Universidade Federal de Minas Gerais

Rumen-protected lysine for dairy cows: stability, bioavailability and lactation performance

Victor Marco Rocha Malacco

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Versão final

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ATA DE DEFESA DE TESE DE VICTOR MARCO ROCHA MALACCO

Às 09:00min horas do dia 13 de dezembro de 2019, reuniu-se, na Escola de Veterinária da UFMG a Comissão Examinadora de Tese, indicada pelo Colegiado em reunião no dia 10/10/2019, para julgar, em exame final, a defesa da tese intitulada: Rumen-protected lysine for dairy cattle: stability, bioavailability and lactation performance, como requisito final para a obtenção do Grau de **Doutor em Zootecnia área de concentração Produção de Ruminantes.**

Abrindo a sessão, o Presidente da Comissão, Prof. Ronaldo Braga Reis, após dar a conhecer aos presentes o teor das Normas Regulamentares da Defesa de Tese, passou a palavra ao (a) candidato (a), para apresentação de seu trabalho. Seguiu-se a argüição pelos examinadores, com a respectiva defesa do candidato (a). Logo após, a Comissão se reuniu, sem a presença do candidato e do público, para julgamento da tese, tendo sido atribuídas as seguintes indicações:

	Aprovada	Reprovada
Prof.(a)/Dr.(a) Ronaldo Braga Reis	X	
Prof.(a)/Dr.(a) Hélton Mattana Saturnino	X	
Prof.(a)/Dr.(a) MARINA DE ARRUDA CAMARGO DANES	X	
Prof.(a)/Dr.(a) Maria L. MARIUS NEVES PEREIRA	X	
Prof.(a)/Dr.(a) SANDRA GESTEIRA COELHO	X	

Pelas indicações, o (a) candidato (a) foi considerado (a): [X] Aprovado (a)

[ ] Reprovado (a)

Para concluir o Doutorado, o(a) candidato(a) deverá entregar 10 volumes encadernados da versão final da tese acatando, se houver, as modificações sugeridas pela banca, e a comprovação de submissão de pelo menos um artigo científico em periódico recomendado pelo Colegiado dos Cursos. Para tanto terá o prazo máximo de 60 dias a contar da data defesa.

O resultado final, foi comunicado publicamente ao (a) candidato (a) pelo Presidente da Comissão. Nada mais havendo a tratar, o Presidente encerrou a reunião e lavrou a presente ata, que será assinada por todos os membros participantes da Comissão Examinadora e encaminhada juntamente com um exemplar da tese apresentada para defesa.

Belo Horizonte, 13 de Dezembro de 2019.

Assinatura dos membros da banca:

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“A colheita é comum, mas o capinar é sozinho…”

João Guimarães Rosa, 1956.

“You gain strength, courage, and confidence by every experience in which you really stop to look fear in the face. You are able to say to yourself, 'I lived through this horror. I can take the next thing that comes along.”

Eleanor Roosevelt

DEDICATÓRIA a minha família.

SUMMARY

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LIST OF ABBREVIATIONS

AA – Amino acid
AASS - Aminoadipic semialdehyde synthase
ADF – Acid detergent fiber
ATCC - American Type Culture Collection
AUC - Area under curve
BCS – Body condition score
BW- Body weight
$BW^{0.75}$ – Metabolic body weight
CON – Control
CP - Crude protein
CR – Clearance rate
CV – Coefficient of variation
DIM – Days in milk
DM – Dry matter
DMI – Dry matter intake
EAA – Essential amino acids
ECM – Energy correct milk
FA - Fatty acids
FCM – Fat correct milk
IVGTT – Intra venous glucose tolerance test
LKR - Lysine ketoglutarate reductase
Lys – Lysine
MCP – Microbial crude protein
MDBK - Madin-Darby bovine kidney epithelial
Met – Methionine
MP – Metabolizable protein
MUN - Milk urea nitrogen
N – Nitrogen
NUE – Nitrogen use efficiency
RDP – Rumen degradable protein
RP – Rumen protected
RP Lys - Rumen protected Lys
RUP – Rumen undegradable protein
SCC – Somatic cell count
SD – Standard deviation
SDH - saccharopine dehydrogenase
SI – Small intestine
TAA – Total amino acids
TMR – Total mixed ratio

