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Assessing the Bioavailability of Infused Lysine and Rumen-Protected Lysine Supplements Using the Area Under the Curve Technique and the Plasma Free Amino Acid Dose-Response Method

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Abstract

The milk production of lactating dairy cows is dependent on factors such as housing conditions, lineage, climate, and health, but the quality of their diets is generally the most influential. Maintaining a proper balance of nutrients is necessary to achieve the greatest milk production at the lowest cost. Maximum feed efficiency is not only critical for increasing the economic profits of an individual farm, but also for increasing food supply without increasing environmental demand. Supplementing cows' diets with lysine (Lys), an essential amino acid (AA), can aid in maximizing protein synthesis. Providing this nutrient in a rumen-protected (RP) coating can increase its bioavailability by delaying its degradation in the rumen. Using a reliable method to assess the bioavailability of an AA is important for ensuring accurate results that can be utilized to yield further improvements in the development of these supplements.

The objective of this experiment was to determine the bioavailability of RP-Lys supplements using plasma Lys concentrations for analysis via the area under the curve (AUC) method and the plasma dose response (PDR) method. Lactating Holsteins, fitted with rumen cannulas, were used in two experiments, through which they received varying forms of Lys. Blood samples were collected, and plasma concentration was measured for each cow. Feed intake, milk yield, and milk components were observed. Data was analyzed to determine average milk yield and dry matter intake, and AUC and PDR statistical analyses were performed to measure the bioavailability of each treatment. While no statistical significance was seen among the Lys prototypes, Prototype II exhibited a higher bioavailability via the AUC method, while Prototype IL exhibited a higher bioavailability via the PDR method. Ultimately, the PDR method appears to be a more effective strategy for determining the bioavailability of Lys.

Chapter 1: Review of Literature

Introduction

The amino acid (AA) concentration of a diet is especially important to consider when formulating rations, as the synthesis of milk protein in the mammary gland utilizes AAs in the blood. These organic molecules composed of carbon, hydrogen, oxygen, and nitrogen atoms, that each contain an amino and a carboxyl functional group, as well as a unique side chain joined to the central carbon atom. AAs are termed by the sequences of their atoms, with over 700 existing in nature and 20 of those serving as the building blocks of protein (Wu, 2013). Those which are substrates of polypeptide biosynthesis are referred to as protein amino acids and compose 98% of the total amino acid concentration of most feed ingredients sourced from plant and animal origins (Wu, 2013).

Proteins are the fundamental components of tissues in animals, with roles that include catalyzing chemical reactions in cells, contributing to cell structure, initiating contractions for cell motility, and transporting nutrients and wastes in and out of cells (NASEM, 2021). These molecules also function as antibodies and hormones, and are responsible for regulating gene expression (NASEM, 2021). Thus, both proteins and amino acids are vital components of life for all mammals.

Essential amino acids are those that cannot be synthesized in animal tissues nor at rates sufficient to meet specific requirements. In contrast to non-essential amino acids, which are readily synthesized from each other, from metabolites of intermediary metabolism, or from an excess of essential amino acids, these amino acids are considered indispensable and must be consumed via dietary intake. The essential amino acid supplied in the smallest amount relative to an animal's requirements is known as the first limiting amino acid, while the essential amino

acid supplied in the second smallest amount is referred to as the second limiting amino acid. Lysine and methionine have been identified as the first limiting amino acids for dairy cattle that are fed a common corn/grain-based diet in North America, with histidine identified as a first limiting amino acid for cows fed typical grass-based diets (NASEM, 2021).

Lysine

Lysine was first discovered as an alkaline substance present in casein hydrolysates by E. Drechsel in 1889 and later discovered in the hydrolysates of conglutin, gluten fibrin, and egg albumin (Wu, 2013). The name "lysine" was given by Ernst Fischer due to the release of urea that resulted when this substance was hydrolyzed in alkaline conditions by barium hydroxide (Wu, 2013). The primary function of this essential amino acid is protein synthesis, though it contributes to the structure and function of collagen via the form of hydroxylysine, and serves to regulate nitric oxide synthesis, antiviral activity, protein methylation, and protein acetylation (Broderick & Schwab, 2017).

Lysine is strictly a ketogenic amino acid because the process of its catabolism produces acetyl coenzyme A, but it does not contribute to gluconeogenesis (Wu, 2013). It is degraded in the liver via the mitochondrial saccharopine pathway and the peroxisomal pipecolate pathway (Wu, 2013). Therefore, promoting the efficiencies of these pathways is important to ensure adequate lysine absorption.

Protein Digestion & Absorption

Lactating dairy cows require high levels of dietary protein due to the significant demand of amino acids necessary to synthesize large amounts of milk protein. All dietary protein is first denatured in the abomasum, the true stomach, by hydrochloric acid, before digestion by various

proteases begins. This involves hydrolysis by pepsins, which forms peptides that are absorbed by the lumen of the small intestine, where they are then further hydrolyzed into peptidases (Wu, 2013). These peptidases then produce small peptides and free amino acids, which can be taken up by different microbes to generate numerous products, including ammonia, microbial protein, nitrogenous substances, pyruvate, short-chain fatty acids, and branched-chain fatty acids (Wu, 2013). Short-chain fatty acids, including acetate, butyrate, and propionate, contribute to glucose and fat production, while branched-chain fatty acids serve as growth factors for microorganisms, as well as precursors of long-chain branched fatty acids (Wu, 2013). However, not all dietary protein passes through the rumen without degradation.

A cow's protein requirement can be determined via the metabolizable protein (MP) system, which measures the concentration of protein that is absorbed from the epithelial cells of the small intestine and that is available to be taken up as amino acids by the blood (Herdt, 2014). Unlike the crude protein system, which only accounts for protein from non-protein nitrogen sources, the MP system recognizes that not all protein provided to cows might be available for absorption. MP consists of microbial protein synthesized in the rumen, referred to as rumen degradable protein (RDP), and proteins in the diet that avoid degradation by the rumen, referred to as rumen undegradable protein (RUP) (Herdt, 2014). The total proportion of dietary protein that is digested in the rumen varies from 30% to 40% for less soluble proteins, to 70% to 85% for most diets (Wu, 2013). The rate at which protein is degraded in the rumen depends on the length of time the protein remains in the rumen, the proteolytic activity of the rumen microbes, and the type of protein supplied (Wu, 2013). Though different, both RDP and RUP are necessary components of a properly balanced diet.

RUP varies from RDP in that it passes through the rumen, reticulum, and omasum without significant alteration, allowing direct digestion by the abomasum and small intestine, and immediate absorption of amino acids. Thus, RUP proportions are greater for cows with higher rates of feed intake because the feed passes through the rumen faster and there is less opportunity for it to be degraded (Herdt, 2014). Feeds that have been processed are typically higher in RUP, especially those that have undergone drying (Herdt, 2014). Generally, RUP should be 45% or less of the total MP of a diet, since more RUP correlates to less RDP (Broderick & Schwab, 2017). Feeding excess RUP also leads to a surplus of non-limiting amino acids and is not a consistent approach to feeding for maximum Nitrogen efficiency (Broderick & Schwab, 2017). However, cows with high requirements for protein and low rates of feed intake, such as cows in early lactation and heifers that are rapidly growing, benefit from diets containing high proportions of RUP (Herdt, 2014).

In contrast, nitrogen from RDP must first be incorporated by microbial protein before it will provide amino acids that are available for absorption (Herdt, 2014). The rate at which this process occurs is dependent on the growth rate of the rumen microbes, which is in turn dependent on the supply of fermentable energy available in the rumen (Herdt, 2014). Therefore, diets consisting of both RDP and a high energy concentration will yield high levels of microbial protein, leading to greater levels of MP and increased amino acid absorption (Wu, 2013). Feeds that are high in both protein and moisture content, such as legume silages, typically yield high concentrations of RDP (Herdt, 2014). Microbial protein, which consists of particle associated bacteria, fluid associated bacteria, protozoa, and fungi, should be 50% or greater of the total MP of a diet (Broderick & Schwab, 2017). Supplying adequate RDP in the diets of dairy cattle is

important because multiple studies have shown that a deficiency of RDP can lead to reductions in fiber digestibility, nitrogen utilization, and dry matter intake (Broderick & Schwab, 2017).

