamins and minerals (Table 1). The metabolizable protein (MP) supply of BD was 860 g/d, which greatly exceeded NRC (2001) recommendations (534 g/d) to ensure that microbial protein synthesis and body protein synthesis were not altered by the treatments. Animals were fed once a day ad libitum from days 1 to 8. On day 6, animals were moved to metabolism stalls at 08:00, and fed at 6-h intervals through day 8. On days 9 and 10, animals were fed every 2 h, and feed offered was restricted to 95% of the ad libitum DMI observed for the previous 3 d to ensure each meal was eaten and minimize variation in AA absorption according to Estes et al. (2018).

On days 7 to 9 of each period, spot fecal samples were collected every 6 h with the collection time rotating forward 2 h on the 2nd day of collection and 4 h on the 3rd day of collection. Samples were stored at -20 °C until analysis. Feed and refusal samples were collected between 07:00 and 08:00 daily from days 6 through10 and at the end of the infusion, dried at 55 °C for 72 h, and stored for further analysis.

On day 8 of each period, each heifer was fitted with two jugular catheters in the same side as described by Estes et al. (2018). In short, the infusion catheter (90 cm × 2.03 mm i.d., Braintree Scientific Inc., Braintree, MA) tip was placed approximately 40 cm downstream of the tip of the sampling catheter (13 cm × 1.6 mm i.d., Jorvet, Loveland, CO) to ensure infusate circulation through the circulatory system prior to blood sampling. Catheters were placed on alternate sides of the neck in successive periods. On day 10, animals were given a constant jugular infusion of 0.2 g of a sterile ^{13}C labelled algal AA mix dissolved in 100 mL saline (U-^{13}C, 97-99% enriched, Cambridge Isotope Laboratories, Andover, MA) over a 2 h period using clinical infusion pumps (LifeCare 5000, Abbott Laboratories, North Chicago, IL). Infusions were initiated at 11:00 and ended at 13:00. Blood samples (8 - 10 mL each) were collected at -15, 15, 30, 45, 60, 75, 90, 105, 120, 135, 150, 165, 180, 195, 210, 240 min relative to the start of the infusion and stored on ice until processing. Plasma was prepared from the blood samples by centrifugation for 15 minutes at 1665 × *g* within 4 h of sampling. Plasma was stored at -20 ˚C until further analysis.

3.3.2 Sample Analysis

Feed and Fecal* Sample *Analysis. Dried TMR and fecal samples were pooled by animal and period and ground to 2 mm (Wiley Mill). Duplicate subsamples (10 g of TMR or 5 g of feces) were placed in 10 × 20 cm dacron bags with 50 ± 15 µm pore size (Ankom, Macedon NY); the ratios of sample size to surface area were 25 and 12.5 mg/cm^2 specifically (NRC, 2001). The bags

were incubated in the rumen of two cows (fed the BD diet; one replicate in each animal) for 12 d to determine indigestible NDF (iNDF) content (Estes et al., 2018). NDF content of the residue was determined using amylase and an Ankom Fiber Analyzer 200, and iNDF was assumed to be the residue after Ankom digestion. Twenty-five to 40 milligrams of dry, ground feed ingredients and fecal samples were analyzed for N content by combustion using a Vario EL cube analyzer (Elementar, Germany). Results were used to calculate fecal DM output and total tract apparent N digestibility as described by Cochran et al. (1986).

Ruminal Protein Degradation of Feed Ingredients. Two subsamples of feed ingredients were sent to Cumberland Valley Analytical Services (Waynesboro, PA) for in vitro rumen and intestinal digestibility analysis of protein (MSPE package). Another subsample was used for in situ ruminal protein degradation analysis. The same cows used for iNDF measurements. All ingredients (SBM, CS, CG, AH, GH, SH, DDGS) were ground to 2 mm, 10 g of which were weighed into dacron bags (10 × 20 cm, 50 µm pore size, Ankom, Macedon NY), and sample containing bags were incubated in the rumen for 0, 3, 9, 12, 15, 24, 36, 48, 72, 96, 108 h. Upon removal from the rumen, samples were rinsed in cold water and then washed through the delicate cold cycle in a washing machine without detergent and frozen at -20˚C followed by freeze drying. Twenty-five to 40 milligrams of ground feed ingredients were used for nitrogen analysis as described above.

The insoluble but degradable (B), and insoluble and undegradable (C) fractions of each ingredient and the rate of N degradation (Kd) were determined by fitting the following model, a modification of that described by Ørskov and McDonald (1979), to the data:

$$N(t)\ (\%\ \text{of initial}) = C + B \times e^{[-Kd \times t]} \quad [1]$$

The soluble fraction (A) was calculated from the model derived B and C fractions as:

$$A = 100 - B - C \quad [2]$$

N(t) was the N remaining at time t (% of initial N content). The A fraction (% of initial N content) represents N escaping from the bag at time 0 which mostly reflects soluble N, but may include some very small particles. The C fraction (% of initial N content) is the non-degradable N at t=108 h. The Kd represents the rate of B fraction N loss from the bag (h^{-1}). We attempted to introduce the lag time, which however made no difference and thus was removed.

Plasma Sample Analysis. All plasma samples were deproteinized by addition of sulfosalicylic acid (8%, w/v) followed by centrifugation at 1,600 x g for 15 mins at 4 °C. To measure the ^{13}C labeled AA, deproteinized samples were desalted by ion exchange chromatography (BioRad Resin AG 50W-X8*, 100-200 mesh; Bio-Rad, Hercules, CA), and eluted using ammonium hydroxide (2N) into silanized glass vials as described by Calder et al. (1999). Desalted samples were freeze dried, and derivatized as described by Walsh et al. (2014b). Measurements of isotopic ratios of ^{13}C labeled AA were performed using an isotope ratio mass spectrometer coupled to a GC by a combustion oven (Thermo Scientific, Waltham, MA).

3.3.3 Amino Acid Entry Rate Derivation

All modeling work was completed in R studio (version 1.0.143) with R 3.2.1 using the 4-pool dynamic model described by Estes et al. (2018). In short, state variables were total AA in fast (QAA_{Fast}) and slow turnover pools (QAA_{Slow}) and labeled AA in fast ($QAAi_{Fast}$) and slow turnover pools ($QAAi_{Slow}$). The fast turnover pool is thought to represent blood, interstitial, and cytoplasmic free AA, but also likely includes some protein with short half-lives, e.g. less than 30 min, while the slow turnover pool should only represent protein-bound AA. Estes et al. (2018) indicated that the size of the slow turnover pool cannot be accurately estimated with a 2-h infusion resulting in underestimates of plateau and overestimates of total AA entry rates. In the current

study, we were also unable to solve for stable slow turnover rates, which reduces the accuracy of the plateau estimate (Figure1) likely resulting in underestimated AA incorporation into the slow turnover pool and overestimated AA entry rates. However, the bias was accommodated in the intercept of the regression model that was fit across the treatments to derive the proportion of feed AA appearing in blood, and thus was not problematic. We did not adopt the longer infusion time recommended by Estes et al. (2018) solely because the animal work for this study was completed before the Estes et al. (2018) work was completed. Because the model explicitly represents exchange of AA with body tissue, the derived AA entry rates represent only absorption (EAA) or absorption plus de novo synthesis (NEAA) minus loss of AA to splanchnic catabolism during the first pass.

Following initial fits of the model to observed data, residuals outliers were checked, and if studentized residuals exceeded 2 in absolute value, the sample was removed, which was the case for 14% of the dataset. These outliers were generally also visually apparent when the observed data were plotted with the predicted values as the data represented a repeated sampling sequence in time, and thus deviations from the pattern in time were clearly evident.

The modeled AA entry rates were used to derive fractional availability values (g AA appearing in plasma/g CP consumed) for each test ingredient by regression as described by Estes et al. (2018). In Estes et al. (2018), dietary RDP was added to accommodate nutrients entry rates derived from the basal diet excluding soybean meal and MCP which varied when ruminally fermentable soybean meal was replaced by post-ruminal infusions of casein and EAA. In our study, substitutions were for BD, and all treatment diets had RDP which greatly exceeded NRC (2001) requirements, thus negating the need to represent potential changes in MCP using RDP.

3.3.4 Statistical Analysis

Statistical analysis was conducted in R Studio (version 1.0.143) with R 3.2.1. Data were analyzed using the mixed model function lmer in the lme4 package of R (version 3.4.3; R Core Team, 2017). Dry matter intake, fecal output, total tract apparent N digestibility and plasma AA entry rates were analyzed using the model:

$Y_{ijk}= \mu+ \text{Diet}_i+ Period_j+ Cow_k+e_{ijk}$

where Y_{ijk} = the dependent variable, μ = population mean of Y, Diet_i = the fixed effect of diet (df=7), $Period_j$ = the random effect of period (df=7), and Cow_k = the random effect of cow (df=6). Main effects were declared significant at $P \leq 0.05$ and denominator degrees of freedom for all tests were adjusted using the Kenward-Rogers option. Outliers were checked, and if studentized residuals exceeded 2 in absolute value, the sample was removed, which was the case for approximate 5% of the dataset. When main effects were significant, post-hoc testing was conducted. The "lsmeansLT" function of the lmerTest package was used with Kenward-Rogers option and Tukey adjust for estimation of least-square means, and the "difflsmeans" function of the lmerTest package with Tukey adjust was used for separation of means (P<0.05).

3.4 Results and Discussion

All 7 animals completed the trial. An important assumption of this study was that the high CP diets exceeded requirements for MCP synthesis and body protein accretion across treatments so that these processes remained constant across treatments. The CP content of BD was 22.7%, which was a little higher than our formulation goal of 20%. BG (22.8%) and DDGS (22.8%) had higher CP content compared with BD, while AH (21.7%), CG (20.8%), GH (21.1%), SH (21.2%), CS (20.8%) had lower CP content, which was anticipated given the different CP content of

replacement ingredients. All of them greatly exceeded NRC (2001) requirements for RDP and MP, which achieved our formulation goal.

Our objective was to assess individual AA availability from various feed ingredients. However, this is generally restricted to the EAA as non-essential AA (NEAA) can be synthesized by the animal which prevents derivation of a unique estimate of absorbed entry. Derived NEAA entry rates from this study had large standard errors, which indicated that post-absorptive synthesis was variable across treatments and animals, although they were well determined in our previous study that used high protein ingredients (Estes et al., 2018). Therefore, only EAA are discussed in this report. In addition, histidine results were near baseline and quite variable due to the AA derivatization method used, and thus were excluded. Amino acids with polar or charged side chains, such as histidine and serine, generally had lower recovery from alkyl chloroformates (Walsh et al., 2014a).

3.4.1 In Situ Protein Degradation for Test Ingredients

The ruminal N digestion results from the 108-h incubation are summarized in Table 2. Fraction A represents soluble N and N in particles smaller than 50 μm which can escape from the nylon bag. The A fraction (58.4%) of DDGS greatly exceeded values reported by Kleinschmit et al. (2007) (15.9 to 19.7%) and by Mjoun et al. (2010) (11.1 to 18.4%). The difference might be related to the amount of solubles added to grains according to Cao et al. (2009), who found a linear increase in fraction A when solubles in dried distillers grain increased. Soybean meal, soyhulls and corn silage also showed higher A fractions compared to NRC (2001). Increased small particle loss might be partially caused by sample preparation (grinding) or bag wash (machine wash), and can result in an overestimation of the A faction (Michalet-Doreau and Ould-Bah, 1992). For example, the dry-grinding process would cause fracture of the seed kernels and release protein as

a fine powder. The extent of such loss can be determined (Maxin et al., 2013), and used to correct the data. In the current study, we machine washed the bags, which removed bacteria but also might enhance the escape of small particles. These limitations have been noted as potentially contributing to low repeatability of the mobile bag technique within (Setälä, 1983, Madsen and Hvelplund, 1985, Michalet-Doreau and Cerneau, 1991) and across laboratories (Oldham, 1987). Compared to the soluble protein from in vitro evaluation, the A fraction from in situ evaluation was also much higher, which further indicated the particle loss from bags.

The Kd is affected by many factors including particle size and protein structure (NRC, 2001). Smaller particle size increases the surface area per unit of mass and thus the rate of degradation. Therefore, the higher rate of degradation for soybean meal, soyhulls and corn grain compared to NRC may be indicative of reduced particle size which was consistent with our observations of greater A fractions. The heat processing of dried distillers grain makes it more resistant to ruminal degradation (NRC, 2001, Doiron et al., 2009, Mjoun et al., 2010), which was consistent with its low Kd. The potentially degradable CP (fraction B) was low for soybean meal, corn silage, soyhulls and distillers compared to NRC (2001), which was consistent with their high A fraction content. Fraction C is the non-degradable N. The fraction C value may be related to heat process of the protein, which can vary widely within feedstuffs (Kleinschmit et al., 2007, Mjoun et al., 2010). For example, the degradation of individual AA in corn silage during 12 h rumen incubation varied from 19% (Taghizadeh et al., 2005) to 71% (Van Straalen et al., 1997). In the current study, the rumen protein degradation parameters of dry hays (alfalfa hay and grass hay) were more consistent with NRC (2001), which was expected considering small variation in processing and protein structure (Janicki and Stallings, 1988).

The calculated RUP for soybean meal, distillers gain, soyhulls, corn grain and corn silage were all lower than values from NRC (2001) and previous studies (Paz et al., 2014b), which is consistent with larger A fractions and smaller B and C fractions in the current work. The differences among studies could be caused by variability in feedstuff composition or feed and sample processing (Maxin et al., 2013). Compared to in situ and NRC values, the RUP results from in vitro test were much greater for all ingredients except for soybean meal and grass hay. This is due primarily to reduced soluble protein suggesting that particle loss from the bags was a problem. However, there was still significant deviations in RUP estimates from the in vitro evaluations as compared to the NRC value. The potential reason is the in vitro method cannot mimic the bio environment of rumen, for example, a single incubation time may be too short or the ruminal microbes are not less active in incubation flask, which is likely underestimated the RDP and overestimated RUP. Regardless of the reason for the differences among methods, the variation across methods is clear.

3.4.2 Apparent Total Tract Digestibility of N

Feed intake and fecal output, and apparent total tract digestibility observations are summarized in Table 3. The average DMI was 6.68 ± 0.17 kg, which was not significantly affected by treatments. Zhang et al. (2017) also observed that high CP, heifer diets did not affect DMI. The N intake of BD and DDGS were higher than CS, CG, GH and SH, but not significantly different from AH and BG. The total tract apparent N digestibility was not significantly different among treatments with average value 63.1%. Anderson et al. (2015) indicated increasing DDGS in heifer diets from 30 to 50% increased total tract CP digestibility. In this study, we didn't observe significant difference in N digestibility among treatments likely due to small difference in N intake or lack of precision of the measurement.

3.4.3 Plasma Entry Rates of Individual Essential Amino Acid

The standard errors of entry rates for EAA were less than 10% of estimated values (Table 4), which is consistent with Estes et al. (2018). The estimated EAA entry rates were not significantly different among treatments, which is consistent with NRC prediction (Table 4) of no differences in EAA flow to the small intestine among treatments. Compared with NRC estimated duodenal digestible AA flows, the derived AA entry rates were much greater, which was also observed by Estes et al. (2018) using this prediction model . Although the NRC model estimates of EAA flow from the rumen contain as much as 50% errors of prediction (Bateman et al., 2001), they clearly are not in error by 2 or 3 fold. As discussed by Estes et al. (2018), this likely reflected inaccurate estimates of isotope incorporation into slow turnover, body protein pools during a 2 h infusion. Hanigan et al. (2009) was able to describe mammary tissue free AA, fast turnover protein bound AA, and slow turnover protein bound AA using long term infusions of several AA, and found that the fractional incorporation of Leu, Met, Phe, and Val into total mammary tissue protein ranged from a low of 59%/d for Met to 86%/d for Val. Lower activity tissues such as muscle likely would have much lower rates of incorporation, and thus would still be significant sinks for isotope over several days thus explaining the significantly greater estimates of plasma entry versus NRC predicted duodenal digestible flow rates. For example, Garlick et al. (1980) reported that the fractional rates of protein synthesis in muscle of young male rats were from 16.9 to 21.3% per day whereas in active tissues like viscera was as high as 119.2% per day. Hanigan et al. (2009) also indicated the Phe turnover rate in goat mammary was averaged 131% per day. Such bias was expected and was removed when solving for differences between ingredients and BD (Table 4). Since all treatments had the same proportional replacement of BD, the expected entry rate

coefficient for BD should represent the RUP from BD plus MCP contributions to EAA entry plus any bias associated with entry estimates due to loss of label in the slow turnover pool.

The plasma AA entry associated with each ingredient was derived from the dietary entry rates (Table 5), with mean standard error of the estimates of 0.37% of CP across the EAA and ingredients which represented a relative error of 32%. The absolute error was less than the mean of 0.41% of CP reported by Estes et al. (2018), however, because the ingredients generally had lower CP content in the current work (DDGS vs blood meal), the relative error was greater than the 14% reported in the prior work . When the standard errors were compared across EAA, it was the greatest for Phe and Met reflecting the low proportions of these AA in the test ingredients. When the average standard errors of estimated plasma AA entry rates were compared across ingredients, the low CP ingredients, corn silage (49%), corn grain (45%), grass hay (46%) and soyhulls (39%), had greater standard errors than those with greater CP: alfalfa hay (23%), brewers grain (13%) and distillers grain (15%), which was likely due to the difference in proportion of protein contributed by test ingredients. For example, the proportion of total dietary protein contributed by each ingredient was 4.6, 5.1, 5.8, 6.4, 8.4, 12.2 and 13.1 % of CP for corn silage, corn grain, grass hay, soyhulls, alfalfa hay, brewers grain and distillers grain respectively. The correlation between the SE of estimated AA entry rates and protein contribution of test ingredients showed that the dietary true protein proportion contributed by the test ingredient should not be less than 12.1% to obtain results with standard errors less than 15% on a relative basis.

3.4.4 Individual Essential Amino Acid Availability from in Vivo Evaluations of Test Ingredients

The model derived EAA entry rates (% of CP) for Ile, Leu, Lys, Met, Phe, Thr, and Val for each ingredient were then used to estimate AA availability (% ingredient AA) assuming the loss

of EAA during first-pass through the splanchnic bed was the same as reported by Estes et al. (2018), which were calculated based on observed plasma AA availability of infused casein and casein AA digestibility reported by (Rutherfurd and Moughan, 1998)(Table 5). The results indicated AA availability varied across individual AA and feed ingredients from 18.1% of ingredient AA for Leu to 49.6% for Met in corn silage; from 18.4% for Val to 39.4% for Phe in grass hay; from 25.2% for Leu to 50.4% for Met in alfalfa hay, from 23.8% for Thr to 59.2% for Leu in distillers grain; from 20.8% for Val to 27.2% for Met in soyhulls, from 23.9% for Phe to 56.1% for Leu in brewers grain, and from 19.7% for Leu to 46.7% for Ile in corn grain. Research on AA availability of these 7 feed ingredients is limited, thus some calculations were undertaken to make direct comparisons. For example, Taghizadeh et al. (2005) investigated the rumen degradation and intestine digestibility of AA in corn silage, corn grain and alfalfa hay in steers using mobile bags, based on which the AA availability was calculated to vary from 12% for Met to 26% for Lys in corn grain, from 16% for Lys to 25% for Tyr in corn silage, and from 22 for Leu to 25% for Lys in alfalfa hay. Compared to current results, Taghizadeh et al. (2005) had smaller values and variation across individual AA. The potential reason for reduced estimates might be that the nylon bags created a barrier between feedstuffs and chyme, which can cause the underestimation of the nutrient digestibility. In addition, the failure to treat the mobile nylon bags with abomasal pepsin-HCl might also cause the lower values by Taghizadeh et al. (2005). Another potential explanation for the differences is that a 12 h ruminal incubation as used by Taghizadeh et al. (2005) might not reflect the real rumen retention time. Varvikko and Vanhatalo (1991) found that a 16 h rumen incubation was too short for forage. However, the calculated AA availability by using nylon bags from Van Straalen et al. (1997) was from 24% for Tyr to 37% for Met, which was greater than values reported by Taghizadeh et al. (2005). Other possible reason was the

variation in feed sources. Mjoun et al. (2010) reported absorbable AA (g/kg of CP) supplied by distillers grains RUP and the AA composition of the ingredient. The AA availability were calculated to range from 19% for Ile to 40% for Phe. Paz et al. (2014a) reported the AA availability varied from 14% for Lys to 31% for Leu in low fat distillers grain. Previous results showed AA availability varied among different distillers grain sources, which was also reported by Kleinschmit et al. (2005). Usually grass hay is not used as a major source of protein, thus AA digestibility data is limited for it. Borucki Castro et al. (2007) observed a range in AA availability from soybean meal from 31.3% for Met to 40.4% for Thr, and from 50.2% for Val to 71.9% for Met for heat treated soybean meal using in situ methods. However, Titgemeyer et al. (1989) reported the AA availability of soybean meal varied from 27.2% for His to 70.85 for Arg using an in vivo method. The latter values are more consistent with our in vivo observations. The variance is likely caused by different techniques and feed sources. The current isotope technique was found to be accurate and unbiased by Estes et al. (2018) using casein infusions, thus the inconsistent results from in situ and in vitro tests may indicate the inaccuracy of those evaluation methods.

3.4.5 Total Essential Amino Acid Availability from in Vivo, in Situ and in Vitro Evaluations of Test Ingredients

Estimates of least squares means of total RUP-EAA availability are displayed in Table 6. The plasma EAA entry rates derived from our in vivo technique were 30.6, 27.4, 31.3, 37.2, 26.4, 37.8, and 33.5% of EAA in test ingredients for corn silage, grass hay, alfalfa hay, dried distillers grain, soyhulls, brewers grain, and corn grain respectively. If we assume 8.27% utilization by gut tissue, EAA absorbed from the gut lumen (availability) were 33.4, 29.9, 34.1, 40.6, 28.8, 41.2, and 36.5% of EAA in test ingredients. Previous mobile bag studies found that the average TAA availability of RUP for corn silage, grass hay, alfalfa hay, dried distillers grain, soyhulls, brewers

grain, and corn grain were 32.7, 28.5, 26, 52.1,26.0, 45.3, and 35.5% of CP in feed ingredients respectively (NRC, 2001, White et al., 2017b), which is similar to our in vivo results. However, the in vitro and in situ results from single feed ingredient and time point showed great variation. Compared to in vivo results, in vitro tests tended to give lower RUP digestibility for hay and higher values for other feedstuffs, which was inconsistent with previous observations (Berthiaume et al., 2000, Jahani-Azizabadi et al., 2009). The potential reason is in vitro conditions may not mimic the animal digestion system perfectly. The RUP availability from in situ tests in the current study is less than the in vivo results. Berthiaume et al. (2000) compared the in situ and in vivo methods for intestinal digestibility of rumen protected Met and also found in situ techniques underestimated the Met availability (43.6% vs 74.5%), which was hypothesized to be caused by the restricting contact between test feed and duodenal chyme. But this may not be the case for other amino acids and there was discrepancy among studies. Jahani-Azizabadi et al. (2009) found that in vivo total tract nitrogen disappearance was less than indicated by in situ and in vitro methods. This could be due to the lack of correction factors for endogenous crude protein and large intestinal microbial fermentation. The variation might also be due to animal, diets, AA or methods (White et al., 2017b). The in vivo method should be the most reliable since all measurements occur naturally within the animal body. Therefore, it is critical to compare non-in vivo methods with valid in vivo methods across ingredients to verify the in vitro or in situ approaches before application. However, the cost of in vivo work makes it less applicable as a commercial technique. Although White et al. (2017b) compared mobile bags and in vitro methods for RUP digestibility and found more variation associated with the use of mobile bags, we found that the mean for mobile bag RUP and digested RUP were similar to our estimates calculated from AA availability, and thus are potentially useful for assessment of variation among sources. However, that method is subject to

potential bias associated with the choice of residence time in the rumen, where appropriate times likely vary by ingredient.

3.4.6 Future Work

In the future, more feed ingredients can be tested using this method. To obtain results with standard errors less than 15% on a relative basis, the dietary true protein proportion contributed by the test ingredient should not be less than 12.1% of dietary CP. This will help decrease variation in the final EAA availability estimates and ensure robust entry rate derivation for the ingredient. Additional precision and reduced total entry rate bias may be achieved using an infusion time not less than 6 h. The increased observation time will also reduce the chance of entry rate variation affecting the estimates, and will improve estimates of the true plateau state, which should allow derivation of more accurate and precise estimates of true plasma AA entry rates independent of the BD and a better understanding of protein turnover in the body.

Additionally, it may be possible to define the proportion of total EAA entry that is derived from digested MCP if the microbes are labelled with ^{15}N via ruminal ammonium sulfate infusion.

Finally, the protein digestibility of feed ingredients is likely not consistent under different feeding conditions, such as very high or low CP and energy intakes (Yang and Beauchemin, 2004). In addition, ingredient source and degradability of dietary CP are also factors that may introduce variation among sources of a common ingredient (Prange et al., 1984, Mabjeesh et al., 1996, Kleinschmit et al., 2005). Therefore, additional work is required to assess the range in availabilities from different sources of an ingredient with different feeding conditions (e.g. high and low forage), and to further evaluate in situ and in vitro methods as compared to in vivo measurement if we are to develop a real time system that can be used by industry.

3.5. Conclusion

We observed EAA availability for corn silage, grass hay, alfalfa hay, distillers grain, soyhulls, brewers grain, and corn grain of 33.4, 29.9, 34.1, 40.6, 28.8, 41.2, and 36.5% of ingredient EAA respectively assuming 8.27% utilization by gut tissue. Although the general trend (availability of forage and byproducts < availability of grains) was consistent, compared to in vivo results, in vitro evaluations underestimated RUP availability of hay but overestimate other feedstuffs, whereas in situ methods underestimated RUP availability of all test ingredients. The average RUP availability derived from meta-analysis of mobile bag results are representative and can be used to evaluate the variation among sources, but the availability of individual AA from in situ or in vitro vary less compared to in vivo value. Therefore, in vivo studies are necessary to build a matrix of EAA availabilities for representative ingredients that can be used in nutritional models.