RESUMO

A principal hipótese explorada nesta tese é que o valor da lisina protegida da degradação ruminal (**RP-Lis**) é função de sua estabilidade quando pré-misturada em alimentos, inércia no rúmen, biodisponibilidade pós-ruminal e dos potenciais impactos nos parâmetros de produção quando fornecida na dieta de vacas em lactação. A segunda hipótese testada foi que a lisina e o suprimento de energia atuam na regulação de genes importantes para o catabolismo da lisina em bovinos. Para testar essas hipóteses, foram realizados quatro estudos. Os estudos avaliaram a estabilidade, a biodisponibilidade do produto e o desempenho de vacas leiteiras em lactação alimentadas com dieta a base de milho, deficiente em Lis e suplementadas com RP-Lis e determinaram os efeitos dos níveis extracelulares de Lis e do suprimento de energia na expressão do mRNA da enzima aminoadípico semialdeído sintase (**AASS**) como um indicador específico para o catabolismo de Lis. No geral, a associação das práticas de armazenamento antes da suplementação com RP-Lis e a baixa biodisponibilidade observada no estudo de biodisponibilidade podem resultar em menor quantidade de Lis digestível no intestino delgado do que o previsto, o que levaria a ausência de resposta positiva a suplementação nas vacas em lactação. Além disso, vários aminoácidos podem ser limitantes quando a maior parte da proteína da dieta é derivada do milho e seus subprodutos, o que também poderia atenuar o aumento esperado da Lis plasmática e a resposta em produção e componentes do leite. O aumento da Lis no meio de cultura e também o fornecimento de uma fonte de energia adicional não resultou em alterações na expressão da AASS e confirma dados anteriores de estudos *in vivo* com aumento da oferta de lisina pós-ruminal. Juntas, as informações apresentadas nesta tese destacam a importância de se manter a integridade do sistema de proteção da lisina nos produtos em todas as etapas, armazenamento, alimentação e no rumen, o que resultará na correta quantidade de lisina livre pós-ruminal.

Palavras-chave: aminoácidos, proteína, vacas em lactação.

ABSTRACT

The guiding hypothesis explored in this dissertation is that the value of rumen-protected lysine (**RP-Lys**) is a function of its stability when premixed in feeds, inertness in the rumen, postruminal bioavailability, and impact on production parameters when fed to lactating dairy cows. A second hypothesis tested was that lysine and energy supply act to regulate genes that are critical for lysine catabolism in bovine. To test these hypotheses, four related studies were performed. The studies evaluated the rumen-protected Lys product stability, bioavailability and lactating dairy cow's performance due to its use as a supplement in Lys deficient corn-based diets and determined the effects of extracellular Lys levels and energy supply on aminoadipate-semialdehyde synthase (**AASS**) mRNA expression as an indicator of a gene specific to Lys catabolism. Overall, summarizing all those studies related to the RP-Lys we conclude that association of the storage practices before feeding RP-Lys and the low bioavailability observed in the bioavailability trial might result in a lower delivery than predicted for digestible Lys in the small intestine resulting in a lack of response in the lactation trial. In addition, multiple AA may have been limiting when most of the dietary protein was derived from corn products which could also dampen the expected increase in plasma Lys and increase of milk yield and milk components. The increase in the Lys in the media and providing an additional energy source did not translate in alterations in the AASS expression and confirms data from *in vivo* studies with increased postruminal lysine supply. Taken together, the information presented in this thesis highlights the importance of maintaining the integrity of the protection technology for RP-Lys through all sequential segments leading to postruminal presence of free lysine.

Keywords: amino acids, protein, dairy cows.

CHAPTER I– INTRODUCTION

Ration formulation that targets the maximal N efficiency diets for dairy cattle is a desirable objective for achieving optimal nitrogen utilization and improved dairy system profitability given that protein typically represents about 40% of total cost of the diet for lactating dairy cows (St-Pierre and Sylvester, 2005). Furthermore, reductions in nitrogen (**N**) intake and N excretion have environmental benefits as livestock systems impact air and water quality and excess N can contribute negatively to eutrophication and soil acidity (Castillo et al., 2000).

Conversely, several nutrition models indicate that low crude protein (**CP**) diets, especially corn-based diets, commonly result inadequate postruminal flow of lysine (**Lys**), and contribution to metabolizable protein, to the small intestine. Because lactating dairy cows have a high need for protein as a source of amino acids, and supplementation with the optimal amino acid profile in the diet may reduce nitrogen excretion while also not affecting production, it is critical to develop feeding strategies that accomplish both reduced CP intake and optimal postruminal AA supply. Based on these goals the recommended level for Lys and Met in metabolizable protein range from 2.2% to 2.4% for Met and 6.6 to 7.2% for Lys resulting in Lys to Met ratio in the metabolizable protein (MP) of approximately 3 (NRC, 2001a; Arriola Apelo et al., 2014a). Thus, diets balanced to the established AA requirements offer the opportunity to provide similar or greater amounts of limiting AA in the small intestine.