Amino Acid Supplementation

In addition to including sufficient high-quality protein sources in the diets of dairy cattle, limiting amino acids, such as lysine and methionine, must also be provided to achieve optimal growth, reproduction, and lactation performance. This can be accomplished through feeding high-protein ingredients, such as soybean meal, corn, blood meal, and fish meal, but can also be achieved through the use of supplemental amino acid products. However, these products are most effective when stable and protected from rumen degradation. The bioavailability of an amino acid is determined by its ability to escape ruminal degradation, combined with its intestinal digestibility (NASEM, 2021). High-quality proteins can be heated to induce the Maillard reaction, chemically treated to decrease solubility, or exposed to certain phytochemicals, such as tannins, to prevent degradation in the rumen. However, commercially produced amino acids are often physically encapsulated (Wu, 2013). Amino acids can be surface-coated via blood spraying, followed by heating and drying, or coated with hydrogenated lipids, such as lecithin or soy oils, to produce microcapsules (Wu, 2013). Coating combinations include lipids and pH-sensitive polymers, fibers and lipids, or calcium salts and long-chain fatty acids (NASEM, 2021). Although these systems of physical protection must be durable enough to withstand rumen degradation, they must also be susceptible to intestinal release (NASEM, 2021). Thus, achieving an adequate balance is critical to maintain the efficacy of these supplemental products.

Supplementing a diet with encapsulated forms of amino acids is an economically effective method for reaching the requirements of a limiting amino acid, without exceeding

them. Lysine is one limiting amino acid that provides particularly numerous benefits when added to dairy cattle diets. In properly formulated rations, lysine supplementation can increase milk production, milk components, and palatability (Broderick & Schwab, 2017). This first limiting amino acid has demonstrated to be especially important for increasing milk protein (Giallongo, et al., 2016). Furthermore, studies have shown that lysine contributes significantly to promoting nitrogen balance (Morris, 2020). Using a rumen-protected lysine product also alleviates any potential uncertainty related to inevitable variability in the amino acid concentration of feeds derived from animal origins, such as blood and fish meal (Broderick & Schwab, 2017). This is particularly beneficial for diets containing high levels of certain ingredients that are known to be naturally low in lysine content, such as corn and corn byproducts, including distiller's grain and corn gluten meal, that serve as sources of RUP (Broderick & Schwab, 2017).

Chapter 2: Area Under the Curve Experiment

Introduction

The area under the curve (AUC) technique is an *in vivo* method for quantitatively determining the amount of free AA in circulating blood from RP-AA products (Schwab et al., 2001). This process requires ruminally infusing cannulated cows with a single dose of a RP-AA in an amount exceeding that which is normally encountered by the ruminal microbiota. Blood samples prior to experimentation are used to establish baseline values of AA plasma concentration for each cow. The AA treatments are then given as pulse doses, with the test AA administered via abomasal infusion and the RP-AA provided as a bolus dose via the ruminal cannula. Blood samples are taken during and after the infusion periods for plasma AA analysis. These values are graphed and used for trapezoidal AUC analysis by computing the product of the average AA concentration increases between two consecutive sampling times and the duration of the interval. The AUC is determined by calculating the sum of the area of all the trapezoids formed between these two points.

Objective

The objective of this trial is to determine the bioavailability of RP-Lys supplements in lactating Holsteins using plasma Lys concentrations for AUC analysis.

Materials & Methods

Experimental Design and Treatment Diets

This study was conducted using eight multiparous lactating Holsteins (106 ± 32 DIM) at the University of New Hampshire (UNH) Fairchild Dairy Teaching and Research Center. These

cows, equipped with ruminal cannulas, were randomly selected, and used in a replicated 4 x 4 Latin square with 7-day experimental periods. The four experimental treatments (Table 1) were:

1) 95 g Lys from Lysine-HCl
2) 95 g Lys from USALysine
3) 95 g Lys with Prototype I L coating
4) 95 g Lys with Met Prototype II HPO coating

Since 95 g of pure HCL may be too high, and to prevent product rejection, the control treatment (Lys HCL) was supplied directly to the abomasum to produce the AUC for a treatment with 100% bioavailability. The Lys-HCl used for infusion was the same Lys that was used in the production of the RP-Lys supplements. Lys-HCl was diluted in 500 mL of distilled water and the infusion line was properly inserted and unblocked prior to infusion. The Lys infusion was dosed slowly in aliquots of 60 mL, using a 60-mL catheter syringe. To avoid reflux and to ensure that the entire Lys infusion was administered, the infusion line was flushed with 200 mL of tap water. The different RP-Lys treatments were placed in gelatin capsules, which were then placed directly into the rumen at the level of the rumen-omasal orifice.

Management of Cows

All procedures related to animal care were conducted with approval of the UNH Institutional Animal Care and Use Committee (190901). Cows were housed in a naturally ventilated tie-stall barn and fed individually. All cows had continuous and free access to water and were milked twice daily (0430 and 1530 h) in a milking parlor equipped with automatic take-offs and milk meters. Milk weights were recorded at each milking.

All cows were fed a Lys-adequate and Met-adequate basal diet throughout the study (Table 1). The basal diet was fed as a TMR prepared two times daily (0500 and 1600 h) by

weighing each ingredient and mixing them in a mobile paddle mixer (Data Ranger). Samples of TMR and orts were collected daily and composited by cow for each period to allow for determination of DM and calculation of DMI.

Blood Sampling and Analysis

Blood samples were obtained from each cow on the last two days of the experimental periods. Blood samples were collected from the coccygeal vein or artery 0 hr before administration of treatments, to determine basal blood AA concentrations. Blood samples were then taken at 1, 2, 3, 4, 6, 9, 12, 24, 30, and 48 hr post-dosing. Blood was collected in a 10-mL vacutainer tube (Monoject, Mansfield, MA) containing 15% K_3EDTA. Tubes were placed immediately in a Chameleon Cooler and centrifuged within 15 min at 1,200 × g for 20 min at 5°C. Then, 1.0 mL aliquots of deproteinized plasma were removed, placed into 1.8-mL cryovials, and stored at -80°C for plasma AA analysis (Experimental Station Chemical Laboratories, University of Missouri-Columbia, Columbia, MO).

Statistical Methods

The basal plasma Lys concentration (BPLC) value was subtracted from the plasma values obtained after supplementation to yield plasma Lys values only due to supplementation. The adjusted Y (plasma concentrations-BPLC) was subjected to the area of a trapezoid [1/2*h *(a+b)], which was used to calculate the area under the curve [0.5 (time2-time1)*(conc2-conc1)]. The Yadj values were then analyzed using the mixed model of SAS (9.4) and the PDIFF option was used to test treatment differences according to the following model:

Significance $P < 0.05$.

$Y_{adji} = \mu + Li + Ei$

Where:

Y_{adji} = adjusted dependent variable

μ = overall mean

Li = fixed effect of treatment (i = 1...,4)

E_i = residual errors

Results & Discussion

The average DIM of the cows increased from 120 days to 155 days throughout the duration of the trial. The feed component analysis (Table 3) and the AA analysis (Table 4) of the individual feed ingredients, in conjunction with the AMTS evaluation (Table 5), indicate that the diet was not energy deficient, since the NE_L allowable milk or ME allowable milk of 47.0 kg/d is predicted to be slightly higher than the MP allowable milk of 40.7 kg/d. The Lys:Met ratio for the AMTS evaluation is 2.46:1, suggesting that the diet is slightly Lys deficient. The CP of the diet is 15.0%, which is lower than the CP initially formulated at the beginning of the trial. This is likely due to the CP of the corn silage and haylage being lower than expected. Table 6 indicates that the average milk yield and dry matter intake (DMI) values for each cow are not affected by treatments.

The results of the AUC analysis are presented in Table 7. While the infusion treatment varies from the RP-Lys supplements in both Lys µM concentration and AUC value, the three RP-Lys supplements do not differ from each other. The bioavailability of USA Lysine is 68.1%, Prototype IL is 45.3%, and Prototype II HPO is 54.3%. The AUC analysis, presented in Figure 1, depicts a rapid, linear increase in Lys of the infusion treatment, which peaks at 2 hours, and an equally rapid, linear decrease back to its baseline before any of the RP-Lys supplements peak.

After 4 hours, USA Lysine peaks at approximately 185 µM Lys, while Prototype II HPO peaks at approximately 150 µM Lys after 10 hours, and Prototype IL peaks at approximately 125 µM Lys, also after 10 hours.

As demonstrated by the plasma concentrations of AA reported in Table 8, treatment has a significant effect on many of the amino acids. Exceptions include Arg, Trp, Ala, Gln, homocysteine, Tau, and total EAA. However, the treatment x hour interaction is significant for Arg, Lys, and total EAA. The plasma metabolites presented in Table 9 indicate that treatment has a significant effect on α-amino-adipic acid, α-amino-butyric acid, and 3-Methylhistidine. 3-Methylhistidine indicates muscle degradation, and the infusion treatment, which peaks around 800 µM Lys, is significantly different than the three RP-Lys supplements. α-amino-adipic acid is a final oxidation product of Lys and is significantly higher for infusion compared to the other treatments. α-amino-butyric acid serves as an intermediate, occurring in the catabolism of the two essential amino acids, Met and Thr. These are both significantly lower for the infusion treatment than the other RP-Lys supplements, for which the α-amino-butyric acid was also significantly lower. A trend was noted in carnosine and there was treatment x hour interaction observed for α-amino-adipic acid.