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Table 3.1. Diet composition and formulated nutrients. Diets were formulated for a 272 kg BW heifer with a DMI of 6.6 kg/day

Item	Treatment[1]							
	BD	CS	CG	AH	GH	SH	DDGS	BG
Ingredients (% DM)								
Grass Hay, mature	34.7	31.2	31.2	31.2	41.2	31.2	31.2	31.2
Corn Silage, mature	34.7	41.0	31.1	31.1	31.1	31.1	31.1	31.1
Soybean Meal, 48% CP	29.8	26.8	26.8	26.8	26.8	26.8	26.8	26.8
Dried Corn Grain, cracked	—	—	9.9	—	—	—	—	—
Alfalfa Hay	—	—	—	10.0	—	—	—	—
Soybean hulls	—	—	—	—	—	10.0	—	—
Dried Corn Distillers Grain	—	—	—	—	—	—	10.0	—
Wet Brewers Grain	—	—	—	—	—	—	—	9.9
Vitamin and Mineral premix	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9
Nutrient Composition[2]								
CP (%)	22.7	21.3	21.4	22.3	21.5	21.8	23.4	23.3
RDP (g/day)								
Required	653	652	666	645	644	654	662	657
Supplied	923	863	872	916	871	891	940	961
MP (g/d)								
Required	534	537	536	537	536	538	536	529
Supplied	860	841	847	831	844	852	890	832
NDF (%)	42.3	42.5	39	42.2	44.9	44.1	41.9	42.7
ADF (%)	25.8	25.9	23.5	26.5	27.3	27.7	25.2	25.5
NEg (Mcal/kg DM)	1.0	1.0	1.1	1.0	1.0	1.0	1.1	1.0
Ether Extract (%)	2.1	2.2	2.3	2.2	2.1	2.2	2.9	2.4

[1]BD= Basal Diet; CS = Basal Diet + Corn Silage; GH = Basal Diet + Grass Hay; AH= Basal Diet + Alfalfa Hay; DDGS = Basal Diet + Dried Distillers Grain; SH = Basal Diet + Soybean Hulls; BG= Basal Diet + Wet Brewers Grain; CG= Basal Diet + Corn Grain.

[2]Nutrient components were estimated from the (NRC, 2001).

Table 3.2. In situ evaluation of test ingredients during a 108 h ruminal incubation and in vitro evaluation from commercial test

	Ingredients							
	Soybean Meal	Corn Silage	Corn Grain	Alfalfa Hay	Grass Hay	Soyhulls	Dried Distillers Grain	Brewers Grain
In situ evaluation								
A fraction[1], % CP	26.9	79.9	29.0	31.8	26.6	57.1	58.4	21.0
B fraction, % CP	73.1±3.6	20.1±5.4	68.4±5.0	59.4±4.8	57.9±2.1	41.6±3.5	38.6±3.6	73.6±1.5
C fraction, % CP	0.0±1.7	0.0±5.9	2.6±3.4	8.8±3.7	15.5±1.6	1.3±1.7	3.0±3.9	5.5±1.0
Kd, %/h	10.2±1.1	1.4±0.7	5.7±1.1	10.7±1.9	4.2±0.4	3.7±0.8	2.8±0.7	5.3±0.3
RUP[2], % CP	27.1	16.3	37.6	30.1	49.6	27.0	29.3	44.6
In vitro evaluation, n=2								
Soluble protein, % CP	5.9±0.5	49.9±2.4	15.9±2.4	30.2±1.7	26.8±1.2	16.3±1.8	18.2±0.2	3.6±2.9
RDP, % CP	66.8±0.6	19.3±0.1	20.2±0.3	46.2±2.9	51.5±4.4	12.8±1.7	38.8±0.2	27.7±1.2
RUP, % CP	33.2±0.6	80.7±0.1	79.8±0.3	53.9±2.9	48.5±4.4	87.2±1.7	61.2±0.2	72.3±1.2
RUP digestibility, % (NRC, 2001)	79.2±0.2	68.3±5.6	78.1±0.9	38.2±1.4	23.5±7.4	64.1±1.3	72.9±1.8	56.7±0.2
A fraction, % CP	15.0	51.3	23.9	37.9	28.4	22.5	28.5	18.3
B fraction[1], % CP	84.4	30.2	72.5	52.7	52.9	72.2	63.3	64.6
C fraction, % CP	0.6	18.5	3.6	9.4	18.7	5.3	8.2	17.1
Kd, %/h	7.5	4.4	4.9	10.8	5	6.2	3.6	4.7
RUP[2], % CP	38.1	35.7	43.5	28.2	47.6	40.8	47.8	53.3

[1]Defined as 100-B-C.

[2]Predicted with an assumed passage rate of 6%/h (NRC, 2001).

Table 3.3. Least square means for feed intake and fecal output of DM and N and total tract apparent digestibility

	Treatment[1]								
	BD	CS	CG	AH	GH	SH	DDGS	BG	SEM
DM									
Intake(kg/d)	6.79	6.63	6.71	6.77	6.74	6.74	6.62	6.47	0.17
Fecal output(kg/d)	2.58	2.36	2.63	2.78	2.64	2.60	2.52	2.37	0.31
Apparent digestibility (%)	62.1	64.1	60.2	59.4	61.4	61.7	62.3	63.4	3.7
N									
Intake(g/d)	245.1[bc]	221.1[a]	223.4[a]	234.7[b]	227.9[ab]	228.4[ab]	237.1[bc]	236.2[b]	5.7
Fecal output(g/d)	89.5	74.5	87.8	100.9	82.9	88.0	82.7	80.5	12.0
Apparent digestibility (%)	63.1	65.9	60.1	57.7	63.9	61.6	65.9	65.8	5.4
CP of diets (%)	22.1[d]	20.8[a]	20.8[a]	21.7[c]	21.1[b]	21.2[b]	22.8[e]	22.8[e]	0.2

[1]BD= Basal Diet; CS = 90% Basal Diet + 10% Corn Silage; GH = 90% Basal Diet + 10% Grass Hay; AH= 90% Basal Diet + 10% Alfalfa Hay; DDGS = 90% Basal Diet + 10% Dried Distillers Grain; SH =90% Basal Diet + 10% Soybean Hulls; BG= 90% Basal Diet + 10% Wet Brewers Grain; CG=90% Basal Diet + 10% Corn Grain.

[a-e] Least square means within a row with different superscripts are considered significantly different ($P < 0.05$).

Table 3.4. Least square means of plasma entry rates (g/d) for each treatment derived from isotope dilution model and digestible duodenal AA flow predicted by the NRC (2001) model at observed DMI

AA	Treatment[1]								
	BD	CS	CG	AH	GH	SH	DDGS	BG	SEM
Least square means of plasma entry rates (g/d)									
Ile	151	141	146	142	142	143	142	138	5.6
Leu	252	224	234	224	228	228	231	226	9.8
Lys	228	211	213	211	214	214	212	206	8.2
Met	52	48	50	47	49	48	48	47	2.3
Phe	103	93	98	93	96	96	97	94	3.2
Thr	152	142	148	144	145	145	144	142	5.3
Val	234	217	228	214	219	219	219	214	8.4
Predicted total duodenal digestible AA flow[2] (g/d)									
Ile	43	42	42	42	42	43	44	41	1.5
Leu	76	75	76	74	74	76	80	74	2.7
Lys	56	55	56	55	55	57	56	54	1.9
Met	15	15	15	15	15	15	16	15	0.5
Phe	45	44	45	44	44	45	47	44	1.6
Thr	42	41	41	40	41	41	37	40	2.4
Val	47	47	47	46	46	47	49	46	1.6

[1]BD= Basal Diet; CS = 90% Basal Diet + 10% Corn Silage; GH = 90% Basal Diet + 10% Grass Hay; AH= 90% Basal Diet + 10% Alfalfa Hay; DDGS = 90% Basal Diet + 10% Dried Distillers Grain; SH =90% Basal Diet + 10% Soybean Hulls; BG= 90% Basal Diet + 10% Wet Brewers Grain; CG=90% Basal Diet + 10% Corn Grain.
[2]Total AA flow at observed DMI predicted by the NRC model (2001).

Table 3.5. Plasma EAA entry rates for Ile, Leu, Lys, Met, Phe, Thr, and Val and availability for each ingredient

Ingredients	AA	Ingredient AA[1], % of CP	Plasma AA entry, % of CP	SE	*P*>|t|[2]	Plasma availability, % of ingredient AA	EAA availability[3], % of ingredient AA
Corn Silage	Ile	3.34	1.07	0.53	0.05	32.0	40.2
	Leu	8.59	1.39	0.66	0.04	16.2	18.1
	Lys	2.51	0.96	0.41	0.02	38.2	43.6
	Met	1.53	0.67	0.31	0.03	43.8	49.6
	Phe	3.83	-	-	-	-	-
	Thr	3.19	-	-	-	-	-
	Val	4.47	1.02	0.54	0.06	22.8	23.7
Corn Grain	Ile	3.31	1.23	0.56	0.03	37.2	46.7
	Leu	11.2	1.98	0.69	<0.01	17.7	19.7
	Lys	2.84	1.08	0.47	0.03	38.0	43.4
	Met	2.13	0.77	0.32	0.02	36.2	41.0
	Phe	4.62	1.59	0.94	0.09	34.4	39.6
	Thr	3.55	1.35	0.62	0.03	38.0	38.0
	Val	4.02	1.34	0.61	0.03	33.3	34.6
Alfalfa Hay	Ile	3.98	1.09	0.29	<0.01	27.4	34.4
	Leu	7.71	1.74	0.34	<0.01	22.6	25.2
	Lys	4.34	1.42	0.21	<0.01	32.7	37.3
	Met	1.46	0.65	0.17	<0.01	44.5	50.4
	Phe	4.89	-	-	-	-	-
	Thr	4.1	1.37	0.31	<0.01	33.4	33.4
	Val	5.01	1.36	0.30	<0.01	27.1	28.1
Grass Hay	Ile	3.32	0.97	0.44	0.03	29.2	36.7
	Leu	6.22	1.38	0.50	<0.01	22.2	24.8
	Lys	3.48	0.71	0.31	0.02	20.4	23.3
	Met	1.3	0.44	0.23	0.06	33.8	38.3
	Phe	3.92	1.34	0.70	0.06	34.2	39.4
	Thr	3.6	1.24	0.50	0.02	34.4	34.4
	Val	4.51	0.8	0.42	0.07	17.7	18.4
Soyhulls	Ile	3.86	1.13	0.37	<0.01	29.3	36.8
	Leu	6.5	2.15	0.45	<0.01	33.1	36.9
	Lys	6.27	1.17	0.28	<0.01	18.7	21.3
	Met	1.16	0.38	0.21	0.07	32.8	37.2
	Phe	4.33	1.11	0.62	0.07	26.1	30.1
	Thr	3.6	0.83	0.40	0.03	25.0	25.0
	Val	4.56	0.91	0.38	0.02	20.0	20.8
Dried Distillers Grain	Ile	3.71	1.39	0.20	<0.01	37.5	47.1
	Leu	9.59	5.09	0.24	<0.01	53.1	59.2
	Lys	2.24	0.73	0.15	<0.01	32.6	37.2
	Met	1.82	0.61	0.11	<0.01	33.5	37.9

	Phe	4.87	1.87	0.31	<0.01	38.4	44.3
	Thr	3.44	0.82	0.20	<0.01	23.8	23.8
	Val	4.7	1.95	0.19	<0.01	41.5	43.1
Brewers Grain	Ile	3.85	1.02	0.19	<0.01	26.5	33.3
	Leu	9.91	4.98	0.23	<0.01	50.3	56.13
	Lys	3.4	1.45	0.15	<0.01	42.6	48.63
	Met	1.93	0.84	0.11	<0.01	43.5	49.3
	Phe	5.57	1.12	0.29	<0.01	20.8	23.9
	Thr	3.61	1.48	0.21	<0.01	41.0	41.0
	Val	5.14	2.06	0.19	<0.01	40.1	41.6

[1]The AA concentration of ingredients were from NRC (2001).
[2]Test of plasma AA entry different from 0.
[3]Values calculated from AA plasma availability and AA first-pass utilization by gut tissue during absorption (Rutherfurd and Moughan, 1998, Estes et al., 2018b).

Table 3.6. Least square means of protein entry rates predicted from entry of Ile, Leu, Met, Lys, Phe, Thr, and Val for each ingredient and calculated digestible RUP and digestibility of the RUP for each ingredient

Ingredients	Plasma EAA availability, % Ingredient EAA	SEM	RUP-EAA availability[1], % ingredient EAA	RUP-TAA availability [2], % CP	RUP-TAA availability[3], % CP	RUP-TAA availability[4], % CP
Corn Silage	30.6	3.4	33.4	55.1	11.1	32.7
Grass Hay	27.4	3.2	29.9	11.4	11.7	28.5
Alfalfa Hay	31.3	3.4	34.1	20.6	11.5	26.0
Dried Distilles Grain	37.2	3.2	40.6	44.6	21.4	52.1
Soyhulls	26.4	3.2	28.8	55.9	17.4	26.0
Brewers Grain	37.8	3.2	41.2	40.9	25.3	45.3
Corn Grain	33.5	3.2	36.5	62.3	29.4	35.5

[1]Values calculated from plasma AA availability and 8.27% utilization of AA by gut tissue during absorption (Estes et al., 2018b).

[2]Total AA availability of RUP from in vitro results: RUP-TAA availability (% CP) = RUP (% CP) × RUP digestibility (%) / 100.

[3]Total AA availability of RUP from in situ results.

[4]Total AA availability of RUP calculated from RUP and it's digestibility reported by (White et al., 2017b).

Figure 3.1. Predicted and observed Leu isotopic ratios versus infusion time for one infusion.

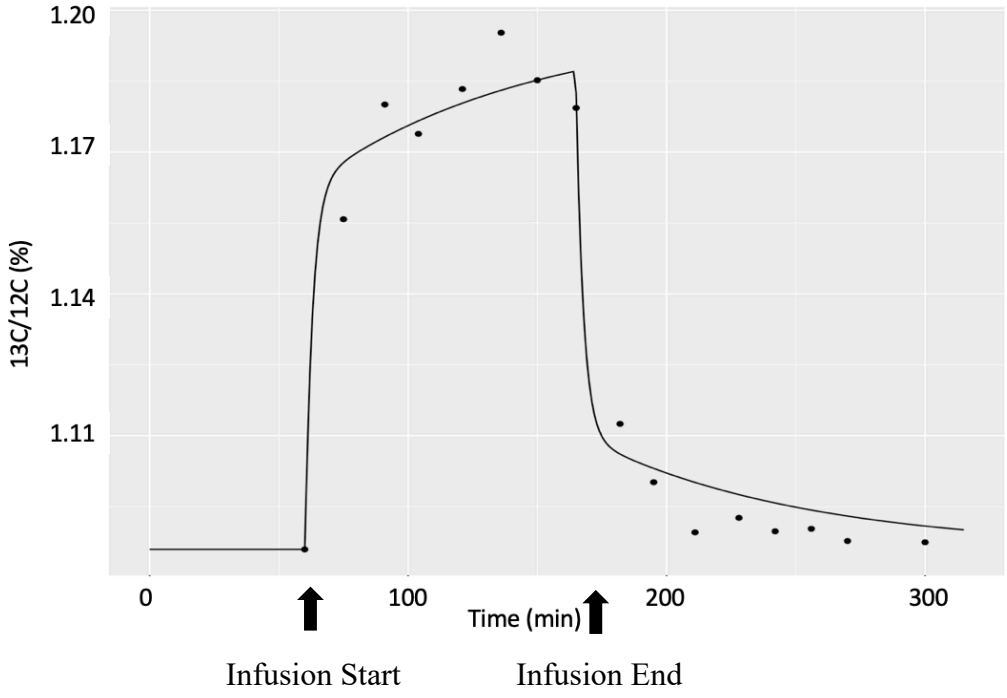
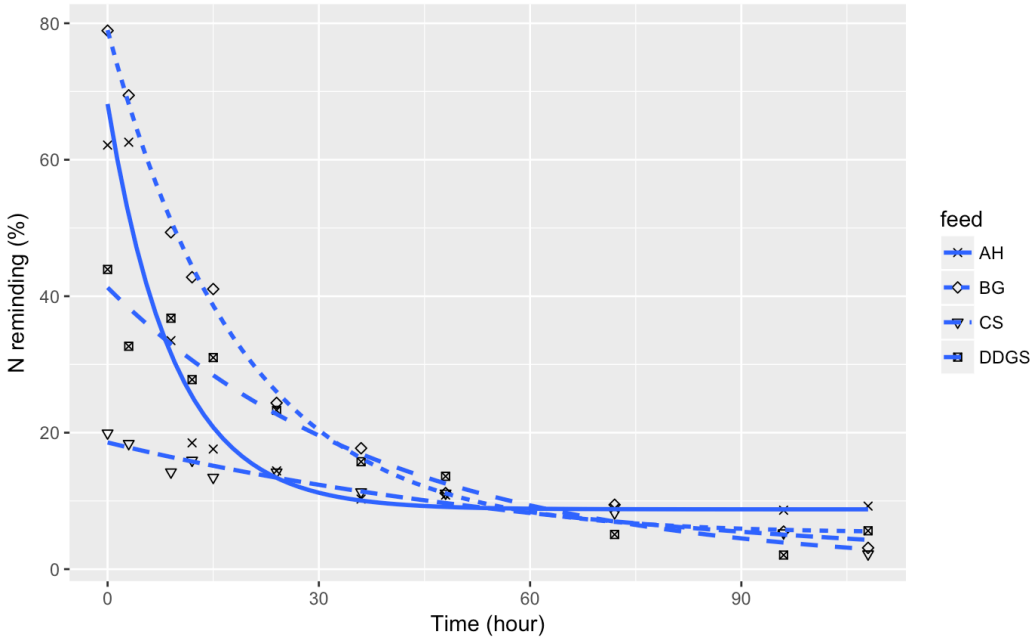
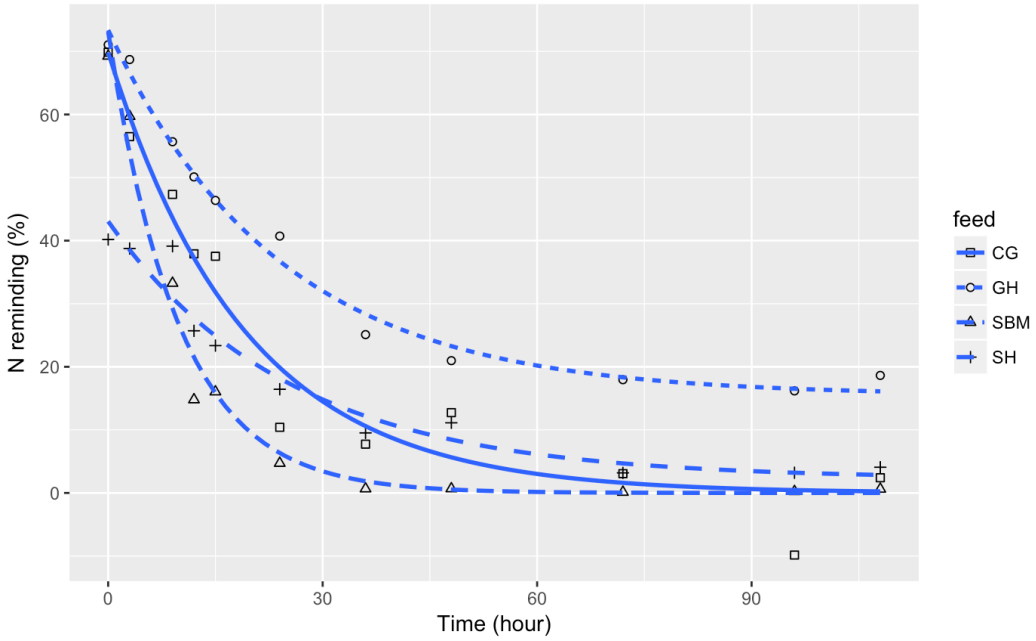


Figure 3.2. Observed and predicted in situ protein degradation for the 8 test ingredients

A) Observed and predicted in situ protein degradation for AH, BG, CS, DDGS



B) Observed and predicted in situ protein degradation for CG, GH, SBM, SH



3.8. Supplemental Materials

Total AA Pools. The differential equations for total AA in fast pool respect to time ($dQAA_{Fast}/dt$, mmol/min) and in slow pool respect to time ($dQAA_{Slow}/dt$, mmol/min) were:

$$dQAA_{Fast}/dt = FAA_{Entry} + FAA_{Infused} + FAA_{SlowFast} - FAA_{FastSlow} - FAA_{Clearance} , \quad [1]$$

$$dQAA_{Slow}/dt = FAA_{FastSlow} - FAA_{SlowFast} , \quad [2]$$

where FAA_{Entry} represented AA absorbed from gut or synthesized in body. $FAA_{Infused}$ was mmol AA infused into jugular vein per minute, which was 0 outside infusion hours. $FAA_{FastSlow}$ was AA from free AA pool used for protein synthesis, which coded as flux from fast pool to slow pool:

$$FAA_{FastSlow} = KAA_{FastSlow} \times QAA_{Fast} , \quad [3]$$

where $KAA_{FastSlow}$ was mass action loss constant, unit is min^{-1}. $FAA_{SlowFast}$ was AA from protein degradation, coded as AA flux from slow pool to fast pool.

$$FAA_{SlowFast} = KAA_{SlowFast} \times QAA_{Slow} , \quad [4]$$

$FAA_{Clearance}$ was AA clearance from the fast pool (utilization other than protein synthesis) and was calculated as:

$$FAA_{Clearance} = KAA_{Clearance} * QAA_{Fast} , \quad [5]$$

where $KAA_{Clearance}$ was mass action loss constant, unit is min^{-1}. The size of fast pool and slow pool at time t can be expressed as:

$$QAA_{Fast} = \int \frac{dQAA_{Fast}}{dt} + iQAA_{Fast} , \quad [6]$$

$$QAA_{Slow} = \int \frac{dQAA_{Slow}}{dt} + iQAA_{Slow} , \quad [7]$$

where the initial size of fast pool ($iQAA_{Fast}$) was calculated from background plasma AA concentration (CAA_{Fast}, mM) and volume of fast pool (VAA_{Fast}, L):

$$iQAA_{Fast} = CAA_{Fast} \times VAA_{Fast} , \quad [8]$$

The initial size of slow pool ($iQAA_{Slow}$) was calculated as:

$$iQAA_{Slow} = BW \times 1000 \times CProt_{BW} \times CAA_{Prot} / MW_{AA} \times KBW_{AA} \times 1000 , \quad [9]$$

where BW was body weight (kg). $CProt_{BW}$ was body protein (% BW), and CAA_{Prot} was AA composition of body protein (g/g) based on study of Williams (1978). MW_{AA} represented the molecular weight of each AA (g/mol). KBW_{AA} was the coefficient used to define the proportion of body protein (g/g) resident in QAA_{Slow}.

Isotopically Labeled Pools. The differential equations for labeled AAs in fast and slow pools respect to time (i represented isotope labeled) were:

$$dQAAi_{Fast}/dt = FAAi_{Entry} + FAAi_{Infused} + FAAi_{SlowFast} - FAAi_{FastSlow} - FAAi_{Clearance} , \quad [10]$$

$$dQAAi_{Slow}/dt = FAAi_{FastSlow} - FAAi_{SlowFast} , \quad [11$$

where $FAAi_{Entry}$, $FAAi_{Infused}$, $FAAi_{SlowFast}$, $FAAi_{FastSlow}$ and $FAAi_{Clearance}$ were calculated from total flux and isotopic enrichment in precursor pools:

$$FAAi_{Entry} = FAA_{Entry} \times eAA_{Background} , \quad [12]$$

$$FAAi_{Infused} = FAA_{Infused} \times eAA_{Infused} , \quad [13]$$

$$FAAi_{SlowFast} = FAA_{SlowFast} \times eAA_{Slow} , \quad [14]$$

$$FAAi_{FastSlow} = FAA_{FastSlow} \times eAA_{Fast} , \quad [15]$$

$$FAAi_{Clearance} = FAA_{Clearance} \times eAA_{Fast} , \quad [16]$$

The size of labeled fast pool and slow pool at time t can be expressed as:

$$QAAi_{Fsat} = \int \frac{dQAAi_{Fast}}{dt} + iQAAi_{Fast} , \quad [17]$$

$$QAAi_{Slow} = \int \frac{dQAAi_{Slow}}{dt} + iQAAi_{Slow} , \quad [18]$$

where the initial labeled fast pool size ($iQAAi_{Fast}$) was calculated from initial total fast pool ($iQAA_{Fast}$) and isotopic enrichment before isotope infusion ($eAA_{Background}$):

$$iQAAi_{Fast} = iQAA_{Fast} \times eAA_{Background} , \quad [19]$$

The initial labeled slow pool size ($iQAAi_{Slow}$) was calculated from initial total slow pool ($iQAA_{Slow}$) and isotopic enrichment before isotope infusion ($eAA_{Background}$):

$$iQAAi_{Slow} = iQAA_{Slow} \times eAA_{Background} , \quad [20]$$

The isotopic ratio in fast pool ($IRAA_{Fast}$) can be calculated as:

$$IRAA_{Fast} = QAAi_{Fast} / (QAA_{Fast} - QAAi_{Fast}) , \quad [21]$$

The isotopic ratio was fitted to the observed values for derivation of model parameters.

Model Inputs and Parameter Estimation. Model inputs in this study included $FAA_{Infused}$ (Equation1); CAA_{Fast} and VAA_{Fast} (Equation 8); BW, $CProt_{BW}$, CAA_{Prot} and KBW_{AA} (Equation 9); $eAA_{Background}$ (Equation 12). Infusion rate, start and stop times were recorded during the trial. $eAA_{Background}$ was determined from background samples collected before isotope infusion. VAA_{Fast} was set to 14.8% of BW according to Estes et al. (2018b) given AA entry rate was not sensitive to it. $CProt_{BW}$ was assumed to be 18.83% of BW, and CAA_{Prot} was AA composition of body protein based on study of Williams (1978). KBW_{AA} was a portion of the total body protein and represented activated protein during infusion period, which was set to 0.00148 according to Estes et al. (2018b).

Model parameters needed to derive included FAA_{Entry} (Equation 1), $KAA_{FastSlow}$ (Equation3), $KAA_{SlowFast}$ (Equation 4), and $KAA_{Clearance}$ (Equation 5). Given the animals were in steady state, we can assume that:

$$FAA_{FastSlow} - FAA_{SlowFast} = 0 , \quad [22]$$

$$dQAA_{Fast}/dt = 0 , \quad [23]$$

$$dQAA_{Slow}/dt = 0 , \quad [24]$$

By combining Equation 1, 2, 22, 23, 24 we can get:

$$KAA_{SlowFast} = \frac{KAA_{FastSlow} \times QAA_{Fast}}{QAA_{Slow}} , \quad [25]$$

$$KAA_{Clearance} = \frac{FAA_{Entry} + FAA_{Infused}}{QAA_{Fast}} \quad , \qquad [26]$$

Then, we can replace $KAA_{Clearance}$ and $KAA_{SlowFast}$ with other variables and parameters and deduced final model to two parameters: FAA_{Entry} and $KAA_{FastSlow}$. All modeling work was completed in R studio (version 1.0.143) with R 3.2.3. The predicted plasma isotopic ratios ($IRAA_{Fast}$) to observed values by a maximized log-likelihood function using the Nelder-Mead optimizer. Figure 2. showed the example of Leu for one infusion. Following the initial fit, residual errors were calculated, and data points were removed where the Studentized residual error exceeded an absolute value of 2, which was the case for 14% of the dataset. These outliers were generally also visually apparent when the observed data were plotted with the predicted values as the data represented a repeated sampling sequence in time, and thus deviations from the pattern in time were clearly evident.