Lysine is an essential amino acid for dairy cattle. Because the rumen microbes are able to effectively hydrolyze, deaminate and decarboxylate amino acids from the diet the success of postruminal Lys supplementation depends on a protective coating to avoid Lys utilization by rumen microorganisms but permits absorption by the cow (Berthiaume et al., 2000). Increasing the supply of Lys to dairy cows, using rumen protection technologies, is a strategy to increase Lys availability for absorption and potential for use by the lactating mammary gland for protein synthesis. Although the potential value of feeding rumen protected Lys has been recognized for some time (Donkin et al., 1989) there are still only a few studies reported using protected Lys (**RP-Lys**) sources (Robinson, 2010). One of the hallmarks of the studies that have been published for RP-Lys is a small nonsignificant response in milk production and milk protein composition

and yield (Mullins et al., 2013; Giallongo et al., 2016; Weiss, 2019). The lack of strong and positive results due to Lys supplementation may have been due to Lys is first limiting but not sufficiently supplied. This may also be related to the requirement calculation, feeding method and strategy, or Lys supplementation alone resulting in an imbalance/deficiency in another essential AA. Therefore, a better understanding of the observed failures in RP-Lys supplementation should be further investigated.

In addition to the resilience against the rumen environment, RP-Lys technologies must also resist breakdown during the mechanical mixing process with common feedstuffs, the impact of exposure to moist feeds in the diet, and the effects of acidic and basic feeds. A lack of protection from these factors may decrease the Lys delivered from a RP-Lys source. In many cases the characterization of the stability of RP-Lys in feeds and the mechanical mixing process are overlooked while rumen stability is documented. It is important to recognize this potential effect of feed mixing in assessing rumen protection strategies and postruminal delivery of AA to dairy cattle.

The duodenum, commonly referred to as the small intestine, is the primary site for AA absorption in dairy cows, therefore, ensuring that dietary AA are able to reach the small intestine for digestion and absorption is crucial to improving AA nutrition and potentiating a production response. Determining the bioavailability of individual essential amino acids (**EAA**) in the small intestine is difficult. The dairy NRC (2001) uses an empirical equation to predict EAA flows, but such an approach likely misses some of the subtler changes in supply and cannot be used outside of the range of the data used to derive the equations. Plasma amino acid concentrations reflect AA supply and rumen-protected aminoacid supplementation and have been used as an index of dietary AA supply but are subject to fluctuations in intake, supply and physiological status (Weekes et al., 2006). Changes in plasma AA with acute changes in postruminal AA are possible (Weekes et al., 2006) and provide a relative assessment of level of rumen-protection combined with digestibility and absorption across the small intestine. All these steps are crucial to increase the postruminal supply of AA and as a strategy to increase N use efficiency and dairy cattle performance.

The work described in this thesis is an evaluation of a rumen-protected Lys product including test of stability in feeds and in the rumen, relative estimates of post ruminal bioavailability, and impact on production parameters when fed to lactating dairy cows receiving a Lys deficient corn-based diet. In supporting a greater understanding of postabsortive lysine metabolism, the effects of lysine and energy supply were explored in

an in vitro system of bovine cellular metabolism. Taken together the information uncovers important criteria necessary for the full evaluation of RP Lys for lactating dairy cows.

CHAPTER II - LITERATURE REVIEW

2.1. Current protein recommendation for dairy cattle

Dietary crude protein **(CP)** for ruminants and nonruminants is expressed as the nitrogen content of feeds multiplied by a factor of 6.25. However, in balancing diets for ruminants the partitioning of CP to rumen degradable (**RDP**) and rumen undegradable (**RUP**) creates a level of complexity necessary in defining and meeting the CP needs of the animal (NRC 2001). Regardless, many practicing nutritionists in the field are still considering CP in balancing diets (Schwab and Broderick, 2017) despite recognition since the 1990s that requirements for dairy cattle are met through a combination of RUP, RDP, and the dynamics between RDP and ruminal energy available for microbial protein synthesis (NRC, 2001). Optimal feeding strategies combine balances for the amount of N and amino acids that maximize the microbial protein synthesis linked to the right amount of rumen undegradable protein and AA profile that meets the requirements for metabolizable protein by the cow (NRC, 2001a; Schwab and Broderick, 2017).