The results of this trial and those from previous research support observations that Lys is a highly bioavailable amino acid in its RP form. The infused Lys HCl was expected to exhibit a high bioavailability, though not quite such a dramatic peak as observed. To possibly prevent this, a future experiment could involve infusion of the Lys HCl at a controlled rate over a period of 3-4 hours. The HPO treatment produced a premature peak in Lys concentration, potentially due to the coating breaking down earlier than expected. This could be investigated further with an experiment testing this supplement with different types of RP coatings. Although the corn silage

was lower in CP than expected, the diet was still energy efficient due to an intentional overcalculation in the initial formulation. The lack of variation in average milk yields indicates that the supplements are relatively equally beneficial, with none becoming detrimental to the production of the cows.

Conclusions

The AUC method was successful in determining the bioavailability of the RP-Lys supplements. However, while the only expectation was for Lys to increase in plasma concentration, several other amino acids decreased in concentration. Thus, a future experiment feeding a lower dose of Lys, such as 60 g instead of 95 g, could be performed. This study could investigate the effect on 3-Methylhistidine at a lower dose of Lys, which would possibly yield a lower value, indicating less muscle degradation as a result of less Lys. The high bioavailability yielded by these RP-Lys products indicate that supplementing the diets of dairy cows, specifically those high in corn and corn byproducts, with encapsulated forms of this essential AA, can serve as an economically effective method for reaching the requirements of this limiting AA, without exceeding them.

Table 1. Calculations for the amounts of RP-AA products to feed.

Lysine Hydrochloride is 80% Lys 95 g/d Lys/0.80= 118.75 g/d Lys-HCl
USALysine is 69.6 % Lys-HCl 69.6 x 0.80 = 55.68 % Lysine 95 g/d Lys/.5568 = 170.62 g/d USALysine
Prototype IL is 79.2 % Lys-HCl 79.2 x 0.80 = 63.36 % Lysine 95 g/d Lys/.6336 = 149.94 g/d Prototype IL
Prototype II HPO is 79.7 % Lys-HCl 79.7 x 0.80 = 63.76 % Lysine 95 g/d Lys/.6376 = 149.00 g/d Prototype II HPO

Table 2. Ingredient composition of basal Lys-adequate diet.

Ingredient	% DM
Corn silage, mature	24.05
Mixed mostly grass silage, mid-maturity	21.23
Steam flaked corn	4.21
Corn meal	15.97
Beet pulp	9.97
Molasses, sugar cane	1.16
Distillers grains with solubles	0.73
Soybean meal, solvent extracted	6.46
Canola meal, solvent	2.16
SoyPlus	7.54
Urea	0.12
BergaFat-100	3.06
Kessent M	0.09
Mineral/vitamin mix	3.24

Table 3. Chemical composition of feedstuffs (% DM unless otherwise noted)

	Corn Silage	Haycrop Silage	Soy Plus	Canola meal	Soybean meal	Steam Flaked Corn	Corn Meal	Corn Distillers Grains	Beet Pulp	Molasses	Urea	Minerals	Nutra	Core
DM	32.7	30.7	90.9	93.8	89.8	86.8	89.3	90.5	91.7	78.8	99.6	94.6		99.5
CP	6.7	12.1	45.6	43.0	50.6	7.7	6.9	32.3	9.9	7.6	273.0	0.1	-	
aNDFom	38.0	56.5	20.8	24.4	13.0	6.9	7.1	39.7	30.9	-	-	-	-	
ADF	23.7	38.6	12.4	21.5	9.3	2.3	2.4	15.5	28.1	-	-	-	-	
NDF-CP	0.6	2.3	9.5	7.0	6.4	0.9	0.7	5.8	5.1	-	-	-	-	
ADF-CP	0.4	1.4	3.3	4.5	5.9	0.4	0.6	2.7	3.4	-	-	-	-	
Lignin	2.5	6.1	2.8	10.1	1.2	0.5	0.5	5.9	6.5	-	-	-	-	
NFC	47.3	16.4	20.9	21.8	27.6	81.5	81.5	6.8	47.2	-	-	-	-	
Starch	41.1	2.1	1.6	2.5	2.1	75.6	80.5	2.3	1.0	-	-	-	-	
Ether extract	4.40	5.39	6.19	4.11	1.80	2.88	3.30	14.51	1.38	1.2	-	-		97.2
Ash	3.62	9.61	6.52	6.69	6.94	1.09	1.22	6.74	10.55	16.85	-	-	-	
Ca	0.18	0.56	0.36	0.64	0.33	0.01	0.02	0.03	1.07	1.04	-	18.17	-	
P	0.27	0.34	0.72	1.06	0.74	0.21	0.26	1.16	0.12	0.16	-	1.12	-	
Mg	0.09	0.29	0.31	0.56	0.30	0.08	0.09	0.35	0.27	0.44	-	4.68	-	
K	1.06	2.67	2.35	1.09	2.42	0.28	0.34	1.36	0.56	4.42	-	0.10	-	
Na	0.009	0.063	0.028	0.074	0.011	0.008	0.006	0.116	0.049	0.168	-	13.470	-	
Cl ion	0.17	0.90	0.03	0.02	0.02	0.06	0.08	0.20	0.01	-	-	7.73	-	
Fe (ppm)	239	540	112	130	92	53	34	110	1,140	160	-	1,970	-	
Zn (ppm)	26	26	48	60	48	17	25	70	28	16	-	1,000	-	
Cu (ppm)	4	8	15	6	14	2	2	6	8	25	-	169	-	
Mn (ppm)	16	53	35	58	32	4	5	14	76	10	-	778	-	
S	0.10	0.22	0.38	0.82	0.43	0.10	0.09	0.68	0.36	1.14	-	0.23	-	

Table 4. Amino acid composition of feedstuffs. (% of CP)

	Corn Silage	Haycrop Silage	SoyPlus	Canola meal	Soybean meal	Steam Flaked Corn	Corn Meal	Corn Distillers Grains	Citrus Pulp	Molasses
EAA										
Arginine	1.61	2.63	6.84	6.01	7.09	4.69	4.52	5.14	1.61	0.17
Histidine	1.29	1.45	2.58	2.73	2.66	2.90	2.96	3.09	2.47	0.17
Isoleucine	4.03	4.17	4.79	4.10	4.81	3.59	3.74	4.57	3.44	1.01
Leucine	10.49	7.16	7.65	6.98	7.74	11.32	10.92	12.81	5.48	1.01
Lysine	3.71	4.44	5.95	5.57	6.54	3.59	3.90	3.90	3.12	0.34
Methionine	1.94	1.45	1.28	1.99	1.37	1.93	1.87	2.02	1.50	0.00
Phenylalanine	4.52	4.44	5.14	4.05	5.19	4.83	4.68	5.55	3.33	0.68
Threonine	3.23	3.44	3.79	4.12	3.90	3.59	3.59	4.20	3.98	1.01
Tryptophan	0.50	0.51	1.21	1.01	1.36	0.87	0.83	0.81	0.38	0.34
Valine	5.32	5.44	5.00	5.27	5.00	4.83	4.99	5.85	5.48	1.86
Total	36.65	35.13	44.23	41.84	45.65	42.14	42.01	47.94	30.79	6.59
NEAA										
Alanine	9.52	7.61	4.30	4.32	4.34	7.18	7.02	7.73	4.19	4.22
Aspartic Acid	5.97	6.43	10.95	6.83	11.16	6.90	7.18	6.76	6.34	23.48
Cysteine	1.77	1.00	1.28	2.61	1.41	2.21	2.34	2.18	1.18	0.34
Glutamic Acid	10.81	6.89	17.19	16.94	17.59	17.39	17.32	14.49	8.17	5.41
Glycine	4.52	4.80	4.37	5.04	4.16	4.14	4.21	4.47	3.76	1.01
Ornithine	0.97	0.63	0.12	0.05	0.08	0.00	0.00	0.17	0.11	0.00
Proline	7.75	4.35	5.07	6.19	5.04	8.56	8.58	8.37	4.08	0.68
Serine	2.90	2.63	4.09	3.50	4.26	4.55	4.52	4.81	3.44	1.52
Tyrosine	2.26	0.91	0.23	0.20	0.23	1.38	1.72	0.20	2.69	2.03
Taurine	1.45	1.99	3.53	2.66	3.52	2.76	2.18	4.07	3.12	0.84
Total	47.92	37.24	51.14	48.35	51.80	55.07	55.07	53.25	37.07	39.52
Total AA	84.57	72.37	95.37	90.19	97.45	97.21	97.08	101.18	67.86	46.11