Plasma EAA Entry Rates Assessment. The estimated FAA_{Entry} (mmol/min) can be converted to g/d as following:

$$FAA_{Entry}\ (g/d) = FAA_{Entry} / 1000 \times (24 \times 60) / MW_{AA}\ , \qquad [27]$$

Then fractional AA availability for each test ingredient can be derived by using linear mixed model (lmer) in R studio (1.0.143):

$$FAA_{Entry}\ (g/d) = (K1 \text{ x } CP_{ingredient\ 1}) + \ldots + (Kn \text{ x } CP_{ingredient\ n}) + Period + Animal, \qquad [28]$$

where $CP_{ingredient}$ was observed CP intake from BD, AH, BG, CS, GH, DDGS, CG or SH. Period and Animal were set as random effects.

The AA entry rates were used to derive fractional availability values (g AA appearing in plasma/g CP consumed) for each test ingredient by regression as described by Estes et al. (2018b). In Estes et al. (2018b), RDP was added to accommodate entry rates derived from the basal diet excluding soybean meal and MCP which varied when ruminally fermentable soybean

meal was replaced by post-ruminal infusions of casein and EAA. In our study, substitutions were for BD, and all treatment diets had RDP which greatly exceeded NRC (2001) requirements, thus negating the need to represent potential changes in MCP using RDP.

$$AA_{Entry}i\ (\%\ of\ Ingredient\ AA) = Ki\ /\ Ingredient\ AAi \times 100 \quad [29]$$

RUP AA availability in plasma (% of CP) was derived as the ratio of the sum of Ki over the sum of Ingredient AAi:

$$RUP_{Entry\_i}\ (\%\ of\ CP) = \frac{\sum Ki}{\sum Ingredient\ AAi} \times 100 \quad [30]$$

NEAA entry rates assessment in this study had large standard error, which indicated inconsistent results cross treatments and animals, although they were well determined in previous study that used high protein ingredients (Estes et al., 2018b).

3.8.1 References

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CHAPTER 4: Assessing Essential Amino Acid Availability from Microbial and Rumen Undegraded Protein in Lactating Dairy Cows

4.1. Abstract

The objective of this study was to assess a stable isotope technique for determination of essential AA availability from microbial protein in response to starch and rumen degraded protein. The study was designed as a 2 x 2 factorial arrangement of treatments applied in a repeated 4 x 4 Latin square design with 4 periods. Factors were high and low rumen degraded protein, and high and low starch. Twelve lactating cows were blocked into 3 groups according to days in milk, and randomly assigned to the 4 treatment sequences. Each period was 14 days in length with 10 days of adaption followed by 4 days of ruminal infusions of ^{15}N labeled ammonium sulfate. On the last day of each period, a ^{13}C-labeled AA mixture was infused into the jugular vein over a 6 h period to assess total AA entry. Rumen, blood, and urine samples were collected during the infusions. Ruminal microbes and blood samples were assessed for AA enrichment. Total plasma AA absorption rates were derived for each essential AA from the plasma, ^{13}C-AA enrichment. Essential AA absorption from microbial protein was calculated from total AA absorption based on ^{15}N enrichment in blood and rumen microbes. Microbial AA absorption rates derived from the isotope dilution model were greater for the high rumen degraded protein diets and unaffected by starch level, except for Met, which decreased with high starch. Microbial protein outflow estimated from purine derivatives increased with rumen degraded protein and was not significantly affected by starch, which was consistent with estimates from the isotope dilution model. Total AA absorption rates derived from the isotope dilution model were similar to estimates from CNCPS (v6.5), but a lower proportion of absorbed AA was derived from microbial protein for the former method. Microbial protein estimated from White et al. (2017) followed the same trend among

treatments implying it represented ruminal fermentation and microbial growth better than CNCPS. Assuming 7.6% loss during first pass through the splanchnic tissues, the average essential AA digestibility for microbial AA was 82%, which varied across AA and by treatment. The purine derivate-based estimates of microbial CP availability response to rumen degraded protein were similar to estimates derived from the isotope technique suggesting the method is valid. The new method has the advantage of quantifying the essential AA availability for individual EAA from rumen undegraded protein and microbial protein.

Keywords: dairy cows, microbial protein, amino acid availability, stable isotope

4.2. Introduction

It is generally assumed that a more mechanistic representation of AA supply to the mammary glands would improve the accuracy of predictions of milk protein production (Hanigan et al., 1998, Cant et al., 2003, Sok et al., 2017). The first step for developing such a model is an accurate estimate of AA outflow from the rumen and AA digestibility in the small intestine. For most diets, 60% or more of feed protein is degraded in the rumen and used to support microbial protein (MCP) synthesis making MCP an important contributor to the overall supply of AA to the animal (Clark et al., 1992b, Fleming et al., 2019b). If diets are to be formulated so that AA arising from digestion of rumen undegraded protein (RUP-AA) complements AA arising from digestion of MCP (MCP-AA) with respect to animal requirements, it is important to know the contribution of RUP-AA and MCP-AA to the overall metabolizable AA supply (Oltjen, 1969).

There are many factors that can affect MCP-AA, and thus AA availability to the animal. A well-studied nutrient interaction is that of ruminal carbohydrate and N availability. Carbohydrate is an important source of ATP for microbial growth (Nocek and Russell, 1988). To achieve optimal growth, the rate of ATP production from carbohydrate fermentation should be matched to the

needs for protein synthesis (Hespell and Bryant, 1979). Herrera-Saldana et al. (1990) also indicated that MCP synthesis was maximized when starch and protein sources with similar degradation rates were fed. Although the effects of ruminal carbohydrate and degraded protein (RDP) on MCP synthesis and production have been widely studied, observations of the effects on MCP-AA profile and digestibility are limited. Although the AA composition of MCP was initially reported to be constant (Weller, 1957, Purser and Buechler, 1966, Bergen et al., 1968, Ørskow et al., 1986), this was subsequently found to be inaccurate possibly due to varying species composition in the microbiome. In a survey of the AA composition of 441 bacterial samples from 35 experiments, Clark et al. (1992b) found that the reported AA composition varied. In addition, the AA profile of MCP varies between bacteria and protozoa, and may be affected by diets (Sok et al., 2017). Although Clark et al. (1992a) and Rodríguez et al. (2000) indicated that changes in the rumen environment may affect the composition of microbes, and thus the intestinal digestibility of MCP, previous studies observed inconsistent results. Hoogenraad and Hird (1970) found MCP digestibility varied from 79 to 95% using isotopically labeled bacteria. However, Storm et al. (1983) reported true digestibility of MCP of 81 to 82% based on abomasal infusions of isolated MCP in sheep. Most previous studies focused on MCP digestibility from different microbial groups, but studies on the effects of the rumen environment on microbial diversity, and thus MCP digestibility are limited. Therefore, more studies are needed to study the MCP-AA profile and availability under different feeding environments.

Microbial protein outflow is often estimated from observations of urinary purine derivative (PD) excretion (usually allantoin and uric acid in cattle). Although some PD are introduced to the animal in the feed, it is still a useful method for comparison purpose (Hristov et al., 2019). An advantage of this method is that it likely reflects digestible MCP as the PD are only released from

the microbes if they undergo digestion. NRC (2001) assumed the intestinal digestibility of MCP was 80% without considering the individual AA digestibility, which is difficult and expensive to determine. Furthermore, in situ and in vitro techniques such as the mobile bag technique or the modified 3-step procedure are not applicable due to the lack of representative microbes. Therefore, better techniques are required to determine individual AA digestibility of MCP and validate current data used by nutritional models.

Determining the AA digestibility of RUP or MCP in ruminants is technically difficult due largely to the errors of measurement associated with sample collection and animal variation (Titgemeyer et al., 1989, Boucher et al., 2009, Apelo et al., 2014). The cecectomized rooster assay described by Parsons (1985) and Aldrich et al. (1997) seems useful in determining MCP-AA digestibility, but one must assume digestibility in the rooster is representative of the ruminant, and that the microbial sample is not biased by collection and isolation. Estes et al. (2018) and Huang et al. (2019) adapted a stable isotope approach used by Maxin et al. (2013) to assess the RUP-AA availability from 11 individual feed ingredients. This method uses a 2 h or longer constant infusion of a ^{13}C labelled AA mixture derived from enriched algae to assess the plasma absorption rate of each essential AA. Because infusions and sampling are via the jugular vein, measurements can be made with minimal animal preparation. Errors of determination for AA availability from each ingredient are approximately 10% using this method, which is a large improvement compared to measurements of disappearance from the intestine (Titgemeyer et al., 1989). A potential shortfall of this approach is the assumption that microbial contributions to absorbed AA are constant across diets as test ingredients are added and removed which is presumed to be avoided by feeding a high protein diet.

The objective of the current work was to enhance the bioavailability assessment by infusing ^{15}N labeled ammonium sulfate into the rumen, which will be incorporated into microbial AA allowing identification of the proportion of absorbed AA that are derived from MCP. Therefore, the hypothesis of the current study was that MCP-AA availability varied across AA and diets and could be estimated using a stable isotope-based approach. The method was assessed for apparent validity based on MCP and RUP responses to varying dietary starch and RDP concentrations in lactating dairy cows.

4.3. Material and Methods

4.3.1 Animals, Treatments and Sampling

All animal procedures were conducted at the Virginia Tech Kentland Dairy Farm and approved by the Virginia Tech Animal Care and Use Committee. The study was designed as a 2 x 2 factorial arrangement of treatments applied in a replicated 4 x 4 Latin square design with 4 periods of 14 d each. Factors were high and low rumen degraded protein and high and low starch. Twelve lactating cows (629±52 kg BW) were blocked into 3 groups according to days in milk. Eight cows in two blocks had ruminal cannulas. The 4 cows in each block were randomly assigned to treatment sequences consisting of the combination of the 2 factors (RDP and starch): high RDP and high starch (HPHS), low RDP and high starch (LPHS), high RDP and low starch (HPLS) and low RDP and low starch (LPLS). Cows were milked twice daily at 0100 and 1230 h. Cows were housed in a pen fitted with Calan gates, and fed treatment diets 1x/d for the first 10 d of each period. On day 9 of each period, the eight rumen cannulated cows were moved to individual metabolism stalls and fed treatment diets 4x daily with a target minimum refusal of 5%. The other four cows remained in the Calan gate pen with 1x/d feeding until the end of experiment. On day 13 and 14, animals in the metabolism stalls were fed every 2 h at 100% of the observed average DMI for the

previous 3-d to encourage complete meal consumption at each feeding, and variation in AA absorption rates was minimized. Milk yield, feed intake, and body weight of 12 cows were recorded daily.

Ingredient and TMR samples were collected 4x per period on day 11, 12, 13 and 14, and pooled by period. Refusals were sampled on day 14. All samples were stored at -20 °C. Dry matter (DM) of refusals and TMR were determined by drying for 24 h at 100° C. Ingredients were dried at 55 °C for 48 h and sent to Cumberland Valley Analytical Services (Waynesboro, PA) for nutrient analysis (NIR2 package).

4.3.2 Isotope Infusion

From day 10 to 14, ^{15}N labeled ammonium sulfate mixed with pure water was constantly infused into the rumen of each cow in metabolism stall (41.6 mg/h, 9.33 mg ^{15}N /h). During the infusion, blood and whole rumen samples were collected twice per day at 0700 and 1900 h. Rumen samples were collected from three places in the rumen (forward, middle and back) through rumen cannulas. Spot urine samples were collected every 6 h, with the collection time rotating forward 2 h on the second day and 4 h on the third day of sampling. All samples were stored at -20°C until analysis.

On day 9 of each period, cows in metabolism stall were fitted with two jugular catheters (90 cm × 2.03 mm i.d. microrenathane, Braintree Scientific Inc., Braintree, MA) for ^{13}C-lablelled algal AA infusions and blood sampling. The infusion catheter tip was placed approximately 40 cm downstream of the tip of the sampling catheter to ensure infusate circulated through the vascular system prior to sampling as described by Estes et al. (2018). Catheters were placed on alternate sides of the neck in subsequent periods.

On day 14, 8 animals in metabolism stall were given a constant jugular infusion of 1 g of sterile, ^{13}C-labelled AA (U-^{13}C, 97-99% enriched algal AA dissolved in 100 mL of saline, Cambridge Isotope Laboratories, Andover, MA) over a 6 h period using clinical infusion pumps (LifeCare 5000, Abbott Laboratories, North Chicago, IL). Infusions were initiated at 1300 h and ended at 1900 h. Blood samples (8-10 mL each) were collected at -60, 30, 60, 90, 120, 150, 180, 210, 240, 270, 300, 330 min relative to the start of the infusion and stored on ice until processing. Rumen samples were taken every 2 hours during the infusion. Plasma was prepared from blood samples by centrifugation for 15 minutes at 1600 × *g* and stored at -20 ˚C until further analysis.

4.3.3 Sample Analysis

Preparation of bacteria samples. Microbes were isolated from the rumen samples using methods adapted from Cecava et al. (1990). Briefly, samples were thawed and mixed in a 39°C water bath (~1 hour). The liquid fraction was collected by straining through 4 layers of cheesecloth. The reminding particulate fraction was washed from the cheesecloth into a blender with 0.9% saline (1/4 of the liquid fraction volume yielding a ruminal liquid to saline ratio of 3:1). The particulate fraction was blended for 1 min at high speed and then filtered through 4 layers cheesecloth. The combined liquid was divided into 250ml centrifuge bottles and centrifuged for 10 mins at 1000 x *g* at 4 °C. The liquid was transferred into a beaker and the pellet was discarded. The liquid was centrifuged again for 5 mins at 1000 x *g* at 4 °C, and the pellets were discarded. The fluid was divided into centrifuge bottles and centrifuged for 20 mins at 27,000 x *g* at 4 °C. The liquid was decanted, and the pellets were washed with 0.9% saline. Pellets were composited by sample, and centrifuged for 20 mins at 27,000 x *g* at 4 °C. The supernatant was decanted, and the pellets were scraped from the bottom of the bottle, freeze dried, ground to a powder, and stored at -20° C for further analysis.

Isotope ratio analysis. All plasma samples were deproteinized by addition of sulfosalicylic acid (8%, w/v) followed by centrifugation at 16,000 x *g* for 15 mins at 4 °C. Ruminal microbial samples were hydrolyzed in 6N HCl with 0.1% phenol at 90-100 °C for 20 h, and filtered to remove particulate matter. Deproteinized plasma and hydrolyzed microbial samples were desalted by ion exchange chromatography (BioRad Resin AG 50W-X8*, 100-200 mesh; Bio-Rad, Hercules, CA), and eluted using ammonium hydroxide (2N) into silanized glass vials as described by Calder et al. (1999). Desalted samples were freeze dried, and derivatized as described by Styring et al. (2012). Measurements of isotopic ratios of ^{13}C and ^{15}N in each essential AA were performed after gas chromatographic separation of the AA using an isotope ratio mass spectrometer coupled to a GC by a combustion oven (GC-C-IRMS, Thermo Scientific, Sessions, 2006). Amino acids with polar or charged side chains, such as His and Ser, generally had lower recovery from alkyl chloroformates (Walsh et al., 2014). Thus His, Arg and Ser results were near baseline and quite variable due to the AA derivatization method used, and thus were excluded in current study.

Purine derivates analysis. Creatinine and uric acid concentrations in urine were determined using assay kits (ab204537 and ab65344, respectively) from Abcam (Cambridge, MA). Urinary allantoin concentrations were determined by the colorimetric method described by Young and Conway (1942).

4.3.4 Calculations

All modeling work was completed using R studio (version 1.2.135 with R version 3.5.3). The model was that of Estes et al. (2018). Briefly, state variables were total AA in fast (QAA_{Fast}) and slow turnover pools (QAA_{Slow}) and isotope labeled AA in fast ($QAAi_{Fast}$) and slow turnover pools ($QAAi_{Slow}$). The fast turnover pool is thought to represent blood, interstitial, and cytoplasmic free AA, but also likely includes some protein with short half-lives, e.g. less than 30 min, while

the slow turnover pool should only represent protein-bound AA. Estes et al. (2018) indicated that the size of the slow turnover pool cannot be accurately estimated with a 2-h infusion resulting in underestimates of plateau and overestimates of total AA absorption rates. In the current study, we used 6-h infusion in an attempt to address that problem, but we were still unable to solve for stable estimates of the fraction of the estimated total AA pool that is represented in the slow pool. Thus we fixed the fraction of the total protein that was considered in the slow pool to a value that yielded the greatest loglikelihood value across infusions determined by an iterative local sensitivity analysis. Because the model explicitly represents exchange of AA with body tissue, the derived AA absorption rates represent only absorption (EAA) or absorption plus de novo synthesis (NEAA) minus loss of AA to splanchnic catabolism during the first pass through the splanchnic tissues.

Following initial fits of the model to observed data, residuals outliers were assessed, and if studentized residuals exceeded an absolute value of 2, the sample was removed, which was the case for 14% of the dataset except for Met, which had approximately 30% outliers. The latter was due to lower Met concentrations in samples yielding peak areas that were closer to background. This limitation could be alleviated by repeating the analyses at a greater injection volume. However, even at the greater outlier removal rate, enrichment patterns through time were adequate to define the model.

Microbial AA absorption rates were derived from the total AA absorption rates using the ^{15}N enrichment of plasma AA and microbial AA. Plasma ^{15}N enrichment reached a plateau by the start of the ^{13}C infusion (Figure 1), thus ^{15}N exchange with body protein pools was in balance and ^{15}N absorption should equal ^{15}N net use for protein secretion, growth, gestation, and catabolism. As the animals were not pregnant and were mature, clearance reduces to milk protein secretion

and secretion of maintenance related proteins plus catabolism. The balance equation for ^{15}N-AA in the plasma pool can be defined as:

$$\text{Clearance}_{AA} \times Ep = MCP_{AA} \times Em + (\text{Absorbed}_{AA} - MCP_{AA}) \times Eb, \quad [1]$$

where MCP_{AA} represented the absorption rate of each AA in MCP, Absorbed_{AA} represented the total AA absorption rate derived from the ^{13}C data and modeling, Clearance_{AA} represented AA clearance rates (export proteins plus catabolism), Em was the ^{15}N AA enrichment of rumen microbes, Eb was the background ^{15}N AA enrichment, Ep was the ^{15}N AA enrichment in plasma at plateau. Model derived AA absorption rates were compared to the MP-AA predicted from the CNCPS model (version 6.55, (Van Amburgh et al., 2015)) contained in the NDS Professional ration formulation software (version 6.55; RUM&N, 184 NDS Professional, Reggio Nell'Emilia, Emilia-Romagna, Italy) and White's model (White et al., 2017a, b).

The digestibility of MCP-AA was estimated from the isotope-derived MCP-AA absorption corrected for first-pass splanchnic use rates, intestinal MCP flow predicted from PD excretion, and MCP-AA composition reported by Sok et al. (2017):

$$AA_{digest} = (AA_{absorption} + AA_{spluse}) / AA_{intestine} \times 100, \quad [2]$$

$$AA_{intestine} = MCP_{pd} \times Percent_{MCP-AA}, \quad [3]$$

where AA_{digest} represented digestibility of MCP-AA, AA_{entry} represented model derived MCP-AA or absorption rates, AA_{spluse} was AA used by gut and liver tissues at first pass, $AA_{intestine}$ was predicted MCP-AA flow in small intestine, MCP_{pd} was MCP predicted from PD, and $Percent_{MCP-AA}$ was AA composition of MCP reported by Sok et al. (2017).

4.3.5 Estimating MCP from Urinary PD Excretion

The volume of urine excreted ($\mathrm{V}_{urine}, L/d$) was estimated from creatinine concentrations. Creatinine excretion has been observed to be 0.212 mmol/kg BW in lactating Holstein cows and was not affected by milk production (Chizzotti et al. 2008).

$$\mathrm{V}_{urine} = \frac{0.212 \times BW}{C_{creatinine}}, \quad [4]$$

Uric acid and allantoin excretion were thus calculated from their concentration in urine and the estimated urine volume as:

$$\text{Allantoin}_{excreation} = C_{allantoin} \times \mathrm{V}_{urine}, \quad [5]$$

$$\text{Uric Acid}_{excreation} = C_{uric\ acid} \times \mathrm{V}_{urine}, \quad [6]$$

$$\mathrm{PD}_{excreation} = \text{Allantoin}_{excreation} + \text{Uric Acid}_{excreation}, \quad [7]$$

where $C_{creatinine}$, $C_{allantoin}$, and $C_{uric\ acid}$ represented the concentrations of creatinine, allantoin and uric acid in urine (mmol/L), $\mathrm{PD}_{excreation}$ was the total purine derivates excreted in urine. Ruminal MCP outflow was estimated from urinary PD excretion based on an adaption of the method by Chen and Gomes (1992). About 85% of absorbed purines are recovered as PD in urine. The endogenous contribution is calculated as 0.385 mmol /kg $W^{0.75}$ per day. Microbial purine flow in the small intestine was thus calculated assuming a mean digestibility of microbial purine of 83%. Assuming that microbial purines have a N content of 70 g/mol purine, and a purine-N/total-N ratio of 0.116 g/g:

$$MPD_{flow} = \frac{PD_{excretion} - 0.385 \times BW^{0.75}}{0.85}, \quad [8]$$

$$Microbial\ N_{flow}(g\ per\ d) = \frac{PD_{microbes} \times 70}{0.116 \times 0.83 \times 1000}, \quad [9]$$

where MPD_{flow} represented the absorbed purine from microbes (mmol), $0.385 \times BW^{0.75}$ reflected PD originating from endogenous metabolism (mmol), the N content of microbial purine was 70 mg per mmol purine, and purine-N/total-N gram ratio was 0.116.

4.3.6 Statistical Analysis

Statistical analysis was conducted using R Studio (version 1.2.1335; version 3.5.3 of R), and the mixed model function lmer in the lme4 package of R (version 3.5.3; R Core Team, 2019). The effect of treatments on intake, milk and milk components production, nutrient efficiency, purine excretion, MCP and AA absorption rates were analyzed using the model:

$$Y_{ijklm} = \mu + RDP_i + Starch_j + RDP_i * Starch_j + Block_k + Period_l + Cow(Block)_{m(k)} + e_{ijklm}, \quad [10]$$

where Y_{ijklm} = the dependent variable, μ = population mean of Y, RDP_i = the fixed effect of RDP (df=1), $Starch_j$= the fixed effect of starch (df=1), $RDP_i * Starch_j$= interaction of RDP and starch, $Block_k$ = random effect of block (df=1/df=2), $Period_l$ = the random effect of period (df=3), and $Cow(Block)_{m(k)}$ = random effect of cow nested within block (df=6/df=9). Intake and production data were from 12 cows in 3 blocks and absorption data were from 8 cows in 2 blocks. Main effects and interactions were declared significant at $P \leq 0.05$ and tendency was declared at $P \leq 0.1$. Outliers were checked, and if studentized residuals exceeded an absolute value of 2, the sample was removed, which was the case for less than 10% of the samples. Post-hoc mean-separation testing was conducted only on significant main effects using the "lsmeansLT" package with degrees of freedom adjusted using the Kenward-Rogers option. When the interaction was significant, only interaction effects were considered during mean-separation tests.

4.4. Results and Discussion

One cow exhibited signs of mastitis during period 4 and her data for that period were discarded. The high starch diet decreased DMI when occurring with low RDP but had no effect on DMI with high RDP ($P < 0.01$). Kalscheur et al. (2006b) reported that RDP concentrations in the diet did not affect DMI in dairy cows. Other studies also indicated that DMI was not changed when 7.4% RDP diet (Gressley and Armentano, 2007) and 6.8% RDP diets (Reynal and Broderick, 2005, Kalscheur et al., 2006a) were fed. However, Cyriac et al. (2008) found that a 7.6% RDP diet decreased DMI, whereas diets with 8.8% RDP or greater had no effect. The potential reason is that inadequate RDP can reduce ruminal ammonia concentrations, which may depress fiber degradation and reduce DMI (Firkins et al., 1986, Allen, 2000). Therefore, the effect of RDP on DMI may depend on ammonia concentrations which is a function of feed N degradation, urea recycling from blood, and absorption rates (Leng and Nolan, 1984). The effect of dietary starch on DMI in previous studies was also inconsistent. Oba and Allen (2003) found high starch concentrations were negatively related to DMI. White et al. (2016) also reported a negative relationship between starch and DMI. However, Fredin et al. (2015) and Pirondini et al. (2015) did not observe any effect of starch concentration on DMI. Other work indicated that DMI effects were dependent on high starch fermentability yielding increased blood propionate and hypophagia (Albornoz and Allen, 2018, Albornoz et al., 2019). Farningham and Whyte (1993) found that intraruminal infusions of propionate decreased DMI, which supported the hypophagic effects of propionate. Inconsistent effects might be due to a threshold effect for propionate on DMI. The greatest DMI in the current study occurred with LSLP which likely resulted in the lowest propionate production due to low starch and high NDF.

Another possible factor that can affect DMI is the insulin level in blood. Bradford and Allen (2007) and Sheperd and Combs (1998) indicated that high plasma insulin concentrations

may be the signal of adequate nutritional status and may provide negative feedback on hepatic gluconeogenesis. High dietary starch may increase ruminal starch bypass resulting in increased glucose absorption leading to increased insulin and depressed DMI. However, this cannot explain the low DMI for the LSHP treatment in the current study.

4.4.1 Purine Derivatives Excretion and MCP Synthesis

Purine derivative excretion and estimated ruminal MCP outflow are displayed in Table 4. Moorby et al. (2006) reported a strong linear relationship between total purine excretion and microbial N flow to the duodenum. Other work has demonstrated that urinary excretion of PD was linearly related with duodenal infusions of microbial RNA and yeast RNA (Antoniewicz et al., 1980, Balcells et al., 1991, Boero et al., 2001, Gonzalez-Ronquillo et al., 2003). In the current study, daily excretion of allantoin increased with increased dietary RDP, whereas uric acid excretion was not affected, which is consistent with allantoin contributing more than 80% of total PD excretion. Consequently, calculated MCP production was positively related to RDP. Aldrich et al. (1993) reported passage of MCP to the duodenum was the greatest when dietary RDP and nonstructural carbohydrate were high, and the least when high nonstructural carbohydrate was combined with low RDP, which is consistent with the current findings (1513 vs 1192 g/d). The effect of starch on MCP synthesis and urinary PD excretion was not significant, which is consistent with results from Krause et al. (2003). However, if we assumed that digestible starch in the rumen increased with increased dietary starch concentration, one would expect increased MCP (White et al., 2016). A potential explanation is that the reduced DMI and CP intake for the HSLP treatment resulted in less overall fermentable carbohydrate than might be expected for that diet, and the low ruminally available N limited MCP synthesis. The significant increases in urine volume with

increased dietary RDP were expected due to the increased volume of urinary water required to dilute greater quantities of N metabolites (Valadares et al., 1999, Sannes et al., 2002).