Meeting the amino acid requirements for dairy cows hinges on optimizing the rumen environment to maximize the synthesis of microbial protein and flow of amino acids from microbial protein to the small intestine (**SI**) and then augmenting with rumen undegradable protein and amino acids to match the post-absorptive requirements of the cow for maintenance, growth, pregnancy, if applicable and lactation. The NRC (2001) model divides dietary protein into RDP, and RUP. Both microbial CP and RUP flowing out the rumen and are digested in the abomasum and small intestine, the AA from which are then absorbed in the SI for host utilization. Thus, conceptually the total supply of MP fulfilling the AA needs of the cow is met from ruminally synthesized microbial CP, RUP and, to a much lesser extent, endogenous CP reaching the SI (Clark et al., 1992; NRC, 2001a). In practice, CP that could be easily measured in the feed, however the contribution of CP to metabolizable protein is dependent of several factors such as total N to fermentable carbohydrate, protein solubility, rumen digestibility and passage rate, and consequently because of this complexity computer models are necessary to estimate

rumen parameters and relationships (Zanton, 2017) and reduction of parameter estimates to measurable chemical and physical attributes in feeds is still a challenge.

Protein requirements of dairy cows have been assessed in dose-response studies (NRC, 2001a). Similar diets are fed to the animals containing increasing amounts of CP and milk production is tracked as the response variable. The point at which in milk volume or milk protein production reaches a plateau with increasing supply of crude protein is identified as adequate, or requirement. The NRC (2001), compared protein milk yield and milk protein yield response to changes in the dietary CP concentration using a data set of 393 means from 82 protein studies. They observed that milk yield increased quadratically as diet CP concentrations increased and the maximum milk production was obtained at 23% of CP. The milk production expected increases is 0.75 kg/d to 1 % of CP increases between 15 to 16 percent of diet CP and increasing dietary CP 1% unit from 19 to 20% would be expected to increase milk yield by 0.35 kg/d.

Increasing dietary crude protein in diets for lactating dairy cattle to support high levels of milk production could increase the N excretion and reduce the nitrogen use efficiency (**NUE**) (Olmos Colmenero and Broderick, 2006). These authors reported an increase in milk yield, with increasing dietary CP concentration up to 165 g/kg of DM, after which point, they observed a decline in milk yield.

In excess, the crude protein is extensively degraded in the rumen exceeding the capacity of rumen microorganism assimilate the amino acid and ammonia, which leads to an increase in ammonia concentration in the rumen. Ammonia can be removed from the ruminal environment, mainly via diffusion, and can subsequently be returned to the rumen or lost as urea through urine, feces and milk (Russell et al., 1991). Overfeeding crude protein often results in a decrease in profit margin due to the high costs of protein supplements and low efficiency of ruminants in using nitrogen (Broderick, 2003; Hristov and Giallongo, 2014).

Cows are inefficient in using N (Hanigan et al., 2018; Vandaele et al., 2019) but it is probably not an inherent limitation of the specie and might be related to the overestimation of the requirements. Ruminants have a nitrogen use efficiency (**NUE**) that is typically 20–35% of intake N. In a metanalytical study Spek et al. (2013) presented NUE for modern lactating dairy cow that averaged 26.1% in North America and 27.4 in Northern Europe. These low efficiency use rates are associated with high CP

supplementation and results in increasing N excretion in urine and feces which may contribute to environmental pollution (Hristov et al., 2011; Powell and Rotz, 2015).

The concentration of N in urine, especially in the form of urea, varies more than N in feces. After excretion, fecal N is relatively stable, but urinary urea N can transform rapidly to ammonium (NH_4+) and can subsequently be lost as NH_3. Ammonium can also nitrify and denitrify, forming NO_3^- and N_2O (the most potent greenhouse gas emitted from agricultural systems) (Edouard et al., 2016). As reducing the environmental footprint of milk production concern (FAO, 2006), especially with the reduction of N pollution, there is a need to increase the efficiency of nutrient utilization so as to minimize nutrient excretion.

The maximum efficiency is not necessarily aligned with the best performance related to cost, animal longevity, efficiency of nutrient use, or with environmental conservation practices. Therefore some degree of deficiency and reduced animal performance may be acceptable in justifying the marginally deficient nutrient supply (Owens et al., 2014).

Chibisa and Mutsvangwa (2013) showed that milk yield decreased by 3 kg per cow per day and milk protein yield decreased by 140 g per cow per day when dairy cows were fed diets containing 15.2% CP compared to 17.3% CP. Lee et al. (2012) observed a reduction in milk yield in dairy cows fed a diet deficient in metabolizable protein compared to a diet adequate in metabolizable protein. Colmenero and Broderick (2006) studied the crude protein content of diets fed to dairy cattle ranging from 13.5 to 19.4 % CP and observed a trend towards decreased DMI of 0.7 kg/d (P = 0.22) and milk production 2 kg (P = 0.1 in a quadratic fashion) with reduced CP feeding.