Table 5. AMTS (Ver.4.17.0.1) evaluation of the basal formulated diet[1]

aNDFom, % DM	28.86	CP, % DM	15.0
Forage NDF, % DM	21.13	RDP, % DM	8.89
NFC, % DM	41.4	RUP, % DM	6.13
ME, Mcal/kg DM	2.69		
NE_L, Mcal/kg DM	1.73	MP-bacterial, g/d	1532
EE, % DM	6.8	MP-RUP, g/d	1121
ME required, Mcal/d	70.3	MP-Lys, g/d	184.16
ME supplied, Mcal/d	69.7	MP-Met, g/d	74.94
ME balance, Mcal/d	-.06		
		MP-Lys, % MP	6.94
MP required, g/d	2951	MP-Met, % MP	2.82
MP supplied, g/d	2655		
MP balance, g/d	-296	MP- Arg, % MP	6.32
		MP- His, % MP	2.60
DM intake-actual, kg/d	25.9	MP - Ile, % MP	5.22
DM intake-predicted, kg/d	27.1	MP- Leu, % MP	7.74
		MP- Phe, % MP	4.97
ME allowable milk, kg/d	47.0	MP- Thr, % MP	4.80
MP allowable milk, kg/d	40.7	MP- Trp, % MP	1.37
Actual milk, kg/d	47.5	MP- Val, % MP	5.68

[1] BW 712 kg, DMI = 25.9 kg/d, milk yield = 47.5 kg/d, milk fat content = 3.90%, and milk true protein concentration 2.90%.

Table 6. Milk and dry matter intake for Holstein cows fed a diet supplemented with RP-Lys supplements.

			Prototype II HP0			
Item	Infusion	USALysine		Prototype IL	SE	Trt
Milk kg/d	46.3	48.5	47.2	47.9	2.02	0.88
DMI, kg/d	25.1	26.5	25.7	26.0	1.37	0.90

Table 7. Lysine concentration, area-under-the-curve and bioavailability for Holstein cows fed a diet supplemented with RP-Lys supplements.

	Lys, μ*M*				
Treatment	μ*M*	SEM	AUC	SEM	Bioavailability
Infusion	244.93[a]	12.324	2027.19[a]	172.18	-
USALysine	122.59[b]	12.324	1380.95[b]	159.88	68.12
Prototype IL	100.64[b]	12.324	917.96[b]	156.85	45.28
Prototype II HPO	113.81[b]	12.324	1101.41[b]	163.04	54.33

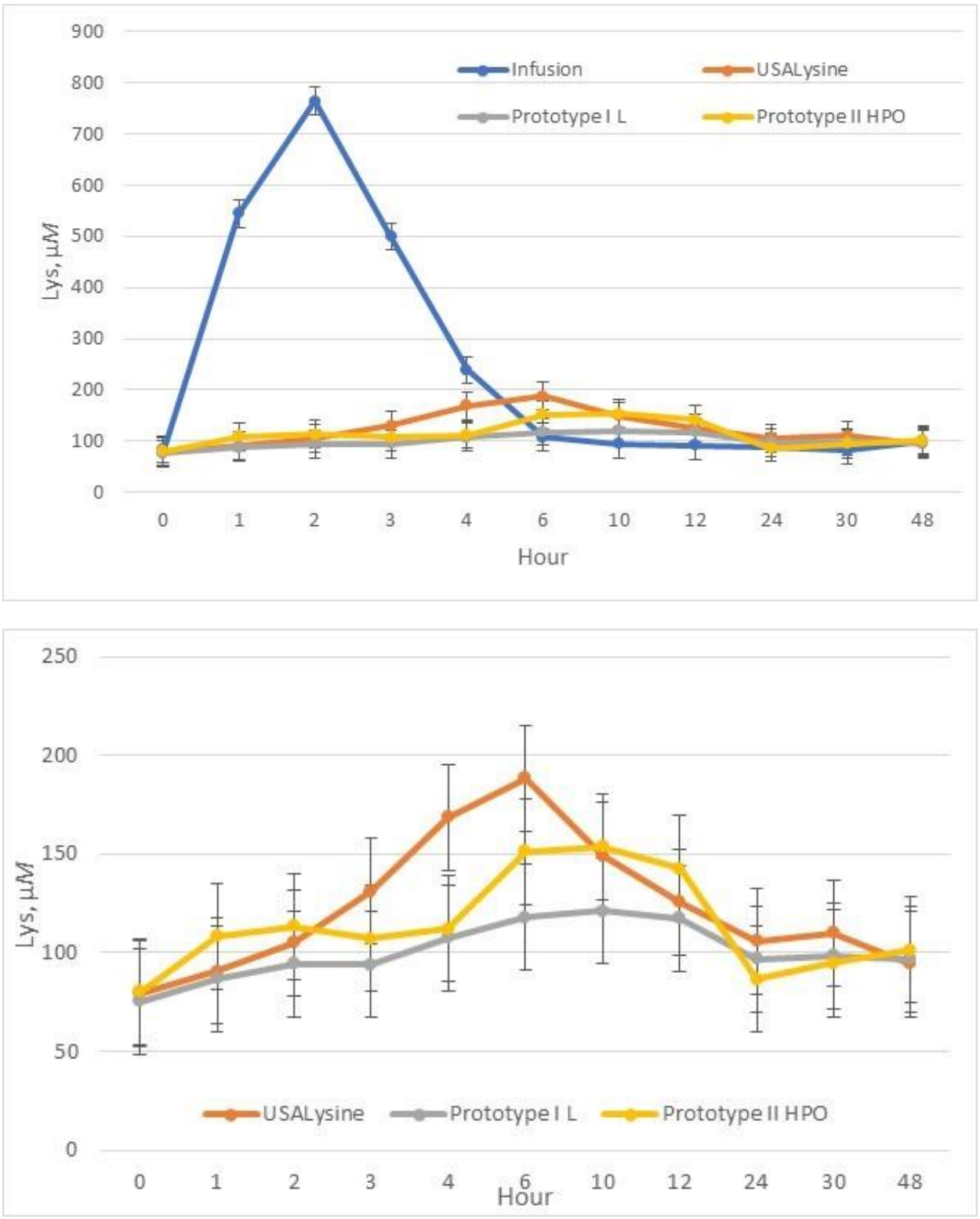

Figure 1. Area-under-the-curve for Holstein cows fed a diet supplemented with RP-Lys supplements.

Table 8. Plasma amino acids (μM) for Holstein cows fed a diet supplemented with RP-Lys supplements.

						P-value	
Item	Infusion	USALysine	Prototype II HP0	Prototype IL	SE	Trt	Trt x Hr
Arginine	85.5	89.0	92.1	87.0	3.52	0.13	0.01
Histidine	52.6^{b}	58.2^{a}	58.8^{a}	60.4^{a}	2.10	<0.0001	0.51
Isoleucine	114.6^{c}	122.5^{b}	129.5^{a}	124.2^{ab}	5.61	0.0002	0.41
Leucine	145.4^{c}	153.3^{b}	161.9^{a}	157.9^{ab}	8.77	0.0004	0.54
Lysine	203.5^{a}	140.3^{b}	137.5^{b}	100.6^{c}	13.91	<0.0001	<0.0001
Methionine	29.4^{c}	32.5^{b}	34.3^{a}	32.7^{ab}	1.56	<0.0001	0.70
Phenylalanine	46.4^{b}	47.5^{ab}	50.1^{a}	48.5^{ab}	2.00	0.01	0.39
Threonine	111.6^{c}	117.7^{b}	123.4^{a}	120.1^{ab}	4.49	0.003	0.60
Tryptophan	49.7	51.4	51.0	50.9	1.30	0.47	0.48
Valine	240.2^{c}	253.1^{b}	267.8^{a}	260.8^{ab}	14.20	<0.0001	0.78
Alanine	319.2	324.7	333.5	321.3	20.27	0.271	0.92
Asparagine	49.9^{b}	57.2^{a}	59.7^{a}	57.1^{a}	1.50	<0.0001	0.85
Aspartic Acid	3.07^{c}	3.26^{ab}	3.32^{a}	3.14^{b}	0.08	0.05	0.86
Citrulline	93.3	98.0^{a}	100.3^{a}	101.1^{a}	5.83	0.003	0.99
Cystine	19.9^{b}	21.2^{a}	21.5^{a}	21.4^{a}	0.89	<0.0001	0.99
Cystathionine/Allocystathionine	2.04^{c}	2.23^{a}	2.19^{ab}	2.13^{bc}	0.11	0.008	0.96
Glutamine	205.9	215.1	209.3	207.4	8.14	0.34	0.89
Glutamic Acid	41.4^{b}	41.6^{b}	43.3^{a}	40.8^{b}	1.46	0.04	0.57
Glycine	342.0^{c}	381.0^{a}	369.3^{ab}	362.3^{b}	11.56	<0.0001	0.62
Homocysteine	3.07	3.19	3.35	3.35	0.35	0.12	0.73
Ornithine	52.8^{b}	55.5^{ab}	58.5^{a}	57.2^{a}	3.90	0.009	0.54
Proline	94.2^{b}	103.6^{a}	106.9^{a}	103.8^{a}	4.98	<0.0001	0.98
Serine	86.9	95.6	98.3	93.6	2.19	<0.0001	0.61
Taurine	48.7	51.2	51.6	51.1	2.76	0.29	0.98
Tyrosine	43.6^{c}	49.1^{b}	52.9^{a}	51.1^{ab}	1.59	<0.0001	0.13
TEAA	1079	1066	1107	1043	42.4	0.17	<0.0001
TNEAA	1406^{b}	1502^{a}	1514^{a}	1477^{a}	33.3	0.0004	0.87
TAA	2485^{b}	2568^{ab}	2620^{a}	2520^{b}	57.4	0.04	0.10
TBCAA	500^{c}	529^{c}	559^{a}	543^{ab}	28.2	<0.0001	0.59
TUCAA	232^{b}	243^{ab}	251^{a}	245^{a}	9.3	0.01	0.35