4.4.2 Amino Acid Absorption Rates

The accuracy and precision of predicted plasma isotope ratios were assessed to ensure that the derived rate constants were representative (Table 5). The RMSE were generally less than 5% for the collection of fits. There was no apparent systematic bias and the error was due mostly to dispersion. The CCC was more than 99%, indicating adequate explanatory power for predictions.

The isotope-derived, total AA absorption rates are displayed in Table 6. The RDP and starch interacted to affect Leu and Lys absorption rates, which decreased when low RDP was fed with high starch, but they were not affected when low RDP was fed with a low starch diet, which is consistent with the changes in DMI. Increased DMI with the LSLP diet provided more degraded protein for MCP synthesis, and thus it is logical to expect greater AA absorption. This is also predicted by the CNCPS model. Phenylalanine absorption was negatively associated with dietary starch, which was consistent with changes in CP intake. Compared to the CNCPS-predicted MP-AA (Table 7), the isotope dilution method resulted in similar values for Leu, Lys, Met and Val. Isoleucine absorption was greater than that predicted by CNCPS whereas Phe absorption was less than predicted by CNCPS. In addition, our results showed larger differences among treatments, which may imply the technique is more sensitive to dietary change. Bateman et al. (2001) compared in vivo data from lactating cows to predicted crude protein and amino acid passage to the duodenum using an older version of the CNCPS model, and found the model poorly predicted the magnitude of change in response to diet, and only predicted the direction of change for the RUP fraction correctly slightly over 50% of the time. The accuracy and precision of the CNCPS system has been improved in the past 2 decades with enhancements in predictions of passage rates,

expanded carbohydrate fractions, and an improved fatty acid submodel. Van Amburgh et al. (2015) evaluated the latest CNCPS model (v 6.5) and reported the flow of microbial N was predicted accurately and precisely with an RMSE of 24.6% and CCC of 0.87. However, the flows of MCP-AA were not thoroughly evaluated. In addition, MCP estimates in CNCPS were more sensitive to rumen degraded carbohydrates (RDCHO) than RDP, which is inconsistent with the current observations.

The isotope-derived, MCP-AA absorption rates are presented in Table 6. Leucine, Lys, Phe and Val absorption rates were positively associated with RDP, which implied more MCP synthesis for higher RDP diets. The results were consistent with MCP results calculated from urinary PD. Starch had no effect on MCP-AA absorption rates, which was not expected considering the large change in dietary starch concentration and the effects of starch on rumen fermentation. One possible explanation is that the depressing effect of high starch on DMI counteracted the positive effect as an energy source for rumen microbes. The CNCPS predicted MCP-AA (Table 7) were similar to our results for the HP diets, whereas predictions for the LP diets were greater, which demonstrated that the model is apparently not adequately sensitive to dietary RDP. Lanzas (2006) observed underestimates of MCP with CNCPS when RDP was limiting, and suggested this may result from an inadequate representation of recycled N, inaccurate predictions of dietary RDP, and inaccurate predictions of the efficiency of MCP synthesis. It however was not the case here as the model overpredicted MCP for the low RDP diet. Although Van Kessel and Russell (1996) reported that the sensitivity of MCP predictions by the CNCPS model to protein supply may be overpredicted with low carbohydrate and underpredicted with high carbohydrate using CNCPS, no interaction of starch and RDP was observed in the current results. Higgs et al. (2015) concluded

that MP predictions by CNCPS (v6.5) were more sensitive to variation in ruminal degradation rates of nutrients than chemical composition of the diets.

The isotope-derived, RUP-AA absorption rates are displayed in Table 6. The RUP-Phe absorption was negatively affected by starch. RUP-Lys, RUP-Phe and RUP-Val were negatively related to RDP and starch. The change in RUP-AA absorption rates was generally the opposite of the MCP-AA change which was consistent with the model predicted RUP concentrations (Table 2). The CNCPS predicted MP-AA from RUP (Table 7) was less than RUP-AA absorption derived by isotope dilution and not different across treatments, which was likely due to the overestimates of MP-AA from MCP by CNCPS. The potential factors that cause the variation in MP-AA estimates from nutritional models are inaccurate dietary RDP and RUP estimates and small intestinal digestibilities of RUP and MCP. Lanzas (2006) indicated the standard deviation for predicted RUP within a high protein diet was as high as 200 g/d, and ruminal degradation rates contributed the most to RUP variability.

Overall, compared to the isotope derived estimates, the CNCPS model predicted total MP-EAA supply with no mean bias, but overpredicted MP-EAA from MCP and underpredicted MP-EAA from RUP. However, there was variation among AA with MP-Lys and MP-Phe being overpredicted and MP-Met and MP-BCAA generally under-predicted. MP-Leu from MCP was minimally biased and the remainder from MCP were generally over-predicted, however, this was more apparent for the HS diets than for the LS diets. MP-Met, MP-Ile, MP-Leu, and MP-Val from RUP were underpredicted, and MP-Lys and MP-Phe from RUP had minimal bias overall, but considerable variation across treatments.

Compared to isotope derived estimates, the White's model (White et al., 2017a, b) tended to slightly underestimate total EAA supply, overestimate MP-EAA from MCP, and underestimate

MP-EAA from RUP, but there was heterogeneity by AA (Table 8). MP-Phe was overestimated; MP-Lys had negligible bias; and the MP-BCAA were underestimated. Conversely, the White's model underpredicted MP-AA from RUP for all EAA except Lys. In particular, predictions of Met supply from RUP were only about half of the values measured by isotope dilution. Fleming et al. (2019b) indicated that the model had non-biased estimates of MCP and NANMN but overestimated outflow of most AA, which they attributed to potentially poor recovery of AA during hydrolysis and poorly specified AA composition of RUP and endogenous protein.

4.4.3 The MCP-AA Digestibility

Calculated MCP-AA digestibilities are displayed in Table 9. The mean total MCP-EAA digestibility was 86±12%, 87±12%, 72±12%, and 78±12% for HSHP, LSHP, HSLP and LSLP. The low RDP diets generally had lower AA digestibility, which was significant for Lys and Phe. The mean EAA digestibility across treatments was 82%, which was identical to the true digestibility of MCP-AA in sheep observed by Storm and Ørskov (1983). Fonseca et al. (2014) reported MCP-EAA digestibility of 77 ± 3% for fluid associated bacteria and 76 ± 3% for particle associated bacteria from lactating Holstein cows. The current estimates for rumen microbes are for a combination of bacteria and protozoa, but Fonseca et al. (2014) only studied bacteria. Differences among studies in AA composition of rumen microbes may reflect analytical variation, which can significantly impact AA digestibility estimates. The individual AA digestibilities herein ranged from 65% for Phe to 116% for Lys, which agreed with Fonseca et al. (2014) and Hvelplund and Hesselholt (1987), who also found Phe had the lowest digestibility (69%). We observed larger digestibility differences among AA with Lys apparently exceeding 100%, which was likely due to an underestimate of microbial Lys flow as PD derived MCP was slightly lower compared to prediction from CNCPS and White's model.

4.4.4 Milk and Components Yield

As we observed changes in AA absorption, one might expect to observe changes in milk and component production. Those results are displayed in Table 10. Milk protein production was positively affected by RDP and starch with no interaction. Increased milk protein for the RDP treatments was apparently due to the increased MCP flow which had a larger positive effect than the reduction in RUP flow. Kalscheur et al. (2006b) reported increased milk protein yield and protein concentration in response to dietary RDP increasing from 6.8 to 11.0%. In vitro continuous culture experiments also showed that dietary RDP concentration should be greater than 9.5% to maximize MCP flow (Stokes et al., 1991). The independent effects of starch are not explained by changes in total EAA supply, nor by the total supply of any single EAA suggesting that it is a direct effect of energy supply on milk protein production. Such an independent effect is consistent with the work of Rius et al. (2010), and further refutes the concept of a single limiting nutrient.

Milk fat production was negatively affected by starch. High dietary starch is usually associated to milk fat depression, however, this does not always equate to a reduced fat production, and is thus due to dilution by more milk volume. All cows had high concentrations of milk fat in this study indicating the starch load was not excessive. Sutton et al. (2003) found that low roughage (high starch) diets decreased rumen pH and acetate production. However, Dann et al. (2014) reported no effect of dietary starch on milk fat yield and concentration, which was likely due to smaller treatment differences (17.7% vs 24.6%). Although milk fat production was unaffected by RDP, fat concentration was negatively affected by RDP. Given the lack of change in production, this clearly represents a dilution effect.

Greater RDP increased milk lactose production which was mirrored in milk yield as would be expected given the osmotic draw of lactose (Ebner and Schanbacher 1974). Kalscheur et al.

(2006b) reported that increasing dietary RDP supply from 6.8 to 9.2% improved milk and milk lactose production, but increasing RDP from 9.6 to 11% had no effect. Armentano et al. (1993) also found that increasing RDP from 9.5 to 11.7% did not increase milk production, indicating that feeding RDP greater than 9.5% had no benefits. However, Stokes et al. (1991) found that greater RDP continuously supported greater microbial abundance if carbohydrate was not limiting in a continuous culture experiment, which should result in higher milk and milk protein production. However, the threshold of milk response to RDP likely depends on the requirements of ruminal microbes for RDP and the presentation of carbohydrate in vivo. In the present study, cows fed higher RDP diets had higher milk production, but the study was not designed to determine if RDP responses were continuous.

Dietary starch concentration had no effect on milk and milk lactose yield, which is consistent with previous findings (Albornoz and Allen, 2018). Piccioli-Cappelli et al. (2014) also indicated that dietary starch did not affect milk production and milk lactose in early lactating cows, although the high starch diet was associated with greater blood glucose concentrations. A possible reason for this could be saturation of glucose transport or intracellular hexokinase activity in mammary glands (Mattmiller et al., 2011). Lactose concentrations in milk was negatively affected by starch, however, lactose production was unchanged. Thus this reflects a change in milk volume driven by factors other than lactose.

Starch and RDP had an interactive effect on MUN with MUN increasing by 2.3 mg/dl with LS on the HP diet but only 1.4 mg/dl on the LP diet. Although starch clearly affected MUN, the dominant effect was exerted by dietary protein with approximately a 5 mg/dl drop in MUN on the low protein diets. There was a negative association between MUN concentrations and the gross efficiency of dietary N utilization for milk protein production, which is consistent with previous

findings (Nousiainen et al., 2004). Higher starch resulted in more milk protein output and thus reduced MUN. The greater DMI with LSLP increased N input without increasing milk N output compared to HSLP, thus caused the increase in MUN, which demonstrated energy deficiency can limit N utilization for milk.

4.4.5 Efficiency of Absorbed EAA Used for Milk Protein Synthesis

Essential AA output in milk and efficiency of absorbed EAA use for milk protein production are displayed in Table 11. Secretion of 6 EAA in milk protein increased with high RDP and starch, which was consistent with changes in milk protein yield. The efficiencies of absorbed EAA used for milk protein synthesis averaged 44, 72, 64, 65, 58, 55% for Ile, Leu, Lys, Met, Phe and Val respectively. Blouin et al. (2002) reported milk AA output represented 52, 60, 49, 68, 38 and 61% of net portal flux for Ile, Leu, Lys, Met, Phe and Val respectively. In the current study, higher efficiencies were observed for Lue, Lys and Phe, which was likely due to higher production compared to previous studies (34 kg/d vs 17 kg/d). If splanchnic use is largely driven by supply (Hanigan et al., 2004, Fleming et al., 2019a), and AA use by peripheral tissues is small, a larger fraction of absorbed AA would be expected to return to the splanchnic bed and be catabolized while use for milk protein production is relatively small resulting in poorer efficiency. The efficiencies of absorbed EAA used for milk protein were not affected by treatments except for Phe, which increased with high starch. This was driven by the negative affect by starch on Phe absorption. If true, less Phe would have been catabolized resulting in greater efficiency. Although not significant, other EAA efficiencies were also numerically higher in animals fed high starch and low RDP diets, which implied a N sparing effect of energy supply when RDP was low. Ruminal infusions of propionate (Raggio et al., 2006) and postruminal infusions of starch and glucose (Reynolds et al., 2001, Rulquin et al., 2004) were found to increase AA uptake in

mammary glands, which supports this hypothesis. Rius et al. (2010) reported a mean MP-N efficiency for milk protein of 37%, which increased with a high energy diet and decreased with a high protein diet. The lower efficiency was likely caused by overfeeding N as the MP was 20% higher than requirement. Overall, starch supply may alter MP-AA efficiency through change in MP-AA supply or postabsorptive metabolism.

4.5. Conclusion

The current study demonstrated that differences in MCP-EAA availability can be accurately measured with reasonable precision by isotope dilution, and the digestibility of individual MCP-EAA can be estimated from urinary PD if the AA composition of MCP is constant. The MCP production responded to varying RDP supply, but not to varying starch supply. Starch and RDP interacted to affect dry matter intake. The mechanism for the interaction was not clear as the greatest DMI occurred with the LSLP diet. The MCP calculated from PD increased with RDP, which was consistent with microbial AA absorption derived by isotope dilution. The AA absorption derived from isotope dilution was similar to estimates from the CNCPS model but the latter predicted a higher proportion from MCP with greater error for the LP diets, which suggests the model overestimates MCP responses to dietary RDP. The MCP model of White et al. (2017a) was more closely aligned with the observed data. The mean EAA digestibility for MCP was 82%, but this varied across individual AA and treatments. Absorbed total EAA derived by isotope dilution were affected by RDP, and there was a starch x RDP interaction. The efficiencies of absorbed AA used for milk protein did not differ by diet, and thus milk protein production largely reflected absorbed EAA supply with the exception of the LSLP diet where milk protein was reduced while absorbed EAA was increased relative to the other LP diet. In conclusion, the

isotope dilution method appears to be a viable in vivo model for determining individual EAA absorption from MCP in addition to RUP.

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Table 4.1. The grain premix composition.

Ingredients, % AF	Treatment[1]			
	HSHP	LSHP	HSLP	LSLP
Corn grain, finely ground	42.36	3.27	42.18	3.26
Heat treated soybean meal[2]	-	-	24.28	24.60
Soybean meal, 47.5 CP solvent	36.11	36.58	-	-
Beet pulp, pelleted	8.24	8.35	8.21	8.32
Soybean hulls, ground	2.96	41.33	15.03	53.40
Palmitic enriched fat supplement[3]	2.53	2.56	2.52	2.55
Calcium carbonate	1.97	1.83	1.64	1.50
Sodium bicarbonate	1.89	1.92	1.89	1.91
Molasses, cane	1.25	1.27	1.25	1.26
Calcium phosphate, monocalcium	0.48	0.64	0.76	0.96
Salt, white	0.47	0.48	0.47	0.48
Sodium bentonite[4]	0.38	0.38	0.38	0.38
Magnesium oxide	0.35	0.35	0.35	0.35
Potassium carbonate[5]	0.32	0.32	0.31	0.32
Magnesium potassium sulfate	0.25	0.25	0.25	0.25
Potassium chloride	0.20	0.20	0.20	0.20
Hydrolyzed yeast[6]	0.09	0.10	0.09	0.10
Tracer minerals[7]	0.16	0.16	0.16	0.16

[1]Treatments: 1) HSHP = high starch and high RDP diet; 2) LSHP = low starch and high RDP diet; 3) HSLP = high starch and low RDP diet; 4) LSLP = low starch and low RDP diet.

[2] SurePro, Purina, Arden Hills, MN

[3] Palmit 80, Global Agri-Trade Corp, Long Beach, CA

[4] AB-20, Prince Agri Products, Inc., Quincy, IL

[5] DCAD PLUS, Church & Dwight Co., Inc. Princeton, NJ

[6] Integral, Sel-Plex 2000, Alltech Inc., Nicholasville, KY

[7]Contained 221 mg of Mn, 140 mg of Cu, 2 mg of Fe, 397 mg, and 15 mg of Co from Availa 4 (Zinpro Corp., Eden Prairie, MN), 10 mg of Se (Sel-Plex 2000, Alltech Inc., Nicholasville, KY).

Table 4.2. Diet composition and formulated nutrients estimated from the CNCPS (version 6.55) model

Ingredients	Treatment[1]			
	HSHP	LSHP	HSLP	LSLP
Corn Silage, %DM	36.5	36.0	36.5	36.0
Alfalfa, %DM	14.5	14.0	14.5	14.0
Grain Premix HSHP, %DM	49.0	0	0	0
Grain Premix LSHP, %DM	0	50.0	0	0
Grain Premix HSLP, %DM	0	0	49.0	0
Grain Premix LSLP, %DM	0	0	0	50.0
Nutrient Composition				
CP, %DM	16.27	16.96	13.91	14.56
RDP, %DM	10.45	11.02	7.30	7.82
RUP, % of DM	5.82	5.94	6.61	6.74
Starch, %DM	30.03	15.74	30.19	15.97
ADF, %DM	17.70	26.50	19.60	29.53
aNDF, %DM	26.51	37.44	30.46	41.40
Lignin, %DM	2.98	3.17	2.95	3.52
Fat, %DM	2.98	2.19	3.21	2.71
NFC, %DM	46.19	34.04	44.83	32.71
ME, Mcal/day	61.75	55.05	57.62	55.07
MP, g/d	2575	2489	2468	2682

[1]Treatments: 1) HSHP = high starch and high RDP diet; 2) LSHP = low starch and high RDP diet; 3) HSLP = high starch and low RDP diet; 4) LSLP = low starch and low RDP diet.

Table 4.3. Effect of treatments on nutrient intake (least-squares treatment means)

Items, kg/d	Treatment[1]				SE	P-Value		
	HSHP	LSHP	HSLP	LSLP		RDP	Starch	RDP x Starch
DMI	22.90[a]	22.80[a]	22.14[a]	23.62[b]	0.75	0.92	0.03	0.01
CP intake	3.73[c]	3.86[c]	3.12[a]	3.44[b]	0.12	<0.01	<0.01	0.05
Starch intake	6.88[d]	3.58[a]	6.68[c]	3.77[b]	0.18	0.99	<0.01	0.02
NDF intake	6.07[a]	8.54[c]	6.74[b]	9.78[d]	0.26	<0.01	<0.01	<0.01
ADF intake	4.05[a]	6.05[c]	4.34[b]	6.98[d]	0.18	<0.01	<0.01	<0.01

[1]Treatments: 1) HSHP = high starch and high RDP diet; 2) LSHP = low starch and high RDP diet; 3) HSLP = high starch and low RDP diet; 4) LSLP = low starch and low RDP diet.

Table 4.4. Effect of treatments on purine excretion through urine and predicted urine volume and microbial protein (least-square treatment means)

Items	Treatment[1]				SEM	P-Value		
	HSHP	LSHP	HSLP	LSLP		RDP	Starch	RDP x Starch
Creatine, mmol/d	129	129	131	130	8	0.10	0.69	0.45
Urine, L/d	20	22	18	20	2	0.05	0.03	0.67
Allantoin, mmol/d	296	298	240	272	27	0.06	0.42	0.46
Uric Acid, mmol/d	34	32	31	34	4	0.89	0.77	0.18
PD[2], mmol/d	330	330	271	306	30	0.07	0.43	0.42
MCP, g/d	1513	1508	1192	1383	161	0.07	0.43	0.41

[1]Treatments: 1) HSHP = high starch and high RDP diet; 2) LSHP = low starch and high RDP diet; 3) HSLP = high starch and low RDP diet; 4) LSLP = low starch and low RDP diet.
[2]PD = allantoin + uric acid

Table 4.5. Evaluations of predictions of essential amino acid model isotope ratios after the model was fit by treatment to the observed data

Item[1]	Treatments[1]	Mean observed	Mean predicted	CCC	RMSE	Mean bias	Slope bias	Dispersion
EaAAi		%	%		% Observed mean	% of Mean squared error		
Ile	HSHP	1.13	1.13	0.97	0.48	2.33	3.62	94.04
	LSHP	1.13	1.13	0.98	0.38	0.79	2.13	97.07
	HSLP	1.14	1.14	0.94	0.53	0.17	0.31	99.53
	LSLP	1.12	1.12	0.98	0.46	6.83	6.55	86.62
Leu	HSHP	1.12	1.12	0.99	0.32	0.84	0.93	98.22
	LSHP	1.13	1.13	0.99	0.24	2.51	4.50	92.99
	HSLP	1.14	1.14	0.95	0.52	0.50	1.58	97.92
	LSLP	1.12	1.12	0.99	0.34	6.47	4.59	88.94
Lys	HSHP	1.10	1.10	0.94	0.25	2.29	5.64	92.07
	LSHP	1.10	1.10	0.99	0.05	4.49	5.44	89.13
	HSLP	1.11	1.11	0.96	0.22	2.28	5.13	92.62
	LSLP	1.10	1.10	0.92	0.22	20.13	16.58	63.49
Met	HSHP	1.11	1.11	0.97	0.31	7.79	9.71	82.50
	LSHP	1.12	1.12	0.96	0.32	2.01	4.25	93.74
	HSLP	1.12	1.12	0.92	0.76	6.98	5.71	87.32
	LSLP	1.11	1.11	0.96	0.30	2.93	2.13	94.94
Phe	HSHP	1.14	1.14	0.99	0.34	2.23	2.76	95.01
	LSHP	1.15	1.15	0.98	0.50	15.07	6.51	78.37
	HSLP	1.18	1.18	0.97	0.67	3.04	5.51	91.45
	LSLP	1.14	1.14	0.97	0.61	5.47	5.13	89.40
Val	HSHP	1.12	1.12	0.99	0.16	4.05	3.79	92.16
	LSHP	1.12	1.12	0.99	0.17	0.15	0.62	99.19
	HSLP	1.13	1.13	0.96	0.35	0.59	1.01	98.40
	LSLP	1.11	1.11	0.99	0.15	7.54	4.25	88.21

[1]Treatments: 1) HSHP = high starch and high RDP diet; 2) LSHP = low starch and high RDP diet; 3) HSLP = high starch and low RDP diet; 4) LSLP = low starch and low RDP diet.

Table 4.6. Absorbed AA (g/d) derived from isotope dilution.

Items	Treatment[1]				SEM	P-Value		
	HSHP	LSHP	HSLP	LSLP		RDP	Starch	RDP x Starch
Total EAA[2]								
Ile	168	184	149	191	25	0.78	0.13	0.49
Leu	243[b]	202[a]	214[a]	226[ab]	16	0.83	0.19	0.02
Lys	175[b]	147[a]	149[a]	167[b]	18	0.81	0.66	0.06
Met	65	70	53	75	15	0.78	0.29	0.50
Phe	106	113	90	125	14	0.83	0.05	0.16
Val	139	145	152	134	10	0.77	0.45	0.23
EAA[3]	886[ab]	806[a]	802[a]	919[bc]	51	0.84	0.69	0.02
MCP-AA[4]								
Ile	71	74	49	66	12	0.14	0.30	0.48
Leu	119	101	95	79	20	0.09	0.22	0.93
Lys	129	132	71	92	16	<0.01	0.25	0.37
Met	24	38	19	31	8	0.37	0.07	0.92
Phe	55	58	26	35	9	<0.01	0.48	0.71
Val	72	57	51	48	9	0.08	0.28	0.52
EAA[3]	471	401	319	350	46	<0.01	0.55	0.13
RUP-AA[5]								
Ile	97	107	101	125	16	0.38	0.16	0.59
Leu	123	105	119	147	21	0.20	0.75	0.12
Lys	46	21	69	75	17	<0.01	0.46	0.21
Met	41	31	34	44	12	0.72	0.98	0.25
Phe	51	55	64	91	14	<0.01	0.08	0.18
Val	73[a]	85[a]	101[b]	87[a]	7	0.03	0.88	0.05
EAA[3]	419	417	485	569	64	<0.01	0.23	0.21

[1]Treatments: 1) HSHP = high starch and high RDP diet; 2) LSHP = low starch and high RDP diet; 3) HSLP = high starch and low RDP diet; 4) LSLP = low starch and low RDP diet.
[2]Toatl EAA absorption from MCP and RUP
[3]EAA calculated as the sum of Ile, Leu, Lys, Met, Phe, and Val
[4]MCP-AA: Absorbed microbial AA
[5]RUP-AA: Absorbed rumen undegradable AA, which was calculated as difference of total AA absorption and MCP-AA absorption

Table 4.7. MP-AA, absorbed MCP-AA, and absorbed RUP-AA (g/d) predicted from CNCPS (version 6.55) resident in the NDS[2] software

Items	Treatment[1]			
	HSHP	LSHP	HSLP	LSLP
Total MP-AA[2]				
Ile	132	127	122	133
Leu	202	186	196	203
Lys	177	175	168	187
Met	54	51	53	55
Phe	130	124	123	132
Val	142	136	131	142
EAA[3]	837	799	793	852
MCP-AA[4]				
Ile	81	76	72	78
Leu	104	97	92	99
Lys	113	106	101	108
Met	37	35	33	35
Phe	71	67	63	68
Val	85	80	76	81
EAA[3]	491	461	437	469
RUP-AA[5]				
Ile	50	51	50	55
Leu	98	89	104	104
Lys	64	69	67	79
Met	17	16	20	20
Phe	58	57	59	64
Val	57	56	55	60
EAA[3]	344	338	355	382

[1]Treatments: 1) HSHP = high starch and high RDP diet; 2) LSHP = low starch and high RDP diet; 3) HSLP = high starch and low RDP diet; 4) LSLP = low starch and low RDP diet.
[2]Metabolizablel EAA absorption from MCP and RUP
[3]EAA calculated as the sum of Ile, Leu, Lys, Met, Phe, and Val
[4]MCP-AA: Absorbed microbial AA
[5]RUP-AA: Absorbed rumen undegradable AA, which was calculated as difference of total AA absorption and MCP-AA absorption

Table 4.8. MP-AA, absorbed MCP-AA, and absorbed RUP-AA (g/d) predicted from White's model (2017a, b)

Items	Treatment[1]			
	HSHP	LSHP	HSLP	LSLP
Total MP-AA[2]				
Ile	124	104	104	100
Leu	187	153	162	100
Lys	161	137	138	137
Met	45	36	36	33
Phe	120	100	103	99
Val	128	108	109	105
EAA[3]	764	638	651	625
MCP-AA[4]				
Ile	80	58	52	42
Leu	105	76	68	55
Lys	107	78	70	57
Met	30	22	19	16
Phe	72	52	47	38
Val	78	57	51	41
EAA[3]	472	343	306	249
RUP-AA[5]				
Ile	44	46	52	58
Leu	82	77	94	97
Lys	53	59	69	80
Met	15	14	16	17
Phe	48	48	57	61
Val	50	51	58	64
EAA[3]	292	295	345	376

[1]Treatments: 1) HSHP = high starch and high RDP diet; 2) LSHP = low starch and high RDP diet; 3) HSLP = high starch and low RDP diet; 4) LSLP = low starch and low RDP diet.
[2]Metabolizablel EAA absorption from MCP and RUP
[3]EAA calculated as the sum of Ile, Leu, Lys, Met, Phe, and Val
[4]MCP-AA: Absorbed microbial AA
[5]RUP-AA: Absorbed rumen undegradable AA, which was calculated as difference of total AA absorption and MCP-AA absorption

Table 4.9. MP-AA intestinal digestibility estimates.