Owens et al. (2014) in order to examine the relationship between performance and dietary protein concentration, used a dataset from 13 published trials. Data were analyzed by regressing the performance data in relation to dietary CP concentration and tested for significance of linear, quadratic and cubic dietary CP responses including diet as a fixed factor. The authors observed with the equations that decreasing dietary protein from 18% to 17, 16, and 15% of diet DM would be expected to decrease expected daily fat corrected milk (**FCM**) yield by an average of 1.4, 3.0, and 5.0 kg/d below FCM yield with 18% CP. Although the changes identified by the authors were not statistically different in single studies the magnitude of the effect, if consistent would be economically important.

A primary goal for the modern dairy nutritionist is formulating diets that meet the metabolizable protein needs of dairy cows by feeding to match the least amount of protein content possible without causing detrimental effects in milk production and on this way reduce the N excretion. Some of the uncertainty in response to reducing CP in diets may be the result of inaccuracies in predicting rumen metabolism and microbial protein synthesis, in predicting RUP, estimating flow and postruminal digestion or a combination of these factors. Furthermore, because AA supply with reduced CP is close to borderline sufficiency the effects of these inaccuracies may be amplified. Although many diets, when fed at low CP levels, may be deficient in Lys and methionine (NRC, 2001a) because of that overfeeding crude protein (> 17% of the diet) has overcome potential amino acid deficiencies for high producing dairy cows. However, it may be possible to achieve high milk yield and milk protein yield in dairy cows with lower CP diets by supplementation using ruminal protected AA especially for those AA that are limiting for milk production and optimal health. Thus, supplementing rumen protected Lys and Met is a strategy to increase or maintain milk yield when dietary CP content are lower (Armentano et al., 1997; Dinn et al., 1998).

2.2. Current amino acid recommendation for dairy cattle

Amino acids are designated as dietary essential and non-essential depending on the body's ability to synthesize it in adequate quantities to meet tissue needs or not. Nine AA cannot be formed in cellular metabolism and must be absorbed from the gastrointestinal tract (His, Ile, Leu, Lys, Met, Phe, Thr, Trp and Val). Three amino acids (Arg, Cys, Tyr) are denoted as “semi-essential” because they can be synthesized but supplementation improve performance, Arg is synthesized in the urea cycle, or because they can spare other AA requirements since they are used partly to synthesize, Cys and Tyr could spare Met and Phe requirements. The remaining 8 AA are referred to as “non-essential” (Broderick, 2016)

High milk production requires high output of amino acids, which means that the CP intake needs to be increased and/or a supplemented, to improve the AA ratio of the protein that reach the small intestine.

A survey conducted by the Dairy Herd Management show that 81 % of the nutritionists and veterinarians formulate diets to balance for AA content (Dairy Herd Network, 2010), aiming to reduce CP level of the diet. The value of feeding diets with reduced CP levels is recognized as a means to reduce feed costs but also as a means to

reduce N output in the manure (Arriola Apelo et al., 2014a). Thus, rumen protected amino acid supplementation could be used as a strategy to avoid the lost in milk and solids production due to the use of the low protein diets (Broderick et al., 2008).

Initial studies explored the sequence of AA limitations in dairy cows fed with corn-based diets and Lys and methionine were identified as the two main limiting amino acids (Schwab et al., 1976; NRC, 2001a). However, histidine may be the first limiting amino acid when high producing dairy cows are fed with high forage diets due to the low content of rumen undegradable in the diet, the reliance in the microbial protein to supply metabolizable protein and the low level of His in microbial protein (Vanhatalo et al., 1999; Lee et al., 2012). Despite recognizing the potential limitation in His with high forage diets current nutritional standards for dairy cows in the US (NRC, 2001) provide recommendations for methionine 2,5 % of MP and Lys 7.2 % of MP only. Histidine has been shown to limit milk production in lactating cows in some studies, but histidine requirements in the diets have not been defined.