TSAA	103[b]	110[a]	113[a]	111[a]	3.6	0.0002	0.99
TAA-Lys	9.18[a]	5.85[b]	5.66[b]	4.13[c]	0.61	<0.0001	<0.0001

[a-c] Means in the same row differ $P < 0.05$.

Table 9. Plasma metabolites (μM) for Holstein cows fed a diet supplemented with RP-Lys supplements.

						P-value	
Item	Infusion	USALysine	Prototype II HP0	Prototype IL	SE	Trt	Trt x Hr
1-Methylhistidine	30.2	23.4	24.4	23.0	4.38	0.55	0.34
3-Methylhistidine	3.88[a]	3.50[b]	3.53[b]	3.44[b]	0.20	<0.0001	0.99
α-amino-adipic acid	17.9[a]	15.5[b]	16.2[b]	14.1[c]	1.00	<0.0001	<0.0001
α-amino-butyric acid	17.5[c]	20.3[a]	19.3[b]	19.9[ab]	0.72	<0.0001	0.99
β-alanine	10.5	11.0	11.1	10.9	0.47	0.15	0.81
Carnosine	15.9	16.6	16.5	16.8	0.94	0.10	0.98
Ethanolamine	6.50	6.36	6.50	6.96	0.49	0.83	0.86
γ-amino-butyric acid	3.60	3.36	3.15	3.51	0.46	0.08	0.47
Hydroxylysine	1.94	1.93	1.87	1.92	0.15	0.99	0.92
Hydroxyproline	11.3	11.5	11.4	11.2	0.93	0.39	0.98
Phosphoserine	5.06	5.20	5.15	4.99	0.30	0.96	0.13
Sarcosine	31.2[b]	33.4[a]	32.0[ab]	32.1[a]	1.05	0.05	0.90

[a-c] Means in the same row differ $P < 0.05$.

Chapter 3: Plasma Dose Response Experiment

Introduction

The plasma dose-response (PDR) technique is a method used to determine the bioavailability of Lys from RP-Lys supplements. Due to variations in the availability, quality, and digestibility of high Lys-containing protein sources, such as blood meal and fish meal, RP-Lys supplements serve as effective alternatives. However, commercial RP-Lys supplements vary in encapsulation technology, size, density, Lys concentration, and availability to ruminants. Thus, a reliable, standardized method for estimating Lys bioavailability is necessary for accurately comparing the ability of different RP-Lys supplements to provide highly metabolizable Lys.

The plasma free Lys-dose response technique is a quantification method that has been refined through several studies (King et al., 1991; Rulquin and Kowalczyk, 2003; Borucki-Castro et al., 2008). This technique is based on the positive linear relationship between infused doses of Lys into the omasum, abomasum, or duodenum, and the concentration of Lys in plasma (Hanigan, 2009). The assumption that increases in plasma Lys concentration reflect increases in net absorption of Lys is relied upon to calculate an estimate of the bioavailability of Lys from RP-Lys supplements. Variation is minimized by providing all treatments in the same Latin square.

Objective

The objective of this trial is to determine the bioavailability of RP-Lys supplements in lactating Holsteins using plasma Lys concentrations for analysis via the PDR method.

Materials & Methods

Experimental Design and Treatment Diets

This study was conducted using eight multiparous lactating Holsteins (128 ± 38 DIM) at the University of New Hampshire (UNH) Fairchild Dairy Teaching and Research Center. These cows, equipped with ruminal cannulas, were randomly selected and used in a replicated 4 x 4 Latin square with 7-day experimental periods. The four experimental treatments (Table 1) were:

5) 0 g/d Lys (negative control)
6) 60 g/d Lys from abomasally infused Lys HCLmater
7) 60 g/d Lys from Prototype II HPO
8) 60 g/d Lys from Prototype IL

The infusion treatment was prepared by dissolving Lys-HCl in 4 L of hot tap water. The Lys solutions were continuously infused into the abomasum via the rumen cannula using a peristaltic pump (Masterflex, Cole-Parmer, Vernon Hills, IL). Fresh infusion solutions were prepared daily at 1300 h and pumping rates were closely monitored and adjusted to ensure treatments were completely and uniformly infused. The pump was disabled and the infusion lines were disconnected twice daily when the cows were moved from their stalls to the milking parlor.

Immediately prior to each feeding, the RP-AA supplements were mixed with 1.5 kg of TMR. The RP-Lys supplements were prepared with the same Lys-HCl that was used to produce the infusion treatment. These mixtures were placed in rubber tubs and fed to the cows 30 min before each feeding, to ensure RP-Met supplements were completely consumed. Any TMR/RP-AA mix not consumed by a cow within 15-20 min was manually inserted into the rumen via the ruminal cannula. Cows were fed 1/3 of their daily feed allotment 3 times daily (0500, 1300, and 2100 h). The daily amounts of RP-Lys products were divided into 3 equal portions to ensure that a constant ratio of RP-Lys products to total TMR consumption was maintained.

Cow characteristic before the start of the trial:

	BW, kg	BCS	DMI, kg/d	Milk, kg/d	Fat, %	Protein, %
Block 1	730	3.03	25.8	43.9	3.79	2.92
Block 2	701	2.99	25.1	48.4	3.69	2.89

Management of Cows

Cows were housed in a naturally ventilated tie-stall barn and fed individually, with continuous and free access to water. All procedures related to animal care were conducted with approval of the UNH Institutional Animal Care and Use Committee (190901). Cows were milked twice daily (0430 and 1530) in a milking parlor equipped with automatic take-offs and milk meters. Milk weights were recorded after each milking.

All cows were fed a Lys-adequate and Met-adequate basal diet throughout the trial (Table 2). The basal diet was fed as a TMR and prepared 3 times daily (0500, 1300, and 2100 h) by weighing each ingredient and mixing them in a mobile paddle mixer (Super Data Ranger, Calan Inc., Northwood, NH). Cows were fed *ad libitum* for feed intake, but with minimal orts (2 to 4%). Samples of TMR and orts were collected daily and composited individually by cow for each period for determination of dry matter (DM) and dry matter intake (DMI) calculations.

Feed Sampling and Analysis

Samples of corn silage and haycrop silage were collected daily for DM analysis. AminoMax Berga Fat-100, hay, and the mineral mix were sampled once per week. The other grains were sampled by Poulin Grain and sent to the UNH Fairchild Dairy Teaching and Research Center with each load of grain. All samples were freeze-dried (Labconco Model 5, Kansas City, MO) for 48 h following collection. Samples were stored in glass jars until the

completion of the study and were ground to pass through a 1-mm screen using a Wiley Mill (Thomas Scientific, Swedesboro, NJ). Composites were made of each feedstuff for analysis of DM, CP, NDF, ADF, ADI-CP, NSC, Ca, P, Mg, K, Na, Fe, Zn, Cu, Mn, Mo, S, (DHI Forage Testing Laboratory, Ithaca, NY) and AA content (Experimental Station Chemical Laboratories, University of Missouri-Columbia, Columbia, MO).

Blood Sampling and Analysis

Blood samples were collected from each cow on the last 3 days of the covariate period and the last 3 days of the experimental periods. Each day, 4 blood samples were collected from the coccygeal vein or jugular vein at 2-h intervals, starting at 0700 h. Blood was collected in 10-mL vacutainer tubes (Monoject, Mansfield, MA) containing 15% K3EDTA. The tubes were placed immediately in a Chameleon Cooler, centrifuged within 15 min at 1,200 × g for 20 min at 5°C. A 4-mL aliquot from each sample was placed in a labeled glass test tube containing 1.0 mL of 15% SSA. The tubes were allowed to sit for 10 min in the centrifuge before spinning at 1,200 × g for 20 min at 5°C. A 0.45 mL aliquot of deproteinized plasma was removed and placed into 1.8-mL cryovials and stored at -80°C for plasma AA analysis (Experimental Station Chemical Laboratories, University of Missouri-Columbia, Columbia, MO).