Items	Treatment[1]				SEM	P-Value		
	HSHP	LSHP	HSLP	LSLP		RDP	Starch	RDP x Starch
Ile	73	78	68	80	16	0.89	0.56	0.79
Leu	82	86	93	72	16	0.83	0.61	0.32
Lys	116	128	76	111	26	0.09	0.6	0.49
Met	69	107	69	75	20	0.35	0.18	0.35
Phe	65	76	40	51	14	0.05	0.40	0.99
Val	70	62	67	59	12	0.77	0.47	0.99
EAA[2]	86	87	72	78	12	0.32	0.75	0.84

[1]Treatments: 1) HSHP = high starch and high RDP diet; 2) LSHP = low starch and high RDP diet; 3) HSLP = high starch and low RDP diet; 4) LSLP = low starch and low RDP diet.

[2]EAA = the mean of Ile, Leu, Lys, Met, Phe and Val.

Table 4.10. Effect of treatments on milk production, and nutrient efficiency (least-squares treatment means)

Items	Treatment[1]				SE	P-Value		
	HSHP	LSHP	HSLP	LSLP		RDP	Starch	RDP x Starch
Milk yield, kg/d	35.3	34.9	34.0	33.6	1.88	<0.01	0.30	0.96
ECM, kg/d	37.3	39.7	36.6	38.6	1.63	0.24	<0.01	0.83
Lactose, kg/d	1.7	1.7	1.7	1.7	0.10	<0.01	0.59	0.44
Protein, kg/d	1.2	1.2	1.1	1.1	0.04	<0.01	0.01	0.81
Fat, kg/d	1.5	1.6	1.5	1.6	0.09	0.58	0.01	0.17
Lactose, %	4.9	4.9	4.9	4.9	0.06	0.8	<0.01	0.43
Protein, %	3.5	3.4	3.3	3.2	0.16	<0.01	<0.01	0.05
Fat, %	4.3	4.6	4.6	4.7	0.32	0.01	<0.01	0.25
MUN, %	11.3	13.6	6.8	8.2	0.41	<0.01	<0.01	0.03
N efficiency[2], g/100 g	32.8	30.1	35.8	32.1	1.62	<0.01	<0.01	0.32

[1]Treatments: 1) HSHP = high starch and high RDP diet; 2) LSHP = low starch and high RDP diet; 3) HSLP = high starch and low RDP diet; 4) LSLP = low starch and low RDP diet.

[2]Nitrogen efficiency calculated as milk protein yield (kg/d) / total CP intake (kg/d) × 100.

Table 4.11. Essential AA output in milk and efficiency of MP-AA excreted into milk (least-squares treatment means)

Items	Treatment[1]				SE	P-Value		
	HSHP	LSHP	HSLP	LSLP		RDP	Starch	RDP x Starch
EAA output in milk, g/d								
Ile	73	68	67	65	4	<0.01	0.04	0.32
Leu	122	114	111	108	6	<0.01	0.04	0.32
Lys	103	97	94	92	5	<0.01	0.04	0.32
Met	36	33	33	32	2	<0.01	0.04	0.32
Phe	61	57	56	54	3	<0.01	0.04	0.32
Val	82	76	74	72	4	<0.01	0.04	0.32
EAA	478	446	434	423	23	<0.01	0.04	0.32
MP-AA Efficiency, g milk AA / 100 g MP-AA								
Ile	47	42	48	37	6	0.61	0.12	0.55
Leu	78	70	79	61	9	0.61	0.12	0.55
Lys	62	66	68	59	9	0.92	0.68	0.25
Met	60	67	78	55	14	0.77	0.43	0.16
Phe	64	55	68	45	7	0.58	<0.01	0.18
Val	59	55	51	55	3	0.15	0.99	0.12
EAA	59	61	61	49	6	0.25	0.23	0.10

[1]Treatments: 1) HSHP = high starch and high RDP diet; 2) LSHP = low starch and high RDP diet; 3) HSLP = high starch and low RDP diet; 4) LSLP = low starch and low RDP diet.

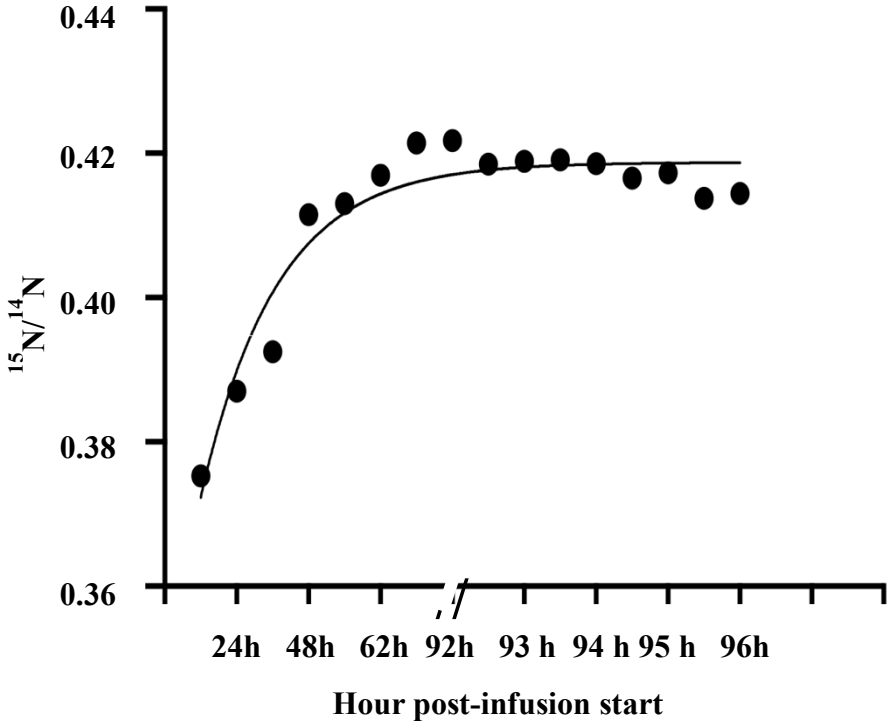

Figure 4.1. The $^{15}N/^{14}N$ isotope ratio of N in plasma over infusion time from day 11 to day 14

CHAPTER 5: Assessing Amino Acid Uptake and Metabolism in Mammary Glands of Lactating Dairy Cows Infused with Methionine, Lysine, and Histidine or Leucine and Isoleucine

5.1. Abstract

The objective of this study was to evaluate the effect of jugular infusion of 2 groups of AA on essential AA (EAA) uptake and metabolism by mammary glands. Four cows (78 ± 10 DIM) were assigned to 4 jugular infusion treatments: saline (CON); methionine, lysine, and histidine (MKH); isoleucine and leucine (IL); or MKH plus IL (MKH+IL) in a 4 x 4 Latin square design. Each period was 16 d in length with 8 d of adaption followed by 8 d of jugular AA infusion. Infusion rates were 10 g of methionine, 38 g of lysine, 20 g of histidine, 50 g of leucine and 22 g of isoleucine per day. Cows were fed a basal diet consisting of 15.2 % crude protein with adequate rumen degradable protein but 15% deficient in metabolizable protein. On the last day of each period, ^{13}C-labeled AA mix was infused into the jugular vein over 6 h. Mammary uptake of essential AA was 100-156 % of milk protein output. Efflux of EAA from mammary to blood represented 13-61% of influx. The fractional use of EAA taken up by the tissue for milk protein and catabolism was affected by EAA infusion. In general, EAA infusion resulted in numerical or significant increases in net uptake, catabolism and incorporation into milk protein, but decreased the efficiency of EAA used for milk protein synthesis, which partially explained the low marginal efficiency of AA conversion to milk protein when each EAA was supplemented.

Key words: Essential AA, stable isotope, metabolism, mammary glands

5.2. Introduction

Current feeding systems used by the dairy industry are mostly empirical and not sensitive to dietary AA other than Met and Lys. This is assumed to contribute to prediction errors for milk

and milk components and low nutrient efficiency (~25% for N) (NRC, 2001). Addressing these limitations requires a more mechanistic understanding of AA supply and utilization in lactating cows.

It is assumed that balancing animal requirements with supply of EAA can optimize milk production in dairy cows (Lapierre et al., 2012). In lactating dairy cows, Met, Lys and sometimes His are considered to be the most limiting EAA in many typical North American diets (NRC, 2001, Lee et al., 2012). However, previous studies reported inconsistent milk responses to Met, Lys, or His (Schwab et al., 1976, St-Pierre and Sylvester, 2005, Appuhamy et al., 2011a, Lee et al., 2012, Giallongo et al., 2017). Zanton et al. (2014) addressed this in a recent meta-analysis in which responses due to supplementation of Met were related to Met sources. This variability in response might also be related to the presence of other limiting AA (Varvikko et al., 1999) and stage of lactation (Schwab et al., 1992, Socha et al., 2008). In addition, the changes in AA uptake of mammary glands may not always translate into comparable changes in milk protein output.

Deletion studies have demonstrated that more severe supply restrictions caused by removal of each AA from a supplemental infusate resulted in decreased milk protein production when the branched chain AA (BCAA) or Leu were removed (Rulquin and Pisulewski, 2006, Doelman et al., 2015, Tian et al., 2017). However, Weekes et al. (2006) found no effect of deletion of BCAA on milk and milk protein yields. Others did not observe increased milk protein yield with infused BCAA (Appuhamy et al., 2011b, Kassube et al., 2017, Curtis et al., 2018). Potential reasons for the inconsistence are not clear yet, which are likely related to deficiencies of other AA or increased non-mammary use of EAA.

The inconsistent effects of EAA supplementation may be due to flexibility in post absorptive utilization. The inability of nutrition models to predict responses to varying nutrient

supplies is partly due to the empirical description of post-absorptive AA metabolism (St-Pierre and Thraen, 1999). For example, nutritional models such as the NRC (2001) use fixed conversion efficiencies for metabolizable protein (MP) to milk protein, which has been demonstrated to be inaccurate with milk protein production an integrated function of protein and energy supplies (Hanigan et al., 1998a). The mammary modeling work of Hanigan et al. (2000) demonstrated that variation in mammary net removal of EAA also existed, which is one of the likely contributors to variable efficiency. Manipulating AA supply to the mammary glands should lead to further improvements in post absorptive AA efficiency, which however cannot be achieved if nutritional models do not accurately represent mammary uptake and metabolism of individual AA. Another reason for variable efficiency of MP-AA utilization in mammary is that mammary AA metabolism is regulated by many factors (Dijkstra et al., 2013). For example, regulating intracellular concentrations of some EAA can affect translation rates thus milk protein synthesis (Apelo et al., 2014a, Apelo et al., 2014b, Ye et al., 2015, Cant et al., 2018). Many studies have been conducted to evaluate milk protein synthesis and AA metabolism in mammary glands using A-V difference, but in the absence of isotope infusions, these only provide estimates of net transport. Hanigan et al. (2001) built a net transport mammary model to predict milk protein based on A-V data, and found that it explained 53% of the observed variation in milk protein output (Hanigan et al., 2002). The effort required a representation of the effects of bidirectional AA transport and assumed mass action catabolism of most AA in the tissue. Those assumptions have not been well explored in vivo.

Stable isotope labeled AA have been used to assess AA availability in blood and utilization by mammary glands (France et al., 1995, Estes et al., 2018). By coupling stable isotope tracers with A-V difference technique and compartmental modelling, Crompton et al. (2014) quantified

Phe and Tyr metabolism in mammary glands of 3 dairy cows (25 kg/d milk production), which demonstrated the utility of the approach. Metabolism of other EAA in mammary glands of lactation cows can be assessed using this technique and related to factors that regulate metabolism to gain a better understanding of the mechanisms and kinetics of AA use.

Our hypothesis was that supplementation of Met, Lys, and His as a group and Ile and Leu as a group would independently affect mammary AA uptake and metabolism and milk protein production. The objective of this study was to assess AA uptake and metabolism in mammary glands that may mediate changes in milk protein yield due to EAA infusion.

5.3. Material and Methods

5.3.1 Animals, Treatments and Sampling

All animal procedures were conducted at the Virginia Tech, Kentland Dairy Farm and approved by the Virginia Tech Animal Care and Use Committee. This work was conducted with a subset of 4 cows (DIM 78 ± 10) within the experiment of Yoder et al. (In press, JDS). The experimental design and diet information are presented in that publication. Briefly, eight Holstein cows in second lactation were blocked by DIM and randomly assigned to 1 of 4 treatment sequences within a 2 x 2 factorial arrangement in replicated 4 x 4 Latin squares. Treatments were jugular infusions of saline (**CON**), methionine, lysine, and histidine (**MKH**), isoleucine and leucine (**IL**), and the combination of MKH and IL (**MKH+IL**). Infusion rates were 10 g of methionine, 38 g of lysine, 20 g of histidine, 50 g of leucine and 22 g of isoleucine per day. Period length was 18 d with 8 d of adaption followed by 10 d of jugular AA infusion. Feeding, feed sampling, and feed analyses were described in the prior work. Cows in 1 block were gave isotope infusion on day 16 (n= 4 cows) to obtain samples of blood and milk, and are the subjects for the results reported herein.

On day 8 of each period, each cow was fitted with a jugular catheter (90 cm x 2.03 mm i.d. microrenathane, Braintree Scientific Inc., Braintree, MA) for AA infusion. Catheters were placed on alternate sides of the neck in subsequent periods. On day 16, animals were given a constant jugular infusion of 1 g of sterile ^{13}C labelled algal AA mix dissolved in 100 mL saline (U-^{13}C, 97-99% enriched, Cambridge Isotope Laboratories, Andover, MA) over a 6 h period using clinical infusion pumps (LifeCare 5000, Abbott Laboratories, North Chicago, IL). Infusions were initiated at 1300 h and ended at 1900 h. Blood samples from the abdominal vein and coccygeal vessel (8 - 10 mL each) were collected at -24, -0.5, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 10 h relative to the start of the infusion and stored on ice until processing. The coccygeal vein blood was considered arterial blood (Hanigan et al., 1991) and will be referenced as arterial blood herein. Cows were milked and milk weight recorded at -1, 2, 4, 6, and 12 h relative to the start of the algal infusion using a portable milker. A 10 mL milk sample was collected at each time point. 5 mL oxytocin (20 USP/mL) was administered intramuscularly to help ensure complete milk removal. Plasma was prepared from the blood samples by centrifugation for 15 minutes at 1600 x *g* immediately after all blood samples were collected. Milk samples were deproteinized by addition of 200 µl sulfosalicylic acid (SSA, 50%, w/v) to 1 ml milk and centrifuged at 10,000 x *g* for 10 min. The resulting protein pellets were washed with SSA (10%, w/v) and centrifuged at 10,000 x *g* for 10 min to isolate milk protein. Plasma and casein samples was stored at -20 ˚C until further analysis.

5.3.2 Sample Analysis

Plasma samples were deproteinized by addition of SSA to achieve a final concentration of 8% (w/v) followed by centrifugation at 16,000 x *g* for 15 mins at 4 °C. Casein samples were hydrolyzed in 6N HCl containing 0.1% phenol at 90-100 °C for 20 h under an atmosphere of N gas to limit oxidation, and filtered to remove insoluble material. Deproteinized plasma and

hydrolyzed casein samples were desalted by ion exchange chromatography (BioRad Resin AG 50W-X8*, 100-200 mesh; Bio-Rad, Hercules, CA), and eluted using ammonium hydroxide (2N) into silanized glass vials as described by Calder et al. (1999). Desalted samples were freeze dried, and derivatized as described by Walsh et al. (2014). Measurements of isotopic ratios of ^{13}C to ^{12}C were performed using an isotope ratio mass spectrometer coupled to a GC by a combustion oven (GC-C-IRMS, Thermo Scientific, Sessions, 2006).

5.3.3 Model Derivation

All modeling work was completed in R Studio (version 1.2.1335; version 3.5.3 of R). The 5-pool dynamic model was modified from the scheme of Hanigan et al. (2009). A schematic of the model is provided in Figure 1. The model represented uptake and metabolism of a single AA. Briefly, state variables were defined for total and isotopically labeled AA in: 1) arterial blood (Q_{aAA}, Q^*_{aAA}), 2) non mammary tissue protein (Q_{btAA}, Q^*_{btAA}), 3) extracellular mammary fluid (Q_{xAA}, Q^*_{xAA}), 4) intracellular mammary fluid (Q_{nAA}, Q^*_{nAA}), and 3) mammary tissue protein (Q_{mtAA}, Q^*_{mtAA}). We attempted to separate body and mammary tissue into slow and fast turnover pools as undertaken by Hanigan et al. (2009), but this resulted in nonunique solutions for parameter estimates due to the shorter infusion time used in the current work. Thus we fixed the fraction of the total protein that was considered in the fast pool to a value that yielded the greatest loglikelihood value across infusions determined by an iterative local sensitivity analysis, and derived fast turnover while assuming the slow pool did not participate.

A full description of the model is provided in the Appendix and stoichiometric constants used in the model are displayed in Supplemental Table 1. We assumed the udder and body protein pools were fixed in size over the course of the infusion, and thus protein degradation was set equal

to synthesis. All of the other fluxes depicted in Figure 1 were explicitly represented as mass action functions.

The FME package of R was used to conduct parameter identifiability and model fitting (Soetaert and Petzoldt, 2010). Model inputs required for the simulation are presented in Supplemental Tables 2 and 3. Mammary plasma flow (MPF) and average AA concentrations in arterial and venous plasma were used to calculate total AA fluxes from arterial to extracellular and from extracellular to venous pools. The MPF was estimated using the Fick principle with Phe and Tyr as the internal markers (Cant et al., 1993). Utilization of AA for milk protein was derived from observed milk protein production rates during the sampling window using standard AA stoichiometries for true milk protein (Lapierre et al., 2012). Amino acid catabolism in mammary glands includes AA oxidation and transamination, which were calculated by difference of AA net uptake and output in milk. Amino acid catabolism in body tissues were calculated by difference of AA absorption and uptake by mammary glands. Initial plasma, extracellular and intracellular AA pools were calculated from average AA concentration in the pool over time and pool volumes. The AA concentrations in the plasma pool were determined from background plasma samples. The AA concentrations in the extracellular pool was assumed to equal AA concentrations in venous plasma (Hanigan et al., 1998b). Intracellular AA concentrations were taken from the literature (Hanigan et al., 2009). Extracellular volume was assumed to be 20% of mammary tissue wet weight and intracellular volume was total wet weight minus extracellular volume and tissue dry matter (Hanigan et al., 2009). Plasma volume was assumed to be 14.8% of BW, which includes both blood and interstitial space. The total protein bound AA pool was estimated from body weight and AA concentrations in body tissue (Supplementary Equation 4). The mammary protein bound AA pool was estimated from mammary weight and the AA concentrations in mammary tissue

(Supplementary Equation 5). The AA composition of tissue protein was as reported by Williams (1978). The initial mass of the isotopically labelled AA pools was set equal to the unlabeled pool sizes multiplied by the observed background enrichments for plasma AA 1 h before the start of the isotope infusions.

Parameter bounds were set to minima of 0 and maxima of 1 for all parameters except for absorption and mammary influx rate constants which were set to 5000 μmol/min and 10 min^{-1} as maxima respectively. Model parameters were derived by fitting the model to observed isotope enrichment in plasma, extracellular, and intracellular pools using modCost and modFit functions within the FME package (Soetaert and Petzoldt, 2010). Isotopic enrichment in the extracellular space was assumed to be equal to venous enrichment (Hanigan et al., 1998b). Intracellular isotopic enrichment is usually obtained by biopsy. In current study, the isotope enrichment in milk casein was used as a substitute by using a lag time of 81 min between synthesis and secretion of casein into milk (Hanigan et al., 2009).

Initial parameter estimates by treatments and AA were derived by maximizing the log-likelihood using the Nelder-Mead algorithm. Following the initial fit, residual errors were calculated, and data points were removed where the Studentized residual errors exceeded an absolute value of 2. These represented 5% of the dataset. These outliers were generally also visually apparent when the observed and predicted data were plotted against sampling time. Final parameter estimates were derived after removal of these outliers.

Root mean squared errors as a percentage of the mean (RMSE) were calculated from mean squared residual errors, and the latter was partitioned into mean bias, slope bias, and dispersion (Bibby and Toutenburg, 1977). The Concordance correlation coefficient (CCC) was also calculated to provide a dimensionless evaluation of precision and accuracy.

5.3.4 Statistical Analysis

Energy-corrected milk yield was calculated as described by NRC (2001):

ECM = [(12.86 × kg of fat) + (7.04 × kg of protein) + (0.3246 × kg of milk)].

Milk AA output was calculated using true milk protein and the AA composition of milk protein from Lapierre et al. (2012).

Milk production, milk composition, DMI, model derived rate constants, and predicted flux rates were analyzed as a 2 x 2 factorial design using the lmer function of the lme4 package in R Studio (version 1.2.1335; version 3.5.3 of R). The model was:

$$Y_{ijkl} = \mu + MKH_i + IL_j + MKH{*}IL_{ij} + Period_k + Cow_l + e_{ijkl}$$

where Y_{ijkl} was the dependent variable, μ was the overall mean of Y, MKH_i the fixed effect of MKH (df=1), IL_j the fixed effect of IL (df=1), $MKH{*}IL_{ij}$ the interaction of MKH and IL (df=1), $Period_k$ the random effect of period (df=3), and Cow_l the random effect of cow (df=3). Main effects and interactions were declared significant at $P \leq 0.05$ and trends at $P \leq 0.10$. Denominator degrees of freedom for all tests were adjusted using the Kenward-Rogers option. Residual errors were evaluated for homogeneity and outliers; and if studentized residuals exceeded an absolute value of 2, the sample was removed from the data statistical analysis, which represented less than 5% of the dataset. When the interaction was significant, a post-hoc means-separation test was conducted using the "lsmeansLT" package with Kenward-Rogers and Tukey options.

5.4. Results and Discussion

One cow was diagnosed with clinical mastitis during period 2 and her data for that period were discarded. Dry matter and dietary CP intake were not affected by treatments (Table 1). The jugular EAA infusions increased total CP intake numerically but not statistically. We did not

observe DMI differences with EAA infusion, which was consistent with prior findings by Yoder et al. (In press, JDS).

5.4.1 Milk and Milk Component Production

Milk protein yield was increased by MKH ($P<0.01$) but not affected by IL ($P<0.14$) (Table 1) with no interaction, which was consistent with the change in milk protein concentration. The MKH results were consistent with those reported by Yoder et al. (In press, JDS) as would be expected given the observations herein are a subset of the results from the larger experiment. However, the lack of an IL effect in current was more likely caused by insufficient power, as we observed increased milk protein production in the large set of observations with 8 animals.

Infusions of MKH and IL had a negative interaction on milk fat yield and concentration. Previous studies indicated that milk fat yield is usually not affected by supplementation of Met, Lys, and His (Lee et al., 2012), or Ile and Leu (Huhtanen et al., 2002, Korhonen et al., 2002). The cause of the fat change in current study is unclear, but may be random as no effects were observed in the full set of animals (Yoder et al., In press, JDS).

Infusion of IL increased milk yield by 1.45 kg/d ($P=0.04$), whereas no effect was observed for MKH (Table 1). Results were consistent with milk production from the set of observations with 8 animals by Yoder et al. (In press, JDS). A recent meta-analysis by Lean et al. (2018) indicated that Leu was positively related to milk yield. Robinson et al. (1999) observed that Ile infusion tended to increase milk yield due to increased lactose production, which is consistent with current findings. There is no demonstrated evidence to suggest that IL involved in lactose synthesis. However, these results may suggest a differential effect of at least IL on factors controlling lactose production such as by stimulation of lactose synthesis related enzymes or stimulation of the metabolic process that allows their synthesis (Robinson et al., 1999) .

5.4.2 AA Concentration in Arterial and Venous Plasma

Arterial plasma concentrations of AA are displayed in Table 2. Results generally agreed with values observed in the full set of animals (Yoder et al., In press, JDS). Arterial plasma concentrations of Met, Lys, His, and Leu increased in response to their infusions, which was expected. The decrease of Phe and Val with MKH+IL was likely driven by increased demand by mammary tissue in support of greater milk protein yield. Bequette et al. (2000) observed that His deficiency increased Phe, Lys and Thr concentrations in arterial plasma, which implied EAA concentrations in plasma are not a simple function of the supply of each, but also a function of other AA and metabolites.

The NEAA concentrations were not affected by treatments except for Ala, Gly and Tyr. Alanine decreased with IL; Gly decreased with MKH; and Tyr decreased with MKH+IL. Total EAA concentrations increased with MKH but decreased in response to MKH+IL, reflecting greater mammary EAA uptake with MKH+IL. Total AA and NEAA were not affected significantly, although they decreased numerically with MKH+IL. Reductions in the concentrations of the non-infused EAA and possibly some NEAA would act to mitigate the stimulatory effects of the infused EAA, which may explain the variability among studies in responses to infused EAA. Studying individual AA uptake and utilization in mammary glands may help us better understand the AA concentration change in blood.

Venous plasma concentrations of AA are displayed in Table 3. In general, total AA, EAA and NEAA decreased in response to MKH+IL, which implied more AA were extracted by mammary glands to support increased milk protein yield. However, interpretation of plasma EAA should be with caution because plasma EAA profiles cannot solely explain AA utilization. Increased EAA concentrations may result from greater intestinal absorption or more degradation

of body protein, whereas low plasma EAA may imply greater use by mammary glands or other tissues or, greater catabolism (Bergen, 1979).

5.4.3 Mammary Uptake of AA

Mammary plasma flow tended to decrease by 83 L/h (11%) in response to MKH but increased by 63 L/h (9%) with IL (Table 4). The effect of MKH might be random as only numerical decrease of 9.5% was observed in the full set of animals (Yoder et al., in press, JDS).