Metabolizable protein, the sum of the microbial protein, protein which escapes degradation in the rumen and endogenous protein secreted into the gastrointestinal tract prior to the duodenum, represents the total protein which is available to the animal for maintenance, growth, fetal growth during gestation, and milk production (NRC, 2001a). Together, RUP and microbial protein have been estimated to represents 50 to 80% of the total protein flow to the small intestine (Bach et al., 2005). The range in this estimate creates an inaccuracy in predicting protein flow from the rumen. However, Pacheco et al. (2012) comparing four rumen sub models predictions with observed values of duodenal flows of EAA concluded that recent commercial models may more accurately predict the flow of EAA to the duodenum. It would appear that additional work is needed to test, refine and implement models that permit greater precision in AA nutrition for dairy cows.

There are two different approaches that have been used to determine the AA requirements of lactating cows. The proportional approach adopted by the (NRC, 2001a) and (INRA, 2007) which includes a broken stick model, a dose-response relationship between the % of individual AA in MP supply and milk protein yield or percentage. The % observed at the breakpoint represents the proportion of this AA in MP supply required to maximize the targeted output (Lapierre et al., 2014). The second is the factorial approach which takes into account the AA needs for each individual function with a defined AA composition and a defined efficiency of transfer. This approach is used by CNCPS (Fox et al., 2004), AMTS and Amino cow, two commercially available models

designed to predict MP and metabolizable (or digested) AA supply with needs using feed input data (Pacheco et al., 2012).

Without quantitative estimates and knowledge of the supply of the amino acids reaching the small intestine coupled with knowledge of the AA requirements of lactating cows the response to AA supplementation might be random and inconsistent (Robinson, 2010; Pacheco et al., 2012). Deficient post-ruminal AA supply, related to the limitation of one or more AA available to tissue, will result in limitations for protein synthesis. According with the limiting AA theory, the most limiting AA dictates the efficiency of utilization of absorbed AA and it could explain the observed performance limitations. When the first limiting AA supply is corrected the performance will be improved until other AA becomes limiting in the synthesis process (Schwab et al., 1992).

Attempts in identify the first single limiting AA for milk protein synthesis or sequentially limiting AA have failed (Cant et al., 2018) leading to the belief that there may be limiting set of EAA that are needed simultaneousness to promote protein synthesis. The fact that milk production could be affected by the same magnitude when different amino acids are removed from duodenal EAA supply or the fact that insulin or glucose could change the milk protein synthesis even when plasma EAA concentrations are reduced tends to support the theory that AA are required as a group (Mackle et al., 2000; Weekes et al., 2006; Toerien et al., 2009; Doelman et al., 2015; Cant et al., 2018).

The optimal amounts of Lys and Met requirements indicated by the NRC (2001) model are 7.2 and 2.4 % of metabolizable protein respectively. Schwab, (2012) presented a compilation of breakpoint estimates for required concentrations of Lys and Met in MP for maximal content and yield of milk protein for the NRC (2001), CPM, and AMTS models using the NRC (2001) database re-evaluated by Schwab et al., (2009) and Whitehouse et al. (2009). The results observed by the authors are different for the updated NRC (2001) version when compared with the first version of the NRC (2001) mostly because of the difference in feed inputs for some of the studies. They also observed differences in the estimative for different models which was expected since the models differ in the approach for predicting supplies of AA.

Knowledge about Lys and methionine requirements in lactating cattle has made significant progress (Schwab et al., 1992; Rulquin et al., 1993) and, by providing recommendations and adequately supplementing the animals, an improvement in performance is observed (Leonardi et al., 2003; Noftsger and St-Pierre, 2003). The same effort must be made in an attempt to establish requirements for all essential amino acids

and thus be able to replace the crude protein as the main reference in the diets formulation (LaPierre et al., 2019). The use of low crude protein diets supplemented with amino acids protected from ruminal degradation seems to be the best economically productive and environmental approach in milk production systems.

Estimations of the optimal supply of individual AA, as defined in Doepel et al (2004), using a meta-analysis approach are presented in Table 1. These authors used studies where AA or proteins were infused postruminally in order to determine the influence of AA supply on the variation of the efficiency of conversion of AA into milk and to estimate requirements for EAA. The results indicate a similar pattern of amino acid MP regardless of the method used to evaluate the data available in the literature.