Milk Sampling and Analysis

Milk samples were obtained from each cow during the a.m. and p.m. milking on the last 3 days of the covariate period and the last 3 days of each of the experimental periods. Samples were preserved with 2-bromo-2-nitropropane-1, 3-diol (1 tablet per 40 mL of milk) and refrigerated until composited by milk weight and sample date. Samples were analyzed for true protein, fat, SCC, and MUN (Dairy One Milk Laboratories, Ithaca, NY) by using a Foss MilkoScan 4000 infrared analyzer (Foss Electric, Hillerød, Denmark).

Statistical Methods

The RSTUDENT Procedure of SAS 9.2 (2010) was used to determine outlier cows. An observation greater than 2.0 standard deviations from the mean was considered an outlier. Outliers were determined within level and cow to prevent lower or higher overall values from being removed from the dataset. The variables used for outlier analysis were Lys (µ*M*), total AA-Lys (µ*M*), and milk yield and milk protein percent. Outliers were reviewed and removed for reasons such as issues with the pump or cows off feed due to sickness. All data from cows designated as outliers was removed from both the milk and plasma data sets. Covariate data was used in the statistical analysis and the data reviewed to ensure there was no carryover of treatment when cows transitioned from the infusion of the RP-Lys treatment to the control.

To determine if day and day × treatment interaction was significant, plasma Lys concentrations, (µ*M*), total AA-Lys (µ*M*) concentrations, milk yield, and milk protein content were used with PROC MIXED and the REPEATED procedure of SAS 9.4 (2010) according to the following model:

$$Y_{ijklm} = \mu + L_i + P_j + B_k + D_l + LD_{il} + KC_{ilm} + E_{ijklm}$$

Where:

Y_{ijkl} = is the dependent variable

μ = overall mean

L_i = is the fixed effect of the i^{th} treatment; i = 1…4

P_j =is the fixed effect of the j^{th} period; j =1…4

B_k = is the fixed effect of the k^{th} block; k =1…2

D_l = is the fixed effect of the l^{th} day; l = 1…3

LD_{il} = is the fixed effect of the interaction between the i^{th} treatment and the l^{th} day

K = is the regression coefficient of the covariate C

C_{ilm} = is the value of the covariate variable for the m^{th} cow within the l^{th} day of the i^{th} treatment, l = 1 …4

E_{ijklm} = is the random residual ~N $(0,\sigma_e^2)$

The effect of day and day x treatment interaction was established using the random effect of cow(block) as the error term in this model. Degrees of freedom was calculated using the Kenward-Roger option of MIXED procedure (SAS, 2010). Significance was noted at $P \leq 0.05$. Day and day × treatment level interactions were not significant, therefore the means for plasma AA concentrations and milk parameters for the 3 days were calculated and the day and day × treatment were dropped from the model. The data was weighted for any missing values. The random effect of cow(block) was used as the error term for the effect of treatment level. Least square means were determined for treatment and treatment means were separated. Significant level effects were noted at $P \leq 0.05$ and trends were noted $P > 0.05$ to $P < 0.10$.

Using the slope ratio assay (Finney, 1978), the least square means generated from the MIXED procedure were subjected to the PROC REG procedure to generate the linear regression variables and r^2.

Results & Discussion

The flow rate for the pump averages 43 +/- 1 rpm with a flow rate of 161 +/- 8 mL/hr. Cow 915 and Cow 962 of the covariate period exhibit extremely high Lys concentrations on Day 2 and Day 3 respectively and are determined as outliers by the RSTUDENT analysis. For the II HPO treatments, Cow 1020 exhibits a low Lys concentration on Day 2, while Cow 888 exhibits a high Lys concentration on Day 3. For the IL treatment Cow 888 exhibits a low Lys concentration. All of these values fall outside the 2.0 standard deviation range. The covariate

data used in the statistical analysis is also used to ensure that the plasma AA values of the cows returned to their baselines for the control treatment when cows were treated with a RP-Lys supplement or infusion immediately prior to receiving the control treatment. The average DIM of the cows increased from 141 to 181 throughout the duration of the trial.

Tables 3 and 4 contain the feed component and AA analysis respectively, of the individual feed ingredients. These, in conjunction with Table 5, which contains the AMTS diet evaluation, indicate that the diet is not energy deficient, since the NE_L allowable milk or ME allowable milk is predicted to be slightly higher when compared to the MP allowable milk. The Lys:Met ratio of the AMTS evaluation is 2.46:1, which indicates that the diet may be slightly Lys deficient. The CP of the diet is 15.0%, which is lower than the value initially formulated at the beginning of the trial. This is possibly due to the corn silage and haylage being lower in CP than expected.

Table 6 contains DMI, milk yield, and milk composition values. There are no effects of treatment on DMI, milk yield, milk fat composition and yield, milk protein composition and yield, lactose composition and yield, total solids composition and yield, linear SCC score, MUN, 4% FCM, ECM, and milk yield/DMI.

Table 8 presents the plasma AA concentrations. There are significant differences in treatments for His ($P = 0.04$), Lys ($P < 0.0001$) and Thr ($P = 0.005$), with the infusion treatment being significantly lower for His and Thr compared to the other three treatments. The infusion treatment is significantly higher in Lys concentration than the other three treatments, and there are no differences between the II HPO and IL treatments, though both are significantly higher than the control. There is a trend ($P = 0.09$) for Arg, ($P = 0.08$) for Leu, Val, Asn, Asp, Tau, and TUCAA, and ($P = 0.07$) for Orn, Tyr, and TSAA. Plasma metabolites (µM) are reported in Table

9. Ethanolamine is significantly higher for the IL treatment compared to the other three treatments. There is no difference in 3-Methylhistidine among the treatments, indicating that although the diet is low in CP, there is no evidence of muscle degradation.

Lysine as a percentage of total AA minus Lys is used to calculate the bioavailability of Lys presented in Table 10. The bioavailability for the II HPO Prototype is 35.5 ± 1.7 and for the IL Prototype is 49.0 ± 3.0. The statistical summary is presented in Table 11. Figure 1 also provides the equations used to determine the amount of Lys provided by a product at a given level of plasma Lys as a % of (TAA-Lys). Table 11 indicates that the metabolizable Lys provided by the Prototype II HPO treatment is 226.3 g/kg, with a 35.5% bioavailability, while that of the Prototype IL is 310.5 g/kg, with a 49.0% bioavailability.

Although the corn silage was lower in CP than expected, the diet was still energy efficient due to an intentional overcalculation in the initial formulation. The lack of variation in average milk yield and compositions indicates that the supplements are relatively equally beneficial, with none becoming detrimental to the production of the cows.

Conclusions

The Lys infusion treatment yields the greatest Lys concentration, while the Prototype treatments both yield slightly lower Lys concentrations. The Prototype II HPO treatment provides less metabolizable Lys than the Prototype IL treatment, although they are relatively similar in bioavailability.

The high bioavailabilities yielded by these RP-Lys products further support the hypothesis that supplementing the diets of dairy cows, specifically those high in corn and corn

byproducts, with encapsulated forms of this essential AA, can serve as an economically effective method for reaching the requirements of this limiting AA, without exceeding them.

The PDR method is successful in determining the bioavailabilities of the RP-Lys supplements. The PDR method appears more effective than the AUC method, since the AUC method involves amounts of Lys higher than would normally be fed. To further investigate which method is a more reliable means of measuring bioavailability, this experiment could be repeated with a different essential amino acid.

Table 1. Calculations for the amounts of RP-AA products to feed.

Kessent 2M is 75.0 % Met 12 g/d Met/0.75 = 16 g/d Kessent M2
Lysine Hydrochloride is 80% Lys 60 g/d Lys/0.80 = 75 g/d Lys-HCl
Prototype IL is 79.2 % Lys-HCl 79.2 x 0.80 = 63.36 % Lysine 60 g/d Lys/.6336 = 94.7 g/d Prototype IL
Prototype II HPO is 79.7 % Lys-HCl 79.7 x 0.80 = 63.76 % Lysine 60 g/d Lys/.6376 = 94.1 g/d Prototype II HPO

Table 2. Ingredient composition of basal Lys-adequate diet.