Net uptake of Lys and Thr increased in response to MKH, whereas Met and His uptake increased with MKH alone but not MKH+IL (Table 4). The interaction was likely due to opposite change in MPF in response to IL and MKH as no interaction was observed in the full set of animals (Yoder et al., in press, JDS). Net uptake of EAA and NEAA were also not significantly affected, though EAA uptake increased 500 μmol/min in response to MKH. TAA increased with MKH, which was likely due to increased EAA. These results were expected given the relative contribution of the infused EAA to the aggregated categories. However, the EAA uptake cannot be simply explained by AA concentration in plasma, MPF or transport activity. Bequette et al. (2000) observed that mammary glands had a large capacity to extract AA when the supply of an AA was reduced. In addition, EAA uptake could also be facilitated by AA exchange mechanisms. For example, the L system use concentration gradients (i.e., high intracellular concentrations) of other AA to drive L system AA removal.

5.4.4 Efficiency of Mammary Uptake of AA Used for Milk Protein

Efficiency of mammary AA for milk protein is displayed in Table 5. The efficiency of TAA captured into milk protein averaged 100% and was not affected by treatments, which was consistent with findings of Omphalius et al. (2019). The averaged efficiency of His, Met, Phe, and Tyr was close to 100% whereas the average efficiency of other EAA were much lower. As the

TAA efficiency was not different from 100% and was not affected by treatments, the EAA with low efficiency donated their N via transamination to NEAA. The efficiency of Lys and Thr decreased with MKH, which was consistent with previous findings (Lapierre et al., 2009). The efficiency of Met and His use declined with MKH but not with MKH+IL. The numerically greater milk protein output and lower uptake of each result in higher efficiency for MKH+IL. The efficiencies of Ile and Leu use were not affected by treatments suggesting the numerical changes in uptake were matched to the observed changes in milk AA output. The efficiency of Arg was lower than other EAA, which supported previous findings (Doepel and Lapierre, 2010, Omphalius et al., 2019). The potential reason for this includes Arg being used for NEAA synthesis, such as Pro uptake was much less than output in milk protein. The efficiency NEAA was generally much greater than 100%, which demonstrated their synthesis in mammary tissue. The efficiency of NEAA was not affected by treatment although numeric difference. Overall, the changes in efficiency demonstrated metabolic flexibility of mammary glands in response to changes in AA supply.

5.4.5 Model Fit Quality

The accuracy and precision of predicted isotope ratios are shown in Table 7. An example of model fit is provided in Supplemental Figure 1. The average RMSE across treatments was less than 5%, with more than 50 % of MSE segregating into dispersion error. The CCC was more than 90%. The isotope ratios appeared to be predicted with good precision and accuracy. The mean bias of prediction for arterial isotope ratio was a 20%. The overestimate is visually obvious in Supplemental Figure 1 (A). potential reason was in slow turnover pool. As the model must capture the kinetics of the protein turnover in order to recreate the rise to an apparent plateau during the infusion, use of a single pool fails to capture all isotope flow to body tissue. This results in an

overestimate of isotope enrichment in arterial. The high slope and mean bias related to milk protein isotope ratio prediction was likely due to variation in milking effectiveness. For example, if more milk was left in the udder on one milking, it would carry low enrichment protein into the next milking interval. However, employment of oxytocin should minimize the residual milk. France et al. (1995) also reported a rate constant 0.31/h for casein labeling for cow had given oxytocin during a 20-h infusion of ^{13}Leu, which was higher than rate constant of 0.25/h in goats without being given oxytocin (Bequette et al., 1994). Another potential reason is that enrichment in milk protein represents that of loaded tRNA which has been found to have lower enrichment than the general intracellular pool (Wilde et al., 1989). Because the model predicts intracellular enrichment as a well mixed pool with inputs from the extracellular and mammary tissue bound pools, any gradients that may exist relative to the tRNA loading site were not represented, and thus the model could be expected to slightly overestimate milk protein AA enrichment and underestimate venous AA enrichment when minimizing overall error. Consistent with that expectation, the venous AA enrichments were consistently underpredicted, but the milk protein AA do not appear to be overpredicted, but both errors are very small and likely not contributing significantly to parameter estimate bias.

5.4.6 Amino Acid Absorption Rates

Amino acid absorption rates represented absorbed AA plus release from slow turnover protein pools that was not captured in the single protein pool represented in the model (Table 8). As the model must captures the kinetics of the protein turnover in order to recreate the rise toward an apparent plateau during the infusion, use of a single pool fails to capture all of the sequestration of labeled AA in the slow turnover protein which exhibits more linear behavior over that time frame. This results in an overestimate of AA absorption rates, which however can still be used for

comparative purposes. Infusion of MKH, which would appear in the derived absorption rates, increased Met absorption significantly (100 g/d) and Lys numerically (27 g/d). Leucine absorption increased significantly with IL (128 g/d). The changes in total AA absorption except for Lys were larger than infusion difference. Part of the change was likely due to different absorption from small intestine, which however was not large enough to cause the big change in total AA absorption. A possible explanation was the difference among treatments were overestimated due to overestimated absorption rates. Interestingly, absorption of Ile increased significantly with MKH, IL and MKH+IL. The reason for the effect of MKH on Ile absorption is unclear. One potential reason would be that animals had numerically higher CP intake with MKH and because the isotope technique is sensitive, it was able to detect the contribution of the small change in DMI. This may also explain the increased Val absorption with MKH and IL alone. Another potential reason is that MKH altered the rate of AA deposition into the slow protein pool and thus was excluded from consideration.

5.4.7 Body Protein Synthesis and Catabolism

The results of AA used for non-mammary tissue protein synthesis and catabolism are presented in Table 9. The rate constants for Met use for non-mammary protein synthesis decreased significantly with MKH and IL. Although the Met rate constant decreased with Met, the Met flux to body tissue increased, which was mainly due to increased Met concentrations in blood. This may be due to the assumption of mass action kinetics. Non-mammary body tissue synthesis is likely to be saturated or nearly so in these nearly mature animals, and thus an increase in arterial concentrations would not equate to a similar increase in synthesis resulting in an apparent reduction in the mass action rate constant. The reduction in Met flux to body tissue with IL was more surprising as Met concentrations were not affected by IL. It was anticipated that non-

mammary body tissue protein synthesis may be stimulated by IL given the strong effect of at least Leu on the regulation of muscle protein synthesis (Escobar et al., 2006), but the rate constant for Leu use for non-mammary protein synthesis tended to decrease with IL rather than increase, and Leu flux to body tissue was not affected. Curtis et al. (2018) found that infusing BCAA plus glucose directed more BCAA to skeletal muscle. That effect is likely driven more by the insulin response to the glucose infusion than to the BCAA. It is also possible these responses were due to the use of mature cows for the experiment. At maturity, it is assumed the body tissues are programmed to maintain a constant protein to DNA ratio (Oltjen et al., 1986, Di Marco et al., 1989). Increased concentrations of Met and Leu with MKH and IL would have to be offset by a reduction in the synthesis rate constants to maintain the set point for the protein/DNA ratio. Although not definitive for such short periods, the lack of change in body weight is consistent with this hypothesis (Yoder et al., In press, JDS).

A portion of absorbed AA are catabolized in non-mammary tissues (Table 9). However, due to the overestimation of AA absorption herein, AA catabolism in non-mammary tissue was also overestimated. Thus the results can only be used for comparison purposes. Catabolism of Met in non-mammy tissue increased with MKH. Catabolism of Leu increased with IL. Catabolism of Ile increased with IL but not IL+MKH. The MUN decreased with IL, which implied less whole body AA oxidation with IL. Therefore, the increased Ile and Leu catabolism with IL was more likely due to increased transamination to NEAA whereas increased Met catabolism with MKH was likely due to increased Met oxidation. When the non-mammary anabolic and catabolic fluxes were summed, there was little support for the hypothesis of Appuhamy et al. (2011b) that infused BCAA failed to elicit a milk protein response due to stimulation of use of other EAA by non-

mammary tissues. Lysine use was numerically greater, but Met and Val use were numerically reduced and Phe use was unchanged.

Overall, the current results demonstrated that catabolic use by non-mammary tissues increased as supply increased, but total EAA anabolic use remained relatively constant across treatments. When the rate constants for EAA conversion to non-mammary tissue are summed or averaged, there is a clear reduction in the overall affinity for EAA associated with the infusions. Increased catabolic use partially explains the low marginal efficiency of AA use for lactation, and anabolic use by these tissues does not appear to contribute.

5.4.8 Mammary Transport and Uptake of AA

The derived model rate constants (min^{-1}) for AA transport into mammary tissue are displayed in Table 10. In general, rate constants for EAA influx and efflux varied across individual AA, were greater than previous values from studies in cultured mammary cells (Yoder, 2019), but less than values for lactating goats (Hanigan et al., 2009). It is not surprising that the values should differ from in vitro measures or measures in other species, as the measurement is a function of tissue mass, and transport activity is highly regulated.

Bequette et al. (2000) demonstrated the udder can respond to AA limitations by altering transport activity to maintain AA uptake. Such changes in activity could be driven by a change in the affinity and numbers of the transporters, or a change in the capillary surface area being perfused. Modeling work has demonstrated that transport flexibility is required to support metabolic flexibility (Hanigan et al., 2000). In the current work, infusion of MKH and IL had no effect on the Met and Lys influx rate constants, which suggested mammary Met and Lys transport activity was not changed. Yoder (2019) found that Met transport activity was saturated at concentrations that were two-fold greater than typical in-vivo concentrations in dairy cows. Thus

the transport activity with and without infusion would be expected to remain in the responsive range for the current work. Mammary affinity for Lys was previously observed to be unaffected by Lys supply (Guo et al., 2017), but Liu et al. (2019) reported mammary affinity for Met increased when Met deficiency varied from 40%-100%. The lack of a change in affinity in the current study may be due to the smaller Met supply differences among treatments (15%) or a less deficient state for Con than that imposed by the single-limiting AA infusion models used by Bequette et al. (2000) and Liu et al. (2019).

The transport affinities for Leu, Ile and Val in mammary glands were similar to one another. This finding is not surprising given that they are all BCAA and use the same transporters. However, it is inconsistent with previous findings in mammary cells (Yoder, 2019), which reported much lower transport affinity for Val than Ile and Leu.

The rate constant for Ile influx was not affected with IL, but was increased for Leu influx. These are seemingly divergent responses that suggest some diversity among the BCAA transporters. Yoder (2019) reported that Ile transport was saturated in mammary cells at the upper end of in vivo blood concentration range, which was however found to be unsaturated in current study. The Ile flux increased proportional to extracellular concentrations. The increased affinity for Leu with IL suggests that other drivers of transport were complicit. Although Shennan et al. (1997) indicated the L-system has affinity for a broad range of substrates including the BCAA, Met and Phe, the sensitivity of uptake of a single AA was not tested relative to changes in the concentrations of other EAA (Maas et al., 1998). While System L transporters are sodium independent, Jackson et al. (2000) reported that sodium driven AA such as Ala and Gln were important for AA exchange by L system. For example, removal of sodium or Gln from the media inhibited uptake of System L AA substrates and excess Ala, Met, and Gln competitively inhibited

uptake. A difference in extracellular Ala was observed in current study, which however cannot explain the change in Leu transport affinity due to interaction of IL and MKH. Another potential explanation was that increase in use for protein synthesis plus catabolism was greater than the increase in supply elicited by mass action thus eliciting a signal that resulted in greater transport activity. For instance, if Leu depletes inside the cell through protein biosynthesis and metabolism, the chances of Leu being transported out of the cell are minimal, while synthesis of most NEAA is induced and transporters such as SNAT2 are up regulated to help in exchanging Leu (Kilberg et al., 2012, Bröer and Bröer, 2017). The decreased Leu efflux and increased Leu catabolism with IL+MKH verified this. Transport affinity of Val was previously not observed to be affected by EAA infusion although Zhou et al. (2018) indicated increasing extracellular Ile supply decreased Val transport due to decreased mRNA expression of SLCA5 in mammary epithelial cells. Transport affinity for Phe was not changed with AA infusion in the current work, although it was numerically increased with MKH, which was consistent with Phe uptake results. In the absence of more specific knowledge of the interactions among AA within AA transport systems, it is difficult to discern a mechanism.

In general, changes in efflux rate constants across treatments were less evident except for Leu. Efflux rate constant of Leu increased with IL alone but were not affected with IL+MKH, which implied more Leu was captured and metabolized with IL+MKH. (Bröer and Bröer, 2017) also found that the harmonizing effects of System L can correct intracellular deficiencies of AA by decreasing cellular efflux. The higher Leu net uptake and milk protein production with IL+MKH also confirmed this. Efflux transport affinity was demonstrated to be related to influx transport regulation (Yoder, 2019). For example, when transporters on the mammary epithelial cells changed due to a change in extracellular supply, the efflux transport affinity change was

proportional to the influx change. This is expected as transport activity is bidirectional and thus should change in concert when driven by a change in the actual transporter. However, a portion of the exchange is non-saturable and thus a function of cell water movement or diffusion across the cell membrane (Christensen, 1990), and for exchange transporters, such as the L-system, apparent transport activity will also be driven by transport of the exchange AA. The combination of diffusion and exchange effects on influx and efflux movement could result in divergent or mixed activity.

The flux rates for AA transport in mammary glands are also displayed in Table 10. The bidirectional influx and efflux rates of Met were not affected by treatments, although they increased numerically with MKH. The numerical increases in influx and efflux, if real, were driven by higher Met concentrations in extracellular and intracellular pools, as the transport rate constants were not significantly affected. Net uptake of Met was increased with MKH but not MKH+IL with the latter having increased efflux as compared to the former. Influx was similar for the 2 treatments. From 27 to 39% of Met extracted by mammary glands was returned to the extracellular space with the proportion very similar for Con, MKH, and IL treatments (27-30%) and 39% for the MKH+IL treatment. Yoder et al. (In press, JDS) reported from 0-85% of the Met extracted by cultured mammary epithelial cells was returned to the extracellular space. Hanigan et al. (2009) also observed 50% of the Met extracted by mammary glands was returned to the extracellular space in goats. The high rate of exchange results in greater flexibility in regulating AA uptake.

Lapierre et al. (2012) indicated that Met was not taken up in excess when supply increased above mammary demand for milk protein, but the current data do not support that concept as the uptake of Met with MKH alone was 25% greater than output in milk whereas the efficiency of transfer for the other 3 treatments approximated unity. A possible reason for the apparent excess

transport of Met with MKH may be competition for AA transporters given that BCAA and Met share the L-system (Shennan et al., 1997).

The influx and efflux rates of Lys were not affected by EAA infusion, although the numerical changes in influx were similar and consistent with the significant effect of MKH on net transport measured as the difference between influx and efflux (Table 10) or by A-V (Table 4). There was a numerical reduction the rate constant for Lys uptake, but it did not reach a trend suggesting that any change in influx was driven by the significant increases in arterial Lys concentrations. The proportion of extracted Lys returned to the extracellular space was slightly less than for Met at 20-22% for the first 3 treatments and 13% for the MKH+IL treatment.

Although IL increased MPF in the current study, it did not have a significant effect on uptake of any EAA other than Leu. Both the influx and efflux rates for Leu were increased with IL. The rate constant for influx tended to increase, but the efflux rate constant was not affected suggesting at least a portion of the flux changes were driven by concentration changes. The efflux rate of Leu increased more in response to IL alone than to MKH+IL. The potential reason was numerically more Leu was used for milk protein, incorporated into mammary tissue, and catabolized with MKH+IL, thus less Leu was available in the intracellular pool for transport back to the extracellular space.

The net uptake of Ile increased with MKH but not with IL. The lack of change in milk protein production with IL and increased milk protein production with MKH were consistent with this response. Neither EAA infusion had an effect on Phe and Val uptake. Numerically the increased use of Phe for milk protein was derived from a reduction in catabolism of Phe. The variance in Val metabolism was greater than the changes that were required to achieve the increase in milk protein output making the source of the required Val unclear.

The proportion of extracted BCAA returned to the extracellular space was similar for Ile and Leu ranging from 31% to 47%, increasing in both cases with the IL infusions. Return was greater from Val ranging from 54% to 61% with no clear pattern, but the lowest observed occurred with MKH+IL. The Phe return was similar to Val ranging from 49% to 55%. The BCAA return rates were consistent with previous findings (Hanigan et al., 2009, Yoder, 2019), which ranged from 17% to 80% of BCAA uptake. In the latter work, it was observed that the ratio of efflux to influx increased as intracellular AA concentrations increased.

5.4.9 Mammary AA Metabolism

The results of AA metabolism in mammary glands are presented in Table 11. The rate constant for Leu used for mammary protein synthesis decreased with IL alone but not with MKH+IL. Increased concentrations of Leu with IL would have to be offset by a reduction in the synthesis rate constant to maintain the same body protein and set protein/DNA ratio (Oltjen et al., 1986, Di Marco et al., 1989), whereas the MKH+IL may not have increased intracellular Leu as much due to increased catabolism and capture in milk protein. The interaction of MKH and IL on the rate constant for Leu used for mammary protein synthesis might be related to a change in translation based on the work by Yoder (2019). In that work, IL and MKH alone had no effect on mammary tissue mTORC1 phosphorylation or its substrates, S6K1, and rpS6, but positively interacted to increase rpS6 phosphorylation, which presumably contributed to increased protein synthesis rates. However, this does not explain the lack of significant changes in the rate constants and fluxes of other EAA.

Methionine and Lys incorporated into milk protein were not affected by treatments, but the numeric differences were consistent with the changes in milk protein yield. The lack of significant difference was likely caused by increased catabolism of Lys and Met. The average efficiency of

extracted Met and Lys conversion to milk protein was 0.98 and 0.78. Yoder et. al. (In press, JDS) also reported efficiencies of Met and Lys of 0.90 and 0.77 in the larger set of animals from this experiment. These were also similar to findings by Lapierre et al. (2012). Previous studies indicated that His, Met, and Phe extracted from blood was equal to their secretion in milk protein whereas Lys and BCAA were extracted in excess of milk protein output with the excess catabolized within the udder (Guinard and Rulquin, 1994). Lysine may be oxidized in a regulatory or passive manner to provide ketogenic intermediates. During 5 day infusions of Lys (9g/d) in late lactation goats, Lys oxidation increase from 16 to 30%, representing a nearly a twofold increase in the absolute rate of oxidation (Bequette et al., 1998).

Methionine output in milk exceeded Met uptake in animals infused with CON and MKH+IL, which implied other Met sources contributed or that the MBF calculated from Phe and Tyr balance was underestimated. Some Met may arise from peptide uptake and hydrolysis, which is supported by previous findings (Backwell et al., 1994, Wang et al., 1996), although in the former work, the contribution was likely less than observed due to recycled label that was not considered in the model. Another possible source is degradation of blood proteins in the extracellular space. Thus, the total uptake of Met probably exceeded its output in milk protein. Methionine efficiency was significantly decreased to 79% with the MKH infusion alone but not changed with the MKH+IL infusion, which likely suggested that the Met supply with MKH exceeded requirement, but the combination of AA resulted in increased deposition in protein. Another potential reason was a change in demand for transmethylation and transsulfuration, which may alter the availability of Met for protein synthesis. Decreased Met efficiency with the MKH infusion may reflect decreased remethylation of homocysteine to produce Met or increased oxidation (Bequette et al., 1998).

The uptake of BCAA by mammary glands was much higher than BCAA excretion in milk, which is consistent with previous work (Clark, 1975, Mepham, 1982, Hanigan et al., 2001). Studies with bovine mammary slices and isolated goat udder also showed that ^{14}C from labeled BCAA was recovered in NEAA, various organic acids, CO_2 and lipids, which indicates that excess BCAA uptake is catabolized to yield C and N for NEAA synthesis and energy supply (Wohlt et al., 1977, Roets et al., 1979, Roets et al., 1983). The BCAA catabolism depends upon phosphorylation status of keto acid dehydrogenase, which is regulated by insulin and BCAA concentrations. The enzyme is inactive (phosphorylated) when insulin is high and BCAA concentrations are low resulting in reduced catabolism (Hasan et al., 1982). However, no differences in catabolism were observed for BCAA with IL infusion. It is possible that the IL infusion was not enough to elicit a change in insulin (Kuhara et al., 1991) and intracellular Ile and Leu concentrations (Clark et al., 1980). Efficiencies of Ile and Leu averaged 68% and 71%. Efficiency of Ile and Val used for milk protein increased with IL alone but not MKH+IL, which was likely due to decreased oxidation with IL. Decreased MUN with IL also indicated decreased AA oxidation. The interaction cannot be explained. Previous studies have shown that the proportion of Leu that was oxidized by the mammary gland was substantially reduced (0.19 vs. 0.07) by the infusion of AA other than Leu (Bequette et al., 1998), thus we expected higher Leu efficiency with MKH+IL in the current study. Another potential reason was that more Ile and Val may have been transaminated to NEAA with MKH+IL to support increased milk protein.

5.5. Conclusion

Jugular infusions of Met, His and Lys increased milk protein yield in high producing dairy cows. The Ile and Leu infusion increased milk lactose and milk yields. The changes in milk and milk component production were associated with changes in mammary plasma flow, AA uptake

and mammary metabolism. The MKH infusion decreased plasma flow whereas the IL infusion increased it. The EAA transport into mammary cells is bidirectional with EAA efflux ranging from 13 to 61 % of influx. Leucine influx affinity tended to increase with IL and lysine influx affinity tended to decrease with MKH. Generally, efflux of BCAA was higher than Met and Lys. In addition, excessive BCAA influx associated with IL was mostly returned to blood whereas excessive Lys with MKH was catabolized, which implied that mammary glands have different mechanism to maintain intracellular pools in response to varying AA supply. The proportions of AA catabolized and used for milk protein were affected by EAA infusion, which demonstrated plasticity of mammary gland in AA metabolism. Although the general mechanisms confirm previous findings in mammary cells and goats, quantitative aspects of AA transport and metabolism in high producing cows were demonstrated here. The efficiency of uptake AA used for milk protein varied across AA and was affected with essential AA infusion, demonstrating it is inaccurate to use an integrated, constant efficiency for all AA and feeding conditions.

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Table 5.1. Effect of AA infusions on intake, milk production parameters, nutrient efficiency, and body weight. Data are presented as least-squares treatment means.

	Treatment[1]					Effect (P-value)		
Item	CON	MKH	IL	MKH + IL	SEM	MKH	IL	MKH*IL
Intake								
DMI, kg/d	24.3	24.8	24.3	24.0	1.2	0.81	0.54	0.54
Dietary CP, kg/d	3.69	3.77	3.68	3.65	0.18	0.82	0.54	0.54
Infused AA, g/d	0	78.9	71.9	150.3	0.1	<0.01	<0.01	<0.01
Total CP, kg/d	3.69	3.86	3.76	3.78	0.17	0.30	0.95	0.42
Milk production								
Milk, kg/d	50.8	51.0	52.4	52.3	2.5	0.91	0.04	0.80
ECM[2]	50.6	52.8	51.7	51.2	3.8	0.43	0.80	0.18
Milk protein %	3.00	3.16	2.96	3.16	0.11	<0.01	0.57	0.47
Milk protein, kg/d	1.52	1.61	1.54	1.65	0.08	<0.01	0.14	0.64
Milk fat %	3.60[b]	3.68[b]	3.54[b]	3.31[a]	0.41	0.28	<0.01	0.02
Milk fat, kg/d	1.81[ab]	1.90[b]	1.84[ab]	1.75[a]	0.24	0.96	0.22	0.05
Lactose %	5.05	5.05	5.06	4.98	0.08	0.32	0.44	0.43
Lactose, kg/d	2.58	2.55	2.65	2.60	0.11	0.35	0.07	0.75
MUN (mg/dl)	8.08	8.80	8.05	7.79	1.00	0.46	0.09	0.12
Efficiency								
Nitrogen efficiency[3]	41.8	41.7	41.4	44.6	2.53	0.18	0.28	0.15

[1]Treatments: 1) CON = infused 3 liters of 0.9% saline; 2) MKH = infused 21 g/d of Met, 20 g/d of His, and 38 g/d of Lys; 3) IL = infused 22 g/d of Ile, and 50 g/d of Leu; 4) MKH+IL = infused 21 g/d of Met, 20 g/d of His, 38 g/d of Lys, 22 g/d of Ile, and 50 g/d of Leu.

[2]ECM=energy-corrected milk calculated as (12.82 x kg of fat) + (7.13 x kg of protein) + (0.323 x kg of milk).

[3]Nitrogen efficiency calculated as milk protein yield (kg/d) / total CP intake (kg/d).

Table 5.2. Effect of AA infusions on AA concentration (μM) in arterial blood (least-square treatment means)

	Treatment[1]					Effect (*P-value*)		
Item	CON	MKH	IL	MKH + IL	SEM	MKH	IL	MKH*IL
Arg	80	89	77	79	4	0.21	0.16	0.34
His	62[a]	83[c]	63[a]	77[b]	4	<0.01	0.05	0.07
Ile	135	145	152	135	11	0.66	0.70	0.14
Leu	200[a]	209[a]	264[b]	225[a]	16	0.19	<0.01	0.05
Lys	82[a]	124[c]	79[a]	102[b]	7	<0.01	<0.01	0.02
Met	20	41	21	37	3	<0.01	0.43	0.22
Phe	47[ab]	48[b]	49[b]	41[a]	3	0.16	0.32	0.08
Thr	95	89	95	77	7	0.10	0.35	0.35
Trp	31	31	31	31	1	0.99	0.70	0.86
Val	289	288	277	231	20	0.18	0.06	0.19
Ala	289	288	277	231	20	0.18	0.06	0.19
Asn	21	22	21	22	2	0.40	0.68	0.81
Asp	11	13	11	11	2	0.50	0.17	0.33
Gln	152	152	162	152	9	0.46	0.51	0.48
Glu	105	97	92	90	8	0.35	0.11	0.62
Gly	242	207	243	202	23	0.01	0.85	0.80
Pro	98	105	105	99	10	0.90	0.94	0.27
Ser	74	76	86	76	8	0.30	0.18	0.18
Tyr	33[b]	35[b]	34[b]	25[a]	4	0.09	0.08	0.04
TAA[2]	2004	2104	2098	1944	87	0.73	69	0.15
EAA[3]	1039[ab]	1147[b]	1107[ab]	1036[a]	53	0.67	0.62	0.07
NEAA[4]	965	957	992	907	64	0.31	0.79	0.39

[1]Treatments: 1) CON = infused 3 liters of 0.9% saline; 2) MKH = infused 21 g/d of Met, 20 g/d of His, and 38 g/d of Lys; 3) IL = infused 22 g/d of Ile, and 50 g/d of Leu; 4) MKH+IL = infused 21 g/d of Met, 20 g/d of His, 38 g/d of Lys, 22 g/d of Ile, and 50 g/d of Leu.
[2]TAA = total amino acids (EAA + NEAA).
[3]EAA = Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Trp and Val.
[4]NEAA = Ala, Asn, Asp, Gln, Glu, Gly, Pro, Ser, and Tyr.