Table 0-1 Ideal concentrations of Lys and Met in MP for milk protein yield in lactating dairy cows (% metabolizable protein)

Amino acid	Segmented linear model	Logistic model
Arginine	4.8	4.6
Histidine	2.4	2.4
Isoleucine	5.3	5.3
Leucine	9.4	8.9
Lys	7.2	7.2
Methionine	2.5	2.5
Phenylalanine	5.2	5.5
Threonine	5.1	5
Tryptophan	-	-
Valine	6.1	6.5

2.3. Rumen protected amino acid technologies

The great advantage of ruminants, the symbiotic association of rumen bacteria with the animal in the rumen, can be both beneficial with regard to the protein nutrition because of the ability to convert non-protein N into a high-quality protein through microbial protein synthesis but also detrimental due to the degradation of supplemented free amino acids by the rumen microorganisms (Wu and Papas, 1997). Consequently, supplementation in a non-protect form usually results in an AA utilization by the ruminal microorganism and no changes in the metabolizable protein. The initial attempts to supplement unprotected Lys for lactating cows suggested that this could be a viable alternative. Early research (described in Wu and Papas, 1997) evaluated ruminal Lys escape using pulse dose infusion, reported ruminal escape of free Lys that varied from 10 to 29% of the infused dose and suggest that free Lys might represent an option for

enhancing Lys supply. However this approach was later discarded due to the confirmation of the low rumen escape rate especially when the source of Lys was mixed with TMR (Robinson et al. 2004) where ruminal escape rate is only 7.2 % of the free lysine. Consequently, the amount of Lys escaping the rumen relative to the quantity provided in the diet necessitated other approaches to supplementation.

Thus, AA supplementation success depends of the adequate coating protection to avoid the Lys utilization by rumen microorganisms (Berthiaume et al., 2000). The protecting derivate must be then stable in the rumen but release the amino acid in the abomasum/small intestine. The main industrial methods used to protect AA are: 1) the production of AA analogs, which are more stable under ruminal condition; 2) coating with fat, mixtures of fats and proteins, proteins treated with formaldehyde or calcium soaps of long chain fatty acids; 3) encapsulation with polymer compounds resistant to rumen degradation, but which are hydrolyzed in the abomasum (Alves, 2004).

Wu and Papas (1997) defined desirable characteristics of rumen-protected amino acid pellets (Fig 1). This authors also listed the criteria that should be meet for a polymer used to formulate the rumen-stable delivery system: 1. Physiologically inert, non-absorbable, unchanged in excretions in animals such as rats, dogs, cows and steers, 2. Non-mutagenic; 3. Produces no adverse effect following chronic feeding; 4. Thermally stable or non-degradable at temperatures experienced during process and storage; 5. Insoluble in ruminal fluid, but soluble in abomasal fluid; 6. Soluble in common volatile organic solvents for coating applications. The water solubility of Lys is so high that the development of RP-Lys has been a great challenge which to date has favored ruminal protection technologies for Lys that utilize hydrogenated fat and/or minerals. These are advantageous because they are already registered as feed ingredients, regarded as safe and do not require special testing for approval as feed supplements (Watanabe et al., 2006).

A variety of rumen protected products are available in the market with different coated protection forms and amino acid cores. Other factors such as the mechanical mixing process, diet moisture, acidity and the time that RP-Lys product is exposed to feed could decrease the AA retained in the rumen protected products. Several studies have evaluated the stability of the ruminal protected amino acids in the feeding and mixing process, and the bioavailability through the performance response of the cows and also using the plasma amino acid concentrations changes due to supplementation

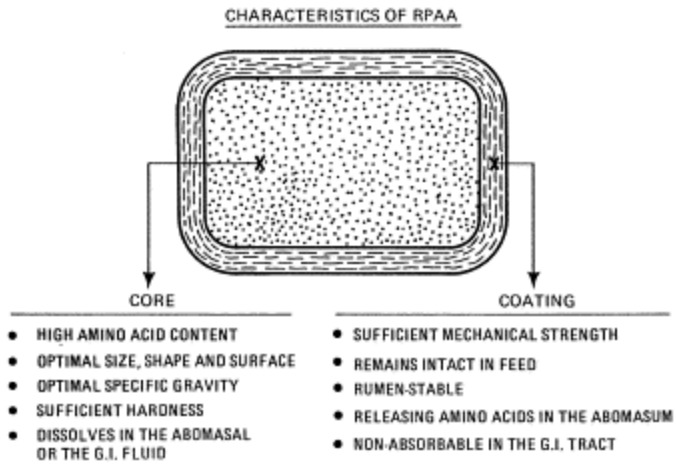

Figure 0-1 Desirable characteristics of rumen-protected amino acid pellets (adapted Wu and Papas, 1997).

As indicated above most of the Lys rumen protected amino acids available in the market are lipid-coated. Studies that evaluated the stability of the Lys coated products exposed to the mixing process concluded that methods of manufacture together with the physical and chemical composition of the diets can affect the amounts of Lys provided from the lipid coated products to the ruminants (Ji et al., 2016; Reiners et al., 2017, 2018). Those studies also found a wide variation in the stability of the products related to the capacity of retain the Lys which clearly impacts the final bioavailability of the product.