Ingredient	% DM
Corn silage, mature	24.05
Mixed mostly grass silage, mid-maturity	21.23
Steam flaked corn	4.21
Corn meal	15.97
Beet pulp	9.97
Molasses, sugar cane	1.16
Distillers grains with solubles	0.73
Soybean meal, solvent extracted	6.46
Canola meal, solvent	2.16
SoyPlus	7.54
Urea	0.12
BergaFat-100	3.06
Kessent M	0.09
Mineral/vitamin mix	3.24

Table 3. Chemical composition of feedstuffs (% DM unless otherwise noted)

	Corn Silage	Haycrop Silage	Soy Plus	Canola meal	Soybean meal	Steam Flaked Corn	Corn Meal	Corn Distillers Grains	Beet Pulp	Molasses	Urea	Minerals	Nutra Core
DM	32.7	30.7	90.9	93.8	89.8	86.8	89.3	90.5	91.7	78.8	99.6	94.6	99.5
CP	6.7	12.1	45.6	43.0	50.6	7.7	6.9	32.3	9.9	7.6	273.0	0.1	-
aNDFom	38.0	56.5	20.8	24.4	13.0	6.9	7.1	39.7	30.9	-	-	-	-
ADF	23.7	38.6	12.4	21.5	9.3	2.3	2.4	15.5	28.1	-	-	-	-
NDF-CP	0.6	2.3	9.5	7.0	6.4	0.9	0.7	5.8	5.1	-	-	-	-
ADF-CP	0.4	1.4	3.3	4.5	5.9	0.4	0.6	2.7	3.4	-	-	-	-
Lignin	2.5	6.1	2.8	10.1	1.2	0.5	0.5	5.9	6.5	-	-	-	-
NFC	47.3	16.4	20.9	21.8	27.6	81.5	81.5	6.8	47.2	-	-	-	-
Starch	41.1	2.1	1.6	2.5	2.1	75.6	80.5	2.3	1.0	-	-	-	-
Ether extract	4.40	5.39	6.19	4.11	1.80	2.88	3.30	14.51	1.38	1.2	-	-	97.2
Ash	3.62	9.61	6.52	6.69	6.94	1.09	1.22	6.74	10.55	16.85	-	-	-
Ca	0.18	0.56	0.36	0.64	0.33	0.01	0.02	0.03	1.07	1.04	-	18.17	-
P	0.27	0.34	0.72	1.06	0.74	0.21	0.26	1.16	0.12	0.16	-	1.12	-
Mg	0.09	0.29	0.31	0.56	0.30	0.08	0.09	0.35	0.27	0.44	-	4.68	-
K	1.06	2.67	2.35	1.09	2.42	0.28	0.34	1.36	0.56	4.42	-	0.10	-
Na	0.009	0.063	0.028	0.074	0.011	0.008	0.006	0.116	0.049	0.168	-	13.470	-
Cl ion	0.17	0.90	0.03	0.02	0.02	0.06	0.08	0.20	0.01	-	-	7.73	-
Fe (ppm)	239	540	112	130	92	53	34	110	1,140	160	-	1,970	-
Zn (ppm)	26	26	48	60	48	17	25	70	28	16	-	1,000	-
Cu (ppm)	4	8	15	6	14	2	2	6	8	25	-	169	-
Mn (ppm)	16	53	35	58	32	4	5	14	76	10	-	778	-
S	0.10	0.22	0.38	0.82	0.43	0.10	0.09	0.68	0.36	1.14	-	0.23	-

Table 4. Amino acid composition of feedstuffs. (% of CP)

	Corn Silage	Haycrop Silage	SoyPlus	Canola meal	Soybean meal	Steam Flaked Corn	Corn Meal	Corn Distillers Grains	Citrus Pulp	Molasses
EAA										
Arginine	1.61	2.63	6.84	6.01	7.09	4.69	4.52	5.14	1.61	0.17
Histidine	1.29	1.45	2.58	2.73	2.66	2.90	2.96	3.09	2.47	0.17
Isoleucine	4.03	4.17	4.79	4.10	4.81	3.59	3.74	4.57	3.44	1.01
Leucine	10.49	7.16	7.65	6.98	7.74	11.32	10.92	12.81	5.48	1.01
Lysine	3.71	4.44	5.95	5.57	6.54	3.59	3.90	3.90	3.12	0.34
Methionine	1.94	1.45	1.28	1.99	1.37	1.93	1.87	2.02	1.50	0.00
Phenylalanine	4.52	4.44	5.14	4.05	5.19	4.83	4.68	5.55	3.33	0.68
Threonine	3.23	3.44	3.79	4.12	3.90	3.59	3.59	4.20	3.98	1.01
Tryptophan	0.50	0.51	1.21	1.01	1.36	0.87	0.83	0.81	0.38	0.34
Valine	5.32	5.44	5.00	5.27	5.00	4.83	4.99	5.85	5.48	1.86
Total	36.65	35.13	44.23	41.84	45.65	42.14	42.01	47.94	30.79	6.59
NEAA										
Alanine	9.52	7.61	4.30	4.32	4.34	7.18	7.02	7.73	4.19	4.22
Aspartic Acid	5.97	6.43	10.95	6.83	11.16	6.90	7.18	6.76	6.34	23.48
Cysteine	1.77	1.00	1.28	2.61	1.41	2.21	2.34	2.18	1.18	0.34
Glutamic Acid	10.81	6.89	17.19	16.94	17.59	17.39	17.32	14.49	8.17	5.41
Glycine	4.52	4.80	4.37	5.04	4.16	4.14	4.21	4.47	3.76	1.01
Ornithine	0.97	0.63	0.12	0.05	0.08	0.00	0.00	0.17	0.11	0.00
Proline	7.75	4.35	5.07	6.19	5.04	8.56	8.58	8.37	4.08	0.68
Serine	2.90	2.63	4.09	3.50	4.26	4.55	4.52	4.81	3.44	1.52
Tyrosine	2.26	0.91	0.23	0.20	0.23	1.38	1.72	0.20	2.69	2.03
Taurine	1.45	1.99	3.53	2.66	3.52	2.76	2.18	4.07	3.12	0.84
Total	47.92	37.24	51.14	48.35	51.80	55.07	55.07	53.25	37.07	39.52
Total AA	84.57	72.37	95.37	90.19	97.45	97.21	97.08	101.18	67.86	46.11

Table 5. AMTS (Ver.4.17.0.1) evaluation of the basal formulated diet[1]

aNDFom, % DM	28.9	CP, % DM	15.0
Forage NDF, % DM	21.1	RDP, % DM	8.7
NFC, % DM	41.4	RUP, % DM	6.3
ME, Mcal/kg DM	2.68		
NE_L, Mcal/kg DM	1.72	MP-bacterial, g/d	1636
EE, % DM	6.8	MP-RUP, g/d	1261
ME required, Mcal/d	67.9	MP-Lys, g/d	199.9
ME supplied, Mcal/d	75.2	MP-Met, g/d	81.2
ME balance, Mcal/d	7.3		
		MP-Lys, % MP	6.90
MP required, g/d	3043	MP-Met, % MP	2.80
MP supplied, g/d	2897		
MP balance, g/d	-146	MP- Arg, % MP	6.30
		MP- His, % MP	2.59
DM intake-actual, kg/d	28.1	MP - Ile, % MP	5.21
DM intake-predicted, kg/d	26.1	MP- Leu, % MP	7.75
		MP- Phe, % MP	4.97
ME allowable milk, kg/d	52.4	MP- Thr, % MP	4.77
MP allowable milk, kg/d	42.5	MP- Trp, % MP	1.36
Actual milk, kg/d	45.7	MP- Val, % MP	5.67

[1] BW 745 kg, DMI = 28.1 kg/d, milk yield = 45.7 kg/d, milk fat content = 3.54%, and milk true protein concentration 3.03%.

Table 6. Milk and dry matter intake for Holstein cows fed a diet supplemented with RP-Lys supplements.

Item	Control	Infusion	Prototype II HP0	Prototype IL	SEM	*P*=
Dry Matter Intake, kg/d	28.1	27.9	27.9	28.2	0.33	0.88
Milk yield, kg/d	45.7	45.4	45.7	44.3	1.09	0.41
Fat, %	3.54	3.50	3.50	3.46	0.100	0.94
Fat yield, kg/d	1.60	1.58	1.59	1.50	0.083	0.59
Protein, %	3.03	3.05	3.03	3.07	0.030	0.42
Protein yield, kg/d	1.38	1.37	1.37	1.34	0.039	0.73
Lactose, %	4.91	4.93	4.92	4.92	0.015	0.91
Lactose yield, kg/d	2.24	2.23	2.25	2.18	0.059	0.55
Total solids, %	12.42	12.43	12.40	12.40	0.092	0.99
Total solids, kg/d	5.64	5.61	5.64	5.44	0.187	0.46
Linear somatic cell score	1.55	1.44	1.61	1.59	0.15	0.81
MUN, mg/dL	11.4	12.1	12.1	12.1	0.41	0.48
Fat Corrected Milk, kg/d[1]	45.7	45.3	45.6	43.5	1.79	0.46
Energy Corrected Milk, kg/d[2]	45.8	45.3	45.6	43.8	1.70	0.47
Milk yield/DMI	1.62	1.62	1.63	1.57	0.044	0.38

[1] 4% FCM = (0.4255 × milk yield, kg) + (16.425 × (milk yield, kg × fat, %))

[2] ECM = [(fat, % × 0.0929) + (protein, % × 0.0563) + (lactose, % × 0.0395) × (milk yield, kg / 0.68605)]

Table 7. Plasma amino acids (μM) for Holstein cows fed a diet supplemented with RP-Lys supplements.