Table 5.3. Effect of AA infusions on AA concentration (μM) in venous blood (least-square treatment means)

	Treatment[1]					Effect (*P-value*)		
Item	CON	MKH	IL	MKH + IL	SEM	MKH	IL	MKH*IL
Arg	38	44	41	37	6	0.64	0.38	0.10
His	50	67	53	64	4	<0.01	0.90	0.22
Ile	86[a]	85[a]	107[b]	76[a]	10	0.06	0.41	0.07
Leu	121[a]	117[a]	191[b]	133[a]	16	0.01	<0.01	0.03
Lys	28[a]	50[c]	33[a]	41[b]	5	<0.01	0.44	0.04
Met	6	19	7	22	3	<0.01	0.21	0.50
Phe	22[b]	19[a]	27[c]	16[a]	3	<0.01	0.41	0.04
Thr	61[b]	53[a]	68[b]	40[a]	6	<0.01	0.59	0.08
Trp	31	31	31	30	1	0.58	0.21	0.49
Val	224[b]	216[b]	225[b]	157[a]	18	0.03	0.08	0.07
Ala	194[ab]	217[b]	210[ab]	183[a]	31	0.85	0.45	0.07
Asn	10[a]	9[a]	14[b]	7[a]	2	0.07	0.46	0.08
Asp	6	7	8	6	1	0.53	0.91	0.14
Gln	117	112	134	117	7	0.14	0.14	0.42
Glu	53	53	53	52	6	0.90	0.98	0.94
Gly	220	191	225	178	23	0.02	0.74	0.45
Pro	76[a]	85[ab]	90[b]	75[a]	9	0.56	0.67	0.04
Ser	53[a]	47[a]	67[b]	45[a]	9	<0.01	0.09	0.03
Tyr	10[c]	5[b]	14[d]	0[a]	5	<0.01	0.46	<0.01
TAA[2]	1406	1426	1597	1278	77	0.04	0.73	0.03
EAA[3]	667[a]	700[ab]	782[b]	615[a]	52	0.11	0.68	0.03
NEAA[4]	739[ab]	726[ab]	814[b]	663[a]	57	0.05	0.86	0.09

[1]Treatments: 1) CON = infused 3 liters of 0.9% saline; 2) MKH = infused 21 g/d of Met, 20 g/d of His, and 38 g/d of Lys; 3) IL = infused 22 g/d of Ile, and 50 g/d of Leu; 4) MKH+IL = infused 21 g/d of Met, 20 g/d of His, 38 g/d of Lys, 22 g/d of Ile, and 50 g/d of Leu.
[2]TAA = total amino acids (EAA + NEAA).
[3]EAA = Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Trp and Val.
[4]NEAA = Ala, Asn, Asp, Gln, Glu, Gly, Pro, Ser, and Tyr.

Table 5.4. Mammary plasma flow and net uptake of amino acids (least-square treatment means)

	Treatment[1]					Effect (*P-value*)		
Item	CON	MKH	IL	MKH + IL	SEM	MKH	IL	MKH*IL
Mammary plasma flow (MPF), L/h								
MPF	706	654	799	685	62	0.01	0.04	0.21
Net mammary uptake, µmol/min								
Arg	473	490	483	478	59	0.86	0.99	0.75
His	125[a]	173[b]	142[a]	148[a]	13	0.02	0.63	0.04
Ile	564	645	592	669	65	0.16	0.61	0.97
Leu	900	996	971	1046	106	0.34	0.49	0.90
Lys	623	786	611	697	35	<0.01	0.12	0.22
Met	162[a]	237[b]	180[a]	177[a]	22	0.03	0.16	0.02
Phe	280	314	289	285	30	0.55	0.69	0.45
Thr	358	397	340	418	41	0.09	0.97	0.53
Trp	9	8	9	12	4	0.78	0.61	0.56
Val	813	774	681	837	109	0.43	0.64	0.21
Ala	300	418	420	609	164	0.14	0.13	0.70
Asn	120	131	100	132	25	0.35	0.67	0.64
Asp	47	57	46	54	9	0.17	0.70	0.92
Gln	406	443	370	400	50	0.29	0.22	0.91
Glu	489	461	528	425	65	0.28	0.98	0.52
Gly	274	189	235	293	101	0.86	0.57	0.36
Pro	276	214	201	268	49	0.95	0.77	0.10
Ser	237	316	242	352	65	0.06	0.63	0.72
Tyr	274	315	268	289	27	0.2	0.5	0.66
TAA[2]	6667	7330	6683	7554	569	0.07	0.73	0.77
EAA[3]	4287	4820	4299	4767	372	0.11	0.94	0.91
NEAA[4]	2401	2511	2384	2787	343	0.23	0.52	0.47

[1]Treatment: 1) CON = infused 3 liters of 0.9% saline; 2) MKH = infused 21 g/d of Met, 20 g/d of His, and 38 g/d of Lys; 3) IL = infused 22 g/d of Ile, and 50 g/d of Leu; 4) MKH+IL = infused 21 g/d of Met, 20 g/d of His, 38 g/d of Lys, 22 g/d of Ile, and 50 g/d of Leu.
[2]TAA = total amino acids (EAA + NEAA).
[3]EAA = Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Trp and Val.
[4]NEAA = Ala, Asn, Asp, Gln, Glu, Gly, Pro, Ser, and Tyr.

Table 5.5 Efficiency of uptake of AA captured in milk protein (%, least-square treatment means)

	Treatment[1]					Effect (*P-value*)		
Item (g milk AA/ 100 g AA uptake)	CON	MKH	IL	MKH + IL	SEM	MKH	IL	MKH*IL
Arg	39	40	38	40	4	0.62	0.94	0.82
His	131[b]	94[a]	115[b]	115[b]	11	0.06	0.73	0.05
Ile	71	63	72	65	7	0.14	0.75	0.86
Leu	75	68	74	69	7	0.31	0.97	0.83
Lys	81	66	86	78	5	<0.01	0.01	0.18
Met	107[b]	75[a]	101[b]	106[b]	8	<0.01	<0.01	<0.01
Phe	94	87	96	100	7	0.83	0.18	0.26
Thr	93	86	102	85	9	0.03	0.50	0.36
Val	67[a]	66[a]	77[b]	65[a]	7	0.02	0.06	0.04
Ala	101	105	90	59	36	0.62	0.25	0.47
Asn	261	213	288	232	43	0.21	0.54	0.92
Asp	517	490	584	498	115	0.47	0.64	0.71
Gln	138	133	159	156	14	0.56	0.02	0.99
Glu	151	188	152	195	38	0.23	0.89	0.92
Gly	91	163	148	112	51	0.66	0.94	0.20
Pro	306	466	437	323	117	0.76	0.94	0.12
Ser	294	182	416	166	146	0.16	0.65	0.56
Tyr	100	89	107	102	6	0.18	0.10	0.54
TAA[2]	107	101	108	102	7	0.14	0.78	0.96
EAA[3]	74	69	65	73	11	0.85	0.81	0.49
NEAA[4]	174	167	179	153	21	0.32	0.75	0.53

[1]Treatment: 1) CON = infused 3 liters of 0.9% saline; 2) MKH = infused 21 g/d of Met, 20 g/d of His, and 38 g/d of Lys; 3) IL = infused 22 g/d of Ile, and 50 g/d of Leu; 4) MKH+IL = infused 21 g/d of Met, 20 g/d of His, 38 g/d of Lys, 22 g/d of Ile, and 50 g/d of Leu.

[2]TAA = total amino acids (EAA + NEAA).

[3]EAA = Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Trp and Val.

[4]NEAA = Ala, Asn, Asp, Gln, Glu, Gly, Pro, Ser, and Tyr.

Table 5.6. Abbreviations and units for model rate constants and fluxes

Variable	Description	Unit
KaAAbtAA	Rate constant for AA incorporation into body tissue protein	min^{-1}
KxAAnAA	Rate constant for AA uptake	min^{-1}
KnAAxAA	Rate constant for AA efflux	min^{-1}
KnAAmtAA	Rate constant for AA incorporation into mammary tissue protein	min^{-1}
Fabsorption(i)	AA absorption	µmol/min
FaAAxAA(i)	AA flux from the arterial pool to the extracellular pool	µmol/min
FaAAbtAA(i)	AA flux from arterial pool to body tissue protein pool	µmol/min
FbtAAaAA(i)	AA flux from body tissue protein degradation to arterial pool	µmol/min
FxAAnAA(i)	AA flux from extracellular pool to intracellular pool	µmol/min
FxAAvAA(i)	AA flux from extracellular pool to vena	µmol/min
FnAAxAA(i)	AA flux from intracellular pool to extracellular pool	µmol/min
FnAAcAA(i)	AA flux from intracellular pool to catabolism	µmol/min
FnAAmtAA(i)	AA flux from intracellular pool to mammary tissue protein pool	µmol/min
FmtAAnAA(i)	AA flux from mammary tissue protein pool to intracellular pool	µmol/min
FnAAmAA(i)	AA flux from intracellular pool to milk	µmol/min

[1]Subscript *i* represents the isotope labeled.

Table 5.7. Evaluations of predictions of essential amino acid isotope ratios after the model was fit by treatment to the observed data

Item[1]	Mean observed	Mean predicted	CCC	RMSE	Mean bias	Slope bias	Dispersion
	%	%		% Observed mean	% of Mean square prediction error		
Ile							
EaAAi	1.130	1.132	0.97	0.63	21.95	6.79	71.26
ExAAi	1.132	1.130	0.96	0.65	13.95	21.24	64.81
EmAAi	1.113	1.113	0.95	0.57	14.66	23.65	61.69
Leu							
EaAAi	1.118	1.121	0.96	0.58	24.19	5.66	70.15
ExAAi	1.121	1.118	0.95	0.62	20.45	25.30	54.25
EmAAi	1.103	1.103	0.93	0.58	7.97	8.09	83.94
Lys							
EaAAi	1.155	1.158	0.96	1.19	27.40	6.58	66.01
ExAAi	1.160	1.147	0.82	1.57	25.73	12.87	61.39
EmAAi	1.120	1.119	0.92	0.89	26.81	15.16	58.03
Met							
EaAAi	1.100	1.101	0.92	0.45	11.78	10.77	77.45
ExAAi	1.099	1.098	0.90	0.56	22.32	22.00	55.68
EmAAi	1.095	1.095	0.90	0.47	19.08	31.80	49.12
Phe							
EaAAi	1.149	1.156	0.96	1.19	27.4	3.58	66.01
ExAAi	1.160	1.149	0.82	1.57	25.73	12.87	61.39
EmAAi	1.120	1.119	0.93	0.89	26.81	15.16	58.03
Val							
EaAAi	1.121	1.124	0.97	0.56	22.76	6.21	71.02
ExAAi	1.122	1.120	0.98	0.59	13.09	31.66	55.24
EmAAi	1.106	1.105	0.94	0.62	21.80	12.09	66.11

[1]EaAAi, EvAAi and EnAAi represent isotope ratio in arterial plasma, extracellular space and intracellular space.

Table 5.8. Least square means for plasma absorption rates of EAA and AA infusion

Item	Treatment[2]				SEM	Effect (P-value)		
	CON	MKH	IL	MKH + IL		MKH	IL	MKH*IL
$F_{absorption}$[1] (g/d)								
Ile	298[a]	352[b]	383[b]	359[b]	17	0.35	0.02	0.04
Leu	495	543	659	635	42	0.76	0.01	0.35
Lys	245	341	297	254	54	0.59	0.72	0.19
Met	158	309	239	287	33	0.02	0.36	0.14
Phe	175	206	215	225	25	0.37	0.22	0.62
Val	386[a]	416[b]	415[b]	375[a]	22	0.78	0.76	0.09
AA infusion (g/d)								
Ile	0	0	22	22	-	-	-	-
Leu	0	0	50	50	-	-	-	-
Lys	0	38	0	38	-	-	-	-
Met	0	10	0	10	-	-	-	-

[1] Plasma absorption rates of EAA

[2]Treatment: 1) CON = infused 3 liters of 0.9% saline; 2) MKH = infused 21 g/d of Met, 20 g/d of His, and 38 g/d of Lys; 3) IL = infused 22 g/d of Ile, and 50 g/d of Leu; 4) MKH+IL = infused 21 g/d of Met, 20 g/d of His, 38 g/d of Lys, 22 g/d of Ile, and 50 g/d of Leu.

Table 5.9. Least square means for rate constants (min^{-1}) and flux (µmol/min) associated with AA exchange and metabolism in non mammary tissue

Parameter	Treatment[1]				SEM	Effect (*P*-value)		
	CON	MKH	IL	MKH + IL		MKH	IL	MKH *IL
Ile								
$K_{aAAbtAA}$, min^{-1}	0.12	0.11	0.12	0.10	0.03	0.65	0.87	0.99
Catabolism, µmol/min	995[a]	1228[ab]	1429[b]	1235[ab]	117	0.85	0.07	0.08
AA to body tissue, µmol/min	1818	1651	2294	1463	574	0.35	0.78	0.52
Leu								
$K_{aAAbtAA}$, min^{-1}	0.16	0.14	0.11	0.10	0.02	0.42	0.09	0.90
Catabolism, µmol/min	1724	1883	2518	2320	219	0.92	0.02	0.37
AA to body tissue, µmol/min	3485	2943	3089	2415	497	0.19	0.30	0.88
Lys								
$K_{aAAbtAA}$, min^{-1}	0.27	0.26	0.41	0.33	0.08	0.58	0.22	0.64
Catabolism, µmol/min	558	978	802	515	265	0.77	0.64	0.16
AA to body tissue, µmol/min	2393	2812	3362	3420	758	0.72	0.27	0.79
Met								
$K_{aAAbtAA}$, min^{-1}	0.73	0.50	0.41	0.33	0.10	0.06	0.01	0.27
Catabolism, µmol/min	594	1209	929	1166	142	0.02	0.29	0.18
AA to body tissue, µmol/min	1487	1779	802	1268	258	0.07	0.03	0.63
Phe								
$K_{aAAbtAA}$, min^{-1}	0.22	0.17	0.19	0.18	0.03	0.27	0.62	0.60
Catabolism, µmol/min	467	556	617	657	97	0.43	0.16	0.76
AA to body tissue, µmol/min	1114	860	968	731	135	0.04	0.20	0.93
Val								
$K_{aAAbtAA}$, min^{-1}	0.10	0.13	0.08	0.10	0.02	0.18	0.21	0.88
Catabolism, µmol/min	1431[ab]	1698[b]	1776[bc]	1399[a]	138	0.59	0.82	0.02
AA to body tissue, µmol/min	3187	3945	2232	2556	777	0.43	0.12	0.75

[1]Treatment: 1) CON = infused 3 liters of 0.9% saline; 2) MKH = infused 21 g/d of Met, 20 g/d of His, and 38 g/d of Lys; 3) IL = infused 22 g/d of Ile, and 50 g/d of Leu; 4) MKH+IL = infused 21 g/d of Met, 20 g/d of His, 38 g/d of Lys, 22 g/d of Ile, and 50 g/d of Leu.

Table 5.10. Least square means for mammary AA transport constants and fluxes

Parameter	Treatment[1]				SEM	Effect (P-value)		
	CON	MKH	IL	MKH + IL		MKH	IL	MKH*IL
Ile								
K_{xAAnAA}, min^{-1}	0.43	0.45	0.69	0.75	0.23	0.83	0.17	0.90
K_{nAAxAA}, min^{-1}	0.58	0.59	0.55	0.62	0.17	0.82	0.96	0.81
Influx, µmol/min	888	927	1014	1104	154	0.64	0.29	0.85
Efflux, µmol/min	307	291	435	438	117	0.95	0.22	0.93
Net uptake, µmol/min	576	636	542	666	54	0.03	0.94	0.36
Leu								
K_{xAAnAA}, min^{-1}	0.42	0.56	0.59	0.64	0.09	0.15	0.07	0.50
K_{nAAxAA}, min^{-1}	0.53^{a}	0.73^{ab}	0.86^{b}	0.63^{ab}	0.15	0.97	0.25	0.09
Influx, µmol/min	1339	1607	1853	1720	154	0.58	0.04	0.13
Efflux, µmol/min	420^{a}	616^{ab}	879^{bc}	679^{b}	148	0.98	0.03	0.07
Net uptake, µmol/min	908	991	1005	1041	100	0.48	0.39	0.77
Lys								
K_{xAAnAA}, min^{-1}	1.48	0.84	1.17	0.95	0.29	0.15	0.72	0.44
K_{nAAxAA}, min^{-1}	0.21	0.23	0.28	0.22	0.09	0.73	0.71	0.55
Influx, µmol/min	801	932	780	793	64	0.16	0.13	0.24
Efflux, µmol/min	166	183	169	101	66	0.43	0.24	0.22
Net uptake, µmol/min	625	756	611	692	30	<0.01	0.16	0.30
Met								
K_{xAAnAA}, min^{-1}	0.45	0.52	0.72	0.47	0.17	0.44	0.37	0.22
K_{nAAxAA}, min^{-1}	0.47	0.56	0.44	0.64	0.29	0.59	0.93	0.85
Influx, µmol/min	225	320	238	290	53	0.11	0.84	0.60
Efflux, µmol/min	68	89	65	112	51	0.38	0.75	0.69
Net uptake, µmol/min	162^{a}	232^{b}	186^{a}	172^{a}	21	0.09	0.26	0.03
Phe								
K_{xAAnAA}, min^{-1}	0.43	0.80	0.46	0.53	0.21	0.24	0.51	0.39
K_{nAAxAA}, min^{-1}	0.98	0.80	1.01	0.86	0.24	0.45	0.85	0.96
Influx, µmol/min	595	608	618	640	106	0.85	0.76	0.96
Efflux, µmol/min	310	296	330	354	104	0.96	0.68	0.84
Net uptake, µmol/min	283	312	288	287	29	0.57	0.68	0.54
Val								
K_{xAAnAA}, min^{-1}	0.46	0.55	0.45	0.56	0.13	0.37	0.99	0.96
K_{nAAxAA}, min^{-1}	1.05	1.07	0.93	0.79	0.22	0.67	0.17	0.58
Influx, µmol/min	1799	1940	1637	1786	361	0.61	0.58	0.99
Efflux, µmol/min	1022	1174	952	964	361	0.74	0.58	0.78
Net uptake, µmol/min	828	767	685	822	105	0.60	0.54	0.20

[1]Treatment: 1) CON = infused 3 liters of 0.9% saline; 2) MKH = infused 21 g/d of Met, 20 g/d of His, and 38 g/d of Lys; 3) IL = infused 22 g/d of Ile, and 50 g/d of Leu; 4) MKH+IL = infused 21 g/d of Met, 20 g/d of His, 38 g/d of Lys, 22 g/d of Ile, and 50 g/d of Leu.

Table 5.11. Least square means for mammary AA metabolism and calculated efficiency

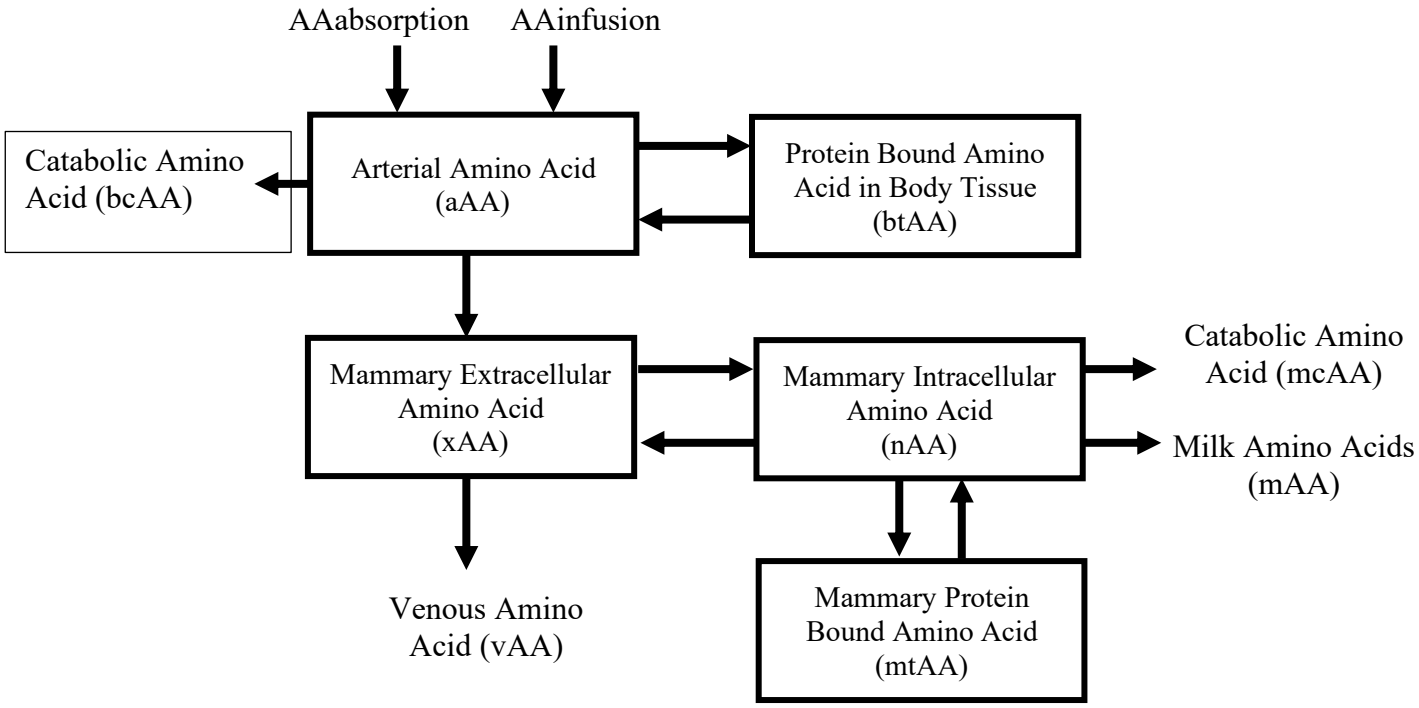
Parameter	Treatment[1]				SEM	Effect (P-value)		
	CON	MKH	IL	MKH + IL		MKH	IL	MKH*IL
Ile								
$K_{nAAmtAA}$, min^{-1}	0.96	0.69	0.53	0.59	0.18	0.47	0.09	0.27
Catabolism, µmol/min	172	226	157	257	56	0.20	0.81	0.99
AA to milk protein, µmol/min	399	410	412	431	32	0.40	0.34	0.81
AA to mammary tissue, µmol/min	541^{b}	331^{a}	344^{a}	390^{ab}	81	0.16	0.23	0.05
Milk protein, % of net uptake	67^{a}	64^{a}	77^{b}	66^{a}	5	0.02	0.03	0.09
Efflux, % of influx	34	29	39	40	8	0.77	0.33	0.67
Catabolism, % of influx	20	26	14	23	5	0.06	0.20	0.58
Milk protein, % of influx	45	45	46	39	8	0.61	0.72	0.63
Leu								
$K_{nAAmtAA}$, min^{-1}	0.92^{b}	0.67^{ab}	0.57^{a}	0.83^{b}	0.16	0.99	0.35	0.04
Catabolism (mammary), µmol/min	239	310	289	325	106	0.53	0.70	0.83
AA to milk protein, µmol/min	663	681	684	715	53	0.40	0.34	0.81
AA to mammary tissue, µmol/min	694	582	580	865	162	0.44	0.45	0.11
Milk protein, % of net uptake	73	69	71	70	9	0.59	0.91	0.73
Efflux, % of influx	29^{a}	38^{ab}	47^{b}	39^{ab}	7	0.83	0.05	0.07
Catabolism, % of influx	20	20	16	18	6	0.82	0.60	0.82
Milk protein, % of influx	51^{b}	42^{ab}	37^{a}	43^{ab}	4	0.62	0.06	0.05
Lys								
$K_{nAAmtAA}$, min^{-1}	0.31	0.25	0.33	0.36	0.13	0.86	0.40	0.52
Catabolism, µmol/min	124^{ab}	251^{c}	90^{a}	147^{b}	32	<0.01	0.01	0.10
AA to milk protein, µmol/min	507	505	522	545	38	0.63	0.25	0.56
AA to mammary tissue, µmol/min	216	280	258	216	129	0.90	0.91	0.57
Milk protein, % of net uptake	80	68	86	79	5	0.01	0.01	0.29
Efflux, % of influx	18	17	19	12	7	0.13	0.41	0.15
Catabolism, % of influx	16	27	12	19	4	0.01	0.04	0.42
Milk protein, % of influx	64	53	69	69	6	0.09	<0.01	0.11
Met								
$K_{nAAmtAA}$, min^{-1}	0.41	0.41	0.31	0.79	0.26	0.21	0.45	0.22
Catabolism, µmol/min	-9^{a}	55^{b}	10^{a}	-13^{a}	15	0.05	0.03	<0.01
AA to milk protein, µmol/min	172	176	177	185	14	0.45	0.42	0.83
AA to mammary tissue, µmol/min	45	59	35	73	20	0.11	0.90	0.43
Milk protein, % of net uptake	106^{b}	79^{a}	99^{b}	108^{b}	8	0.03	0.01	<0.01
Efflux, % of influx	23	25	28	34	13	0.72	0.50	0.88
Catabolism, % of influx	-6^{a}	16^{c}	3^{b}	-4^{a}	6	0.03	0.06	<0.01
Milk protein, % of influx	86	59	69	70	13	0.27	0.78	0.24
Phe								
$K_{nAAmtAA}$, min^{-1}	0.64	0.44	0.63	0.82	0.15	0.97	0.12	0.11
Loss to catabolism, µmol/min	24	39	14	1	20	0.95	0.13	0.33
AA to milk protein, µmol/min	265	272	274	286	21	0.40	0.34	0.81
AA to mammary tissue, µmol/min	183^{a}	176^{a}	205^{a}	277^{b}	34	0.14	0.02	0.09
Milk protein, % of net uptake	93	88	96	100	7	0.90	0.15	0.35
Efflux, % of influx	49	48	53	49	10	0.76	0.76	0.89
Catabolism, % of influx	3	6	2	2	3	0.67	0.40	0.56
Milk protein, % of influx	49	47	45	49	9	0.90	0.91	0.68
Val								
$K_{nAAmtAA}$, min^{-1}	0.68	0.51	0.67	0.75	0.18	0.75	0.40	0.37
Loss to catabolism, µmol/min	329	254	170	283	88	0.71	0.25	0.12
AA to milk protein, µmol/min	499	513	515	539	40	0.40	0.34	0.81
AA to mammary tissue, µmol/min	679	549	558	742	184	0.86	0.81	0.32
Milk protein, % of net uptake	65^{a}	67^{a}	76^{b}	66^{a}	6	0.07	0.03	0.02
Efflux, % of influx	54	59	53	47	12	0.93	0.47	0.53

Catabolism, % of influx	19	13	12	17	6	0.97	0.68	0.16
Milk protein, % of influx	27	28	35	35	8	0.90	0.24	0.99

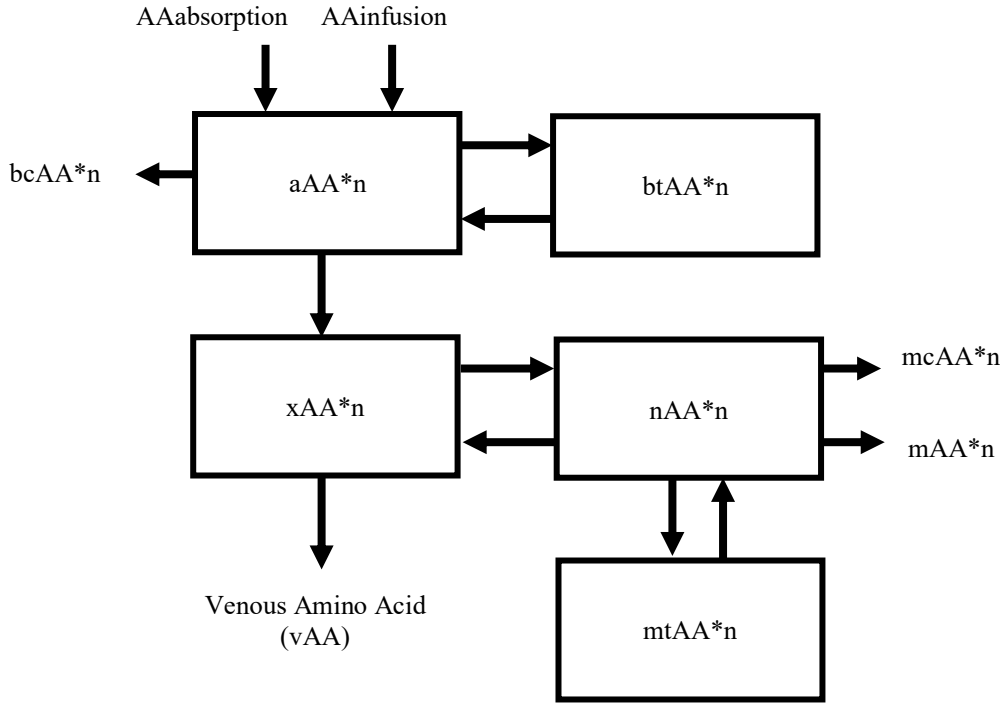
[1]Treatment: 1) CON = infused 3 liters of 0.9% saline; 2) MKH = infused 21 g/d of Met, 20 g/d of His, and 38 g/d of Lys; 3) IL = infused 22 g/d of Ile, and 50 g/d of Leu; 4) MKH+IL = infused 21 g/d of Met, 20 g/d of His, 38 g/d of Lys, 22 g/d of Ile, and 50 g/d of Leu.

Figure 5.1. Flow diagram depicting a model of total (A) and labeled (B) AA flux in mammary tissue.

(A)



(B)



5.8. Appendix

Mass (Q), concentration (C), volume (V), flux (F) and rate constants (k) were expressed in μmol, μmol/L, L, μmol/min and min^{-1} respectively. The unit of time (t) was min. The unit of body weight (BW) was kg. Abbreviations generally followed the form of X_{ab}, where X was the variable type (Q, C, V, F, k, f) with f denoting a fractional proportion. The a represented the precursor (for F and k) or pool of interest (Q, C, V) and b was the reaction product or secondary reference for the parameter, i.e. $f_{btAAbtPrt}$ refers to the fractional proportion of AA in body tissue protein.

The total mass of plasma AA ($Q_{aAA(i)}$), extracellular free AA ($Q_{xAA(i)}$), intracellular free AA ($Q_{nAA(i)}$), body tissue protein bound AA ($Q_{btAA(i)}$) and mammary tissue protein bound AA ($Q_{mtAA(i)}$) for the i^{th} AA (i= Ile, Leu, Lys, Met, Phe and Val) were determined as:

$$Q_{aAA(i)} = V_{a(i)} \times C_{aAA(i)}, \quad (1)$$

$$Q_{xAA(i)} = V_{x(i)} \times C_{xAA(i)}, \quad (2)$$

$$Q_{nAA(i)} = V_{n(i)} \times C_{nAA(i)}, \quad (3)$$

$$Q_{tAA(i)} = BW \times 1000 \times f_{btPrt} \times f_{btAAbtPrt} \times K_{BWAA}, \quad (4)$$

$$Q_{mtAA(i)} = MW \times 1000 \times f_{mtPrt} \times f_{mtAAmtPrt}, \quad (5)$$

where $C_{xAA(i)}$ was assumed to be equal to venous AA concentrations because the exchange between capillary and interstitial space is very rapid and no other sources of AA enter the vessel before sampling (Hanigan et al., 2009). The $C_{aAA(i)}$ and $C_{xAA(i)}$ were the average AA concentration over time in arterial and venous blood. The $C_{nAA(i)}$ was from the literature (Hanigan et al., 2009, Yoder, 2019)). $V_{x(i)}$ was assumed to be 20% of mammary tissue wet weight and $V_{n(i)}$ was total wet weight minus $V_{x(i)}$ and tissue dry matter (Hanigan et al., 2009). The $V_{a(i)}$ was a function of BW, which includes both blood and interstitial space. The $f_{btAAbtPrt}$ represented the

concentration of each AA in body protein (μmol/g), and f_{btPrt} represented the protein fraction in body tissues. The $f_{mtAAmtPrt}$ represented the concentration of each AA in mammary tissue protein (μmol/g), and f_{mtPrt} represented the protein fraction in mammary tissue. These values were from Williams (1978). K_{BWAA} was the proportion (g/g) of body tissue protein considered in $Q_{btAA(i)}$, which is from Huang et al. (2019). A portion of body protein was not considered in the model as the turnover rate was too slow to be uniquely identified during the course of the infusion. The *MW* represents the wet weight of mammary glands.

The mass of each isotopically labeled AA was calculated from the total pool size and background isotopic enrichment:

$$Qi_{aAA(i)} = Q_{aAA(i)} \times E_a, \tag{6}$$

$$Qi_{xAA(i)} = Q_{xAA(i)} \times E_x, \tag{7}$$

$$Qi_{nAA(i)} = Q_{nAA(i)} \times E_n, \tag{8}$$

$$Qi_{btAA(i)} = Q_{btAA(i)} \times E_t, \tag{9}$$

$$Qi_{mtAA(i)} = Q_{mtAA(i)} \times E_m, \tag{10}$$

where E_a and E_x were the background isotopic enrichment for individual AA in arterial and venous blood before the infusion. The E_t was assumed to be equal to E_a. The E_n and E_m were assumed to be equal to background isotope enrichment of individual AA in milk before the infusion.

Fluxes among pools were assumed to be mass action, which included AA exchange between plasma and non-mammary tissue, influx and efflux between the extracellular space and intracellular space, or exchange between intracellular AA and mammary tissue protein.

$$F_{aAAbtAA(i)} = k_{aAAbtAA(i)} \times Q_{aAA(i)}, \tag{11}$$

$$F_{xAAnAA(i)} = k_{xAAnAA(i)} \times Q_{xAA(i)}, \tag{12}$$

$$F_{nAAxAA(i)} = k_{nAAxAA(i)} \times Q_{nAA(i)}, \quad (13)$$

$$F_{nAAmtAA(i)} = k_{nAAmtAA(i)} \times Q_{nAA(i)}, \quad (15)$$

The AA flux from arterial blood to extracellular space and efflux to venous blood were the products of plasma flow (MPF, L/min) and arterial ($C_{aAA(i)}$) and extracellular AA concentrations ($C_{xAA(i)}$). The AA catabolized by body tissue ($F_{aAAbcAA(i)}$) was calculated as the difference of absorption and mammary uptake. The AA catabolized by mammary tissue ($F_{aAAmcAA(i)}$) was calculated as the difference of uptake and AA excreted into milk protein.

$$F_{aAAxAA(i)} = C_{aAA(i)} \times MPF, \quad (16)$$

$$F_{xAAvAA(i)} = C_{xAA(i)} \times MPF, \quad (17)$$

$$F_{aAAbcAA(i)} = F_{absorption(i)} + F_{infusion(i)} + F_{xAAvAA(i)} - F_{aAAxAA(i)}, \quad (18)$$

$$F_{aAAmcAA(i)} = F_{xAAnAA(i)} - F_{nAAxAA(i)} - F_{nAAmAA(i)}, \quad (19)$$

The animals were mature and thus not expected to be growing, or if growing, at a very low rate. Therefore, tissue protein pools were assumed to be in steady state over the 12 h sampling period, protein degradation was set equal to synthesis:

$$F_{btAAaAA(i)} = F_{aAAbtAA(i)}, \quad (20)$$

$$F_{mtAAnAA(i)} = F_{nAAmtAA(i)}, \quad (21)$$

The AA incorporation into milk protein was calculated from milk protein output and the AA composition of milk protein:

$$F_{nAAmAA(i)} = F_{milk} \times f_{mPrt} \times f_{mAAmPrt}, \quad (22)$$

where F_{milk} was milk output (g/min), f_{mPrt} was the fraction of milk as protein (g/g), $f_{mAAmPrt}$ was the fraction of each AA in milk protein (μmol/g), which were from(Lapierre et al., 2012).

The differential equations describing changes in $Q_{aAA(i)}$, $Q_{btAA(i)}$, $Q_{xAA(i)}$, $Q_{nAA(i)}$, $Q_{mtAA(i)}$ with respect time were

$$\frac{dQ_{aAA(i)}}{dt} = F_{absorption(i)} + F_{btAAaAA(i)} + F_{infusion(i)} + F_{xAAvAA(i)} - F_{aAAbcAA(i)} - F_{aAAxAA(i)} - F_{aAAbtAA(i)} \quad (23)$$

$$\frac{dQ_{btAA(i)}}{dt} = F_{aAAbtAA(i)} - F_{btAAaAA(i)}, \quad (24)$$

$$\frac{dQ_{xAA(i)}}{dt} = F_{aAAxAA(i)} + F_{nAAxAA(i)} - F_{xAAnAA(i)} - F_{xAAvAA(i)}, \quad (25)$$

$$\frac{dQ_{nAA(i)}}{dt} = F_{xAAnAA(i)} + F_{mtAAnAA(i)} - F_{nAAxAA(i)} - F_{nAAmtAA(i)} - F_{nAAmcAA(i)} - F_{nAAmAA(i)}, \quad (26)$$

$$\frac{dQ_{mtAA(i)}}{dt} = F_{nAAmtAA(i)} - F_{mtAAnAA(i)}, \quad (27)$$

The differential equations describing changes in $Qi_{aAA(i)}$, $Qi_{btAA(i)}$, $Qi_{xAA(i)}$, $Qi_{nAA(i)}$, and $Qi_{mtAA(i)}$ with respect to time were:

$$\frac{dQi_{aAA(i)}}{dt} = Fi_{absorption(i)} + Fi_{btAAaAA(i)} + Fi_{infusion(i)} + Fi_{xAAvAA(i)} - iF_{aAAbcAA(i)} - Fi_{aAAxAA(i)} - Fi_{aAAbtAA(i)}, \quad (28)$$

$$\frac{dQi_{btAA(i)}}{dt} = Fi_{aAAbtAA(i)} - Fi_{btAAaAA(i)}, \quad (29)$$

$$\frac{dQi_{xAA(i)}}{dt} = Fi_{aAAxAA(i)} + Fi_{nAAxAA(i)} - Fi_{xAAnAA(i)} - Fi_{xAAvAA(i)}, \quad (30)$$

$$\frac{dQi_{nAA(i)}}{dt} = Fi_{xAAnAA(i)} + Fi_{mtAAnAA(i)} - Fi_{nAAxAA(i)} - Fi_{nAAmtAA(i)} - Fi_{nAAmcAA(i)} - Fi_{nAAmAA(i)}, \quad (31)$$

$$\frac{dQi_{mtAA(i)}}{dt} = Fi_{nAAmtAA(i)} - Fi_{mtAAnAA(i)}, \quad (32)$$

The isotopic fluxes were calculated from total fluxes and isotope enrichment as follows:

$$E_a = \frac{Qi_a}{Q_a}, \quad (33)$$

$$Fi_{ab} = F_{ab} \times E_a, \quad (34)$$

where E_a, iQ_a, and Q_a represents the isotope enrichment, mass of the isotopically labeled AA, and mass of total AA for each of the AA pool. The flux of isotopically labelled AA from pool a to b is represented as iF_{ab}. There is one exception when fitting predicted isotope enrichment in intracellular space to observed values due to the time delay of AA from intracellular space to milk protein, thus a delay function was used:

En(t) =Em(t+lag)

The processing time from synthesis of protein in Golgi to release into alveolar space was previously observed to be 81 mins by Hanigan et al. (2009). The En(t) represents the isotope enrichment in intracellular space at time t (min), and Em(t+lag) represents the isotope enrichment in milk at time t+81 (min) relative to the isotope infusion time.

5.8.1 References

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Williams, A. 1978. The amino acid, collagen and mineral composition of preruminant calves. The Journal of Agricultural Science 90(3):617-624.

Yoder, P. S. 2019. Evaluation of amino acid transport and protein metabolism in the mammary gland of dairy cattle. in Dairy Science. Vol. Doctorate Virginia Polytechnic Institute and State University

Supplemental Table 5.1. Model stoichiometric constants and other factors.

Items	Units	Ile	Leu	Lys	Met	Phe	Tyr	Val
f_{btPrt}, f_{mtprt}	g protein/g BW	----------0.1883----------						
$f_{btAAbtPrt}$, $f_{mtAAmtPrt}$	µmol AA/ g protein	173.8	436.1	397.4	133.3	184.0	165.5	283.4
$f_{mAAmprt}$	µmol AA/ g milk protein	368.9	618.9	416.5	124.6	182.2	323.9	510.7
K_{BWAA}	g/g	----------0.01----------						
f_{xVol}	L extracellular space/kg MBW	----------0.2----------						

Supplemental Table 5.2. Model inputs.

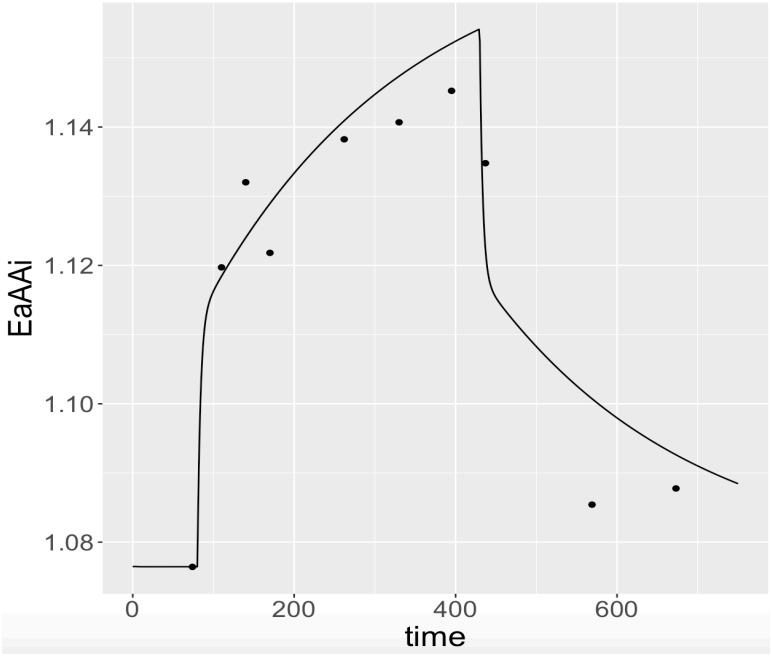
Animal	Period	MPF, L/min	BW, kg
1	1	15.3	622.6
	2	11.3	626.5
	3	10.6	645.7
	4	14.8	639.6
2	1	18.9	716.9
	2	12.9	753.9
	3	12.4	756.7
	4	13.1	788.7
3	1	9.9	718.9
	2	8.1	668.1
	3	9.3	902.0
	4	13.1	709.6
4	1	11.7	703.5
	2	9.4	739.7
	3	12.1	717.6
	4	12.0	730.0

Supplemental Table 5.3. Model inputs.

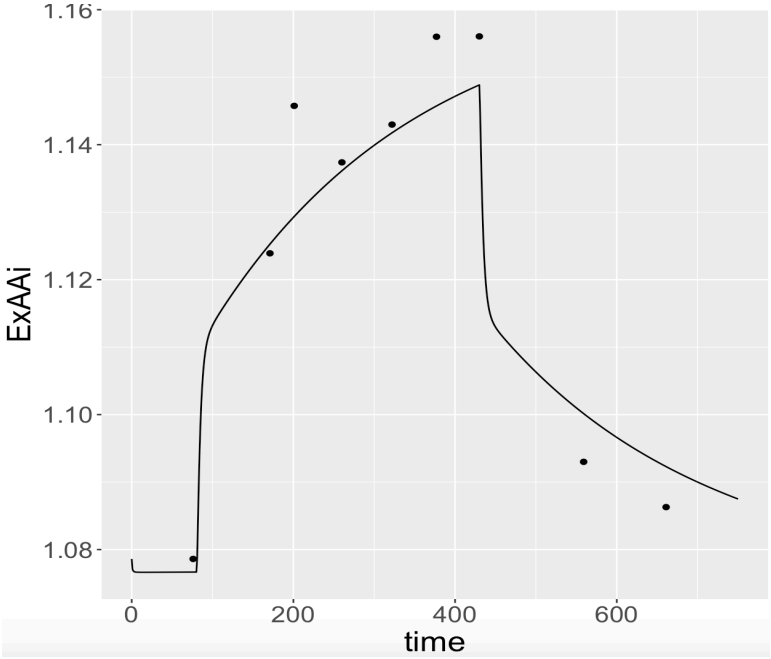
Items	Animal	Period	Ile	Leu	Lys	Met	Phe	Val
C_{aAA}, □mol/L	1	1	163	286	78	22	50	282
		2	147	233	116	35	43	263
		3	190	258	130	35	52	346
		4	153	240	80	14	51	330
	2	1	101	158	62	27	43	219
		2	137	231	74	26	54	285
		3	125	213	85	33	43	218
		4	133	201	108	39	51	266
	3	1	154	264	120	49	49	273
		2	132	188	145	41	48	285
		3	150	221	96	19	48	307
		4	143	273	83	15	48	279
	4	1	121	184	109	48	39	250
		2	138	179	89	21	45	297
		3	164	265	83	21	43	260
		4	115	192	88	33	30	168
C_{xAA}, □mol/L	1	1	122	222	39	13	34	238
		2	75	126	51	22	18	179
		3	122	167	57	17	25	278
		4	104	165	33	3	28	245
	2	1	57	85	19	14	20	176
		2	94	165	25	7	27	229
		3	77	137	31	18	20	163
		4	84	119	46	21	23	201
	3	1	85	154	51	34	19	191
		2	68	87	61	15	16	199
		3	100	146	36	5	26	252
		4	90	177	33	3	27	220
	4	1	64	91	37	24	11	184
		2	78	83	24	3	14	219
		3	122	198	35	6	19	213
		4	68	116	31	17	10	99
C_{nAA}, □mol/L	--	--	153	225	89	48	109	250
F_{nAAmAA}, □mol/min	1	1	393	652	497	169	261	491
		2	458	761	580	197	305	573
		3	436	725	553	187	290	546
		4	464	771	588	199	308	580
	2	1	440	731	557	189	292	550
		2	443	735	560	190	294	553
		3	416	691	526	179	276	520
		4	399	663	505	171	265	499
	3	1	376	624	476	161	250	470
		2	325	540	412	140	216	407
		3	301	500	381	129	200	376
		4	357	593	452	153	237	446
	4	1	479	795	606	206	318	599
		2	432	717	547	186	287	540
		3	456	758	577	196	303	570
		4	473	786	599	203	314	592

Supplemental Figure 5.1. Predicted and observed Leu isotopic ratios versus infusion time for one infusion.

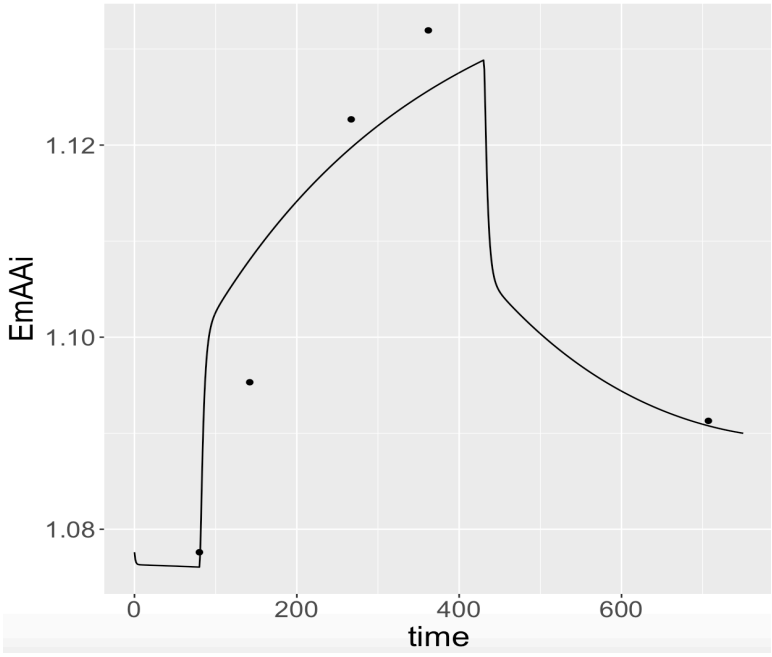
A) Arterial plasma



B) Venous plasma



C) Milk


1.12
1.10
1.08
EmAAi
0
200
400
600
time

CHAPTER 6: Conclusion

It is well recognized that balancing diets for EAA to meet animal requirement and reducing dietary CP can improve N efficiency and maintain the production at the same time. Current nutritional models try to represent processes of N utilization to estimate the N requirement and supply, which however failed to improve the N efficiency beyond 25%. The potential reasons are likely related to the bias existing in estimates of nutrient flows at different sites and inflexible post-absorptive metabolism model of nutrition models. Therefore, it is important to study N digestion and metabolism using more accurate and precise methods to improve the efficiency of N utilization. Determining AA availability for RUP or MCP in ruminants is technically difficult due largely to the errors of measurement associated with sample collection and animal variation. Compared to in-vivo method, porous bags are usually used in in-situ techniques to recover the test feedstuff, which however causes bias due to pore size, wash methods and incubation time. The in vitro technique tried to use enzyme mix to mimic the in vivo digestion activity, which however ignores other biologic factors. Jahani-Azizabadi et al. (2009) compared these three methods and found significant differences in protein digestibility between the methods. By using the stable isotope technique, the total EAA availabilities for corn silage, grass hay, alfalfa hay, dried distillers grain, soyhulls, brewers grain and corn grain were found to be similar to values from meta-analysis of mobile bag studies, but the availabilities of individual AA were more variable compared to in vitro and in situ results. Although the average RUP availability derived from in situ results are representative for compare between feed sources, which cannot be used to determine the availability of individual AA. Therefore, in vivo studies are necessary to build a matrix of EAA availabilities for representative ingredients that can be used in nutritional models. Although the isotope approach has a lot of merits, limitation exists. For example, the AA availability represents

the part of AA in diet RUP that enters the blood. The AA availability may change under different feeding environment, which however cannot be explained by the model since this method doesn't describe the AA digestion in the small intestine. This problem can be solved by introducing correctors under different feeding conditions. Another potential disadvantage of this approach is the assumption that microbial contributions to absorbed AA are constant across diets as test ingredients are added and removed which can be avoided by feeding a high protein diet. We propose to enhance the current bioavailability assessment by infusing ^{15}N labeled ammonia sulfate into the rumen, which will be incorporated into microbial AA allowing identification of the proportion of absorbed AA that are derived from MCP. The PD excretion is used as non-invasive method to estimate the MCP synthesis. Potential challenges existing in this method are urine collection and assumption related to MCP digestibility in small intestine and partition in the body. More variation will be introduced in when the MCP-AA is calculated from MCP due to the bias in AA composition. The CNCPS model also has the same limitations by using fixed AA composition and digestibility. Our isotope model derived MCP-AA availability was consistent with the MCP calculated from purine derivatives and NDS nutritional software. However, our model predicted a lower proportion of metabolizable AA from MCP under diets including low RDP, which implied the nutritional model overestimates MCP under low protein diets. The averaged EAA digestibility from MCP by isotope approach was 82%, which is similar to the values used in the nutrition model (80%). However, the digestibility varied across individual AA and digestibility of some AA was affected by diets. In addition, starch played an important role in N degradation, digestion and metabolism.

Although the difference between treatments was demonstrated, the variation between AA may be caused by the bias in AA composition as we adapted the literature values. In the future,

more research should be conducted to study the AA composition of MCP under different feeding conditions.

The post-absorptive AA utilization in different tissue especially mammary glands is critical in determining AA requirements. Manipulating AA supply to the mammary glands should lead to further improvements in post absorptive AA efficiency, which however cannot be achieved if nutrition models do not accurately represent mammary utilization for each AA. By coupling stable isotope tracers with A-V difference technique and compartmental modelling, current study quantified EAA metabolism in mammary glands of 4 dairy cows (53 kg/d milk production) fed typical TMR for high producing cows, which implied this approach is promising. After absorption, mammary uptake of EAA represented 100-156 % of milk protein EAA output. Cellular efflux represented 13 to 61% of EAA uptake. The proportion of AA catabolized and used for milk protein was affected by essential AA infusion, which demonstrated the plasticity of mammary glands in AA metabolism.

Overall, researchers and nutritionists should take following findings into consideration in future research and diet formulation. Firstly, RUP-EAA availability varied across feed ingredients, individual EAA and feeding conditions. Secondly, MCP-EAA availability varied across individual EAA and can be affected by feeding conditions. Thirdly, starch played an important role in N degradation, digestion and metabolism. Lastly, AA uptake and metabolism in mammary glands are plastic and more EAA than Met, Lys, and His are important for optimizing milk protein production. Therefore, one important thing to do in the future is building a diet library with EAA availability information for commonly used feed ingredients in this field. Based on this library, feed ingredients with high quality RUP can be screened when balancing diets for AA. The ruminal degradation and intestinal digestion and absorption of N are not fixed, which is affected by diet

composition and biological environment. When formulating a diet, nutritionists should also consider feeding conditions, like the energy supply, and inherent flexibility of mammary glands that will result in efficiency of use changes.