Item	Control	Infusion	Prototype II HP0	Prototype IL	SEM	*P*=
Arginine	85.9	99.4	98.7	96.9	4.71	0.09
Histidine	55.5[a]	51.5[b]	57.2[a]	58.4[a]	1.85	0.04
Isoleucine	127.3	133.8	139.6	135.1	4.35	0.18
Leucine	161.5	166.1	178.1	170.8	5.62	0.08
Lysine	89.6[c]	132.2[a]	109.2[b]	110.3[b]	5.16	<0.0001
Methionine	38.4	36.4	41.1	39.0	1.49	0.19
Phenylalanine	47.0	46.6	49.8	47.8	1.38	0.26
Threonine	126.7[a]	117.9[b]	132.0[a]	129.8[a]	4.94	0.005
Tryptophan	45.0	45.6	47.4	45.7	1.30	0.31
Valine	257.8	262.1	278.6	273.7	7.94	0.08
Alanine	315.3	304.6	307.2	312.8	9.97	0.51
Asparagine	60.1	58.9	62.7	63.4	1.55	0.08
Aspartic Acid	1.08	1.12	1.18	0.93	0.070	0.08
Citrulline	102.0	100.4	106.0	107.2	4.23	0.30
Cystine	21.6	21.4	21.9	22.5	0.55	0.19
Cystathionine/Allocystathionine	2.43	2.16	2.36	2.32	0.084	0.15
Glutamine	206.4	214.4	208.5	210.3	10.45	0.78
Glutamic Acid	43.2	43.7	44.4	43.6	0.85	0.45
Glycine	361.1	342.9	358.7	356.7	12.82	0.11
Homocystine	3.21	3.47	3.53	3.07	0.459	0.55
Ornithine	58.1	65.2	66.9	68.3	4.26	0.07
Proline	100.4	96.4	103.9	102.9	2.22	0.11
Serine	88.7	86.2	91.4	90.3	1.65	0.11
Taurine	54.5	50.0	56.3	56.5	2.67	0.08
Tyrosine	50.0	47.2	53.3	52.5	1.79	0.07
TEAA	1035	1092	1132	1107	49.5	0.12
TNEAA	1468	1438	1489	1493	39.7	0.24
TAA	2504	2531	2621	2601	60.3	0.14
TBCAA	547	562	596	580	29.6	0.13
TUCAA	246	265	272	272	11.4	0.08
TSAA	120	113	125	123	3.4	0.07
TAA-Lys	2414	2399	2512	2492	67.5	0.11

Table 8. Plasma metabolites (μM) for Holstein cows fed a diet supplemented with RP-Lys supplements.

Item	Control	Infusion	Prototype II HP0	Prototype IL	SEM	*P*=
1-Methylhistidine	23.2	24.4	24.3	24.2	0.85	0.20
3-Methylhistidine	3.19	2.90	3.22	3.16	0.19	0.13
α-amino-adipic acid	13.7	16.3	14.2	14.5	1.30	0.28
α-amino-butyric acid	21.4	19.8	21.2	21.4	1.32	0.43
β-alanine	10.7	9.7	10.8	10.5	0.55	0.19
Carnosine	16.1	15.7	16.2	16.0	0.48	0.77
Ethanolamine	1.63[b]	1.97[b]	1.79[b]	3.13[a]	0.40	0.05
γ-amino-butyric acid	2.85	3.64	3.52	3.35	0.54	0.38
Hydroxylysine	2.27	2.89	2.43	2.76	0.41	0.63
Hydroxyproline	10.4	10.2	10.5	10.2	0.35	0.92
Phosphoserine	5.03	5.00	5.06	5.07	0.11	0.93
Sarcosine	33.1	29.7	26.5	30.0	4.1	0.47

Table 9. Bioavailability calculation using changes in plasma Lys % of (TAA-Lys) using Commercial values

Item	Infusion	Prototype II HPO	Prototype IL
Slope	0.02846	0.01010	0.01394
Bioavailability of RP-Lys[1]	-	35.5 ± 1.7	49.0 ± 3.0

[1]Calculated as [(slope of RP-Lys /slope Infusion) *100].

Table 10. Statistical summary of trial results using changes in plasma Lys % of (TAA-Lys) using Commercial values

	Maximum value							
	Mean	SE	Slope	SE	Intercept	SE	R^2	RMSE
Infusion	5.347	0.1493	0.02846[1]	0.0007	3.634	0.028	0.99	0.0485
Prototype II HPO	4.246	0.1493	0.01010[1]	0.0005	3.634	0.023	0.99	0.0406
Prototype IL	4.386	0.1493	0.01394[1]	0.0008	3.634	0.029	0.98	0.0529

[1] Slope is different from zero $P < 0.05$.

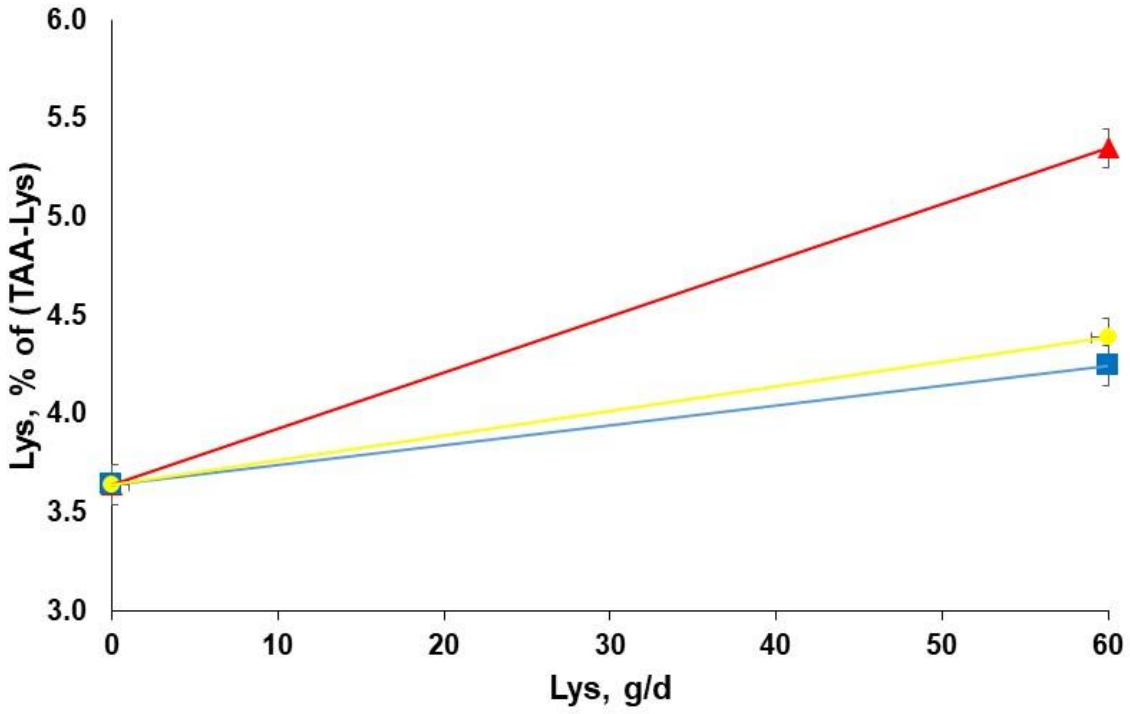

Figure 1. The relationship between Infusion (▲), IL (●), and II HPO (■) in lactating dairy cows using commercial Lys content. Infusion: Y = 3.63 + 0.0285x; slope SE = 0.001, intercept SE = 0.03, r^2 = 0.99. II HPO = 3.63 + 0.0101x; slope SE = 0.001, intercept SE = 0.02, r^2 = 0.99; relative bioavailability = (0.0101 ÷ 0.0285) × 100 = 35.5. IL = 3.63 + 0.0139x; slope SE = 0.001, intercept SE = 0.03, r^2 = 0.98; relative bioavailability = (0.0139 ÷ 0.0285) × 100 = 49.0.

Table 11. Metabolizable lysine for the RP-Lys supplements

	Lys, %	Bioavailability, %	Metabolizable Lys, g/kg
Prototype II HP0	63.76	35.5	226.3
Prototype 1L	63.36	49.0	310.5

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