Increases in the plasma AA concentrations in response to postruminal delivery of a RP AA have been used to determine the ability of RP-Lys products to be absorbed across the duodenum. Whitehouse et al. (2017) using this principle and the dose-response technique determined the relative bioavailability of 27 products tested in 13 different experiments using the free AA dose technique. There was a wide range (5 to 85%) in the bioavailability between products delivered postruminally at different levels. This high variability observed may reflect the difficulties in creating a coating process that effectively protects Lys from ruminal degradation while allowing consistent release of the encapsulated Lys in the intestine. This inability to consistently provide products that deliver predictable payloads of Lys may help to explain the challenges faced by the industry in effectively developing an RP Lys source and documenting the effects of postruminal Lys supply (Robinson, 2010).

2.4. Postruminal amino acids supplementation

Lysine and Met have been the primary focus of the research on postruminal AA supplementation because those two amino acids are identified as the first limiting in a typical corn-based diets in US. Postruminal infusion of 6 to 12 g/d of Met (Pisulewski et al. 1996) increased milk protein yield and content in a linear fashion. A meta-analysis of 129 RP-Met studies determined that supplementation increased milk protein content and yield and slightly increased milk production (Patton, 2010). A more recent metanalysis (Zanton, 2017) indicated that infusing or supplementing with sources of Met which increased Met in MP resulted in increases in both milk protein and fat yield.

In contrast, postruminal methionine supplementation, when combined with Lys, frequently increases milk production (Lean et al., 2017). Post ruminal infusion of a complete mixture of amino acids increases in milk protein yield, although, when Lys was eliminated from the EAA mixture milk production and milk protein production were similar to saline infusion (Weekes et al., 2006; Lapierre et al., 2009). Guo et al. (2017) investigated responses of milk protein synthesis and mammary AA metabolism to a graded decrease of post ruminal Lys supply used 4 lactating goats in a 4 × 4 Latin square experiment. Treatments included a graded decrease of Lys content in the infusate to 100 (complete), 60, 30, or 0% of Lys in casein. When Lys was removed from the infusate there was a linear decrease in milk yield and a tendency for an increase in milk fat to protein ratio. Milk protein content and yield were linearly decreased by graded Lys deficiency. For the authors these data suggest that a decrease in milk protein yield linked to graded Lys deficiency was mainly a result of the varied physiological status, indicated by the elevated circulating glucagon and glucose, rather than a result of the decreased mammary Lys uptake or depressed signals in the mTOR pathway.

In other studies, when Lys was infused postruminally there are inconsistent effects on milk production and composition (Rulquin et al., 1993; Tucker et al., 2017). Increasing post ruminal Lys supply failed to alter mRNA transcripts of key genes in Lys and protein catabolism in liver and mammary tissue despite the fact that these genes are regulated in other species by lysine supply and there was an increase in alpha amino adipic acid, a breakdown product of Lys, with increasing lysine supply (Tucker et al. 2017). The data suggest that hepatic Lys metabolism in bovine is not responsive to Lys supply at the transcription level, and that the availability of Lys to extrahepatic tissue may be determined by hepatic Lys metabolism.

The determination of Lys requirements together with other essential amino acids are the first step to optimize the nutrition and efficiency of N use by dairy cows. However, the profile of amino acids that reach the mammary gland, and are available for milk protein synthesis, is different from that one which is available to be absorbed in the small intestine. If the ultimately goal is to maximize milk protein synthesis and N use efficiency then the next step to be taken is understand the potential modifications of the AA profile supplied, post absorptive metabolism, and also the portioning of the available AA between mammary gland and other tissue (Bequette and Nelson, 2006).

Overall only a small number of studies have evaluated RP Lys (RP-Lys) supplementation alone without another RP AA. For most there is a small or nonsignificant response in milk production and milk protein composition and yield (Mullins et al., 2013; Giallongo et al., 2016; Weiss, 2019). Robinson (2010), systematically evaluated the literature to determine if duodenal Lys level and RP Lys impacts the performance of dairy cows. Results demonstrate that Lys supplementation decreased DM intake as much as 1.9% when compared with control groups and increased the milk/DM ratio. In the 13 studies that were used for the analysis there were no other differences in milk yield, milk protein production or N efficiency due to Lys feeding (Robinson, 2010). Awawdeh (2016) reported an increase in milk production and protein content when cows were supplemented with RP-Lys and RP-Met compared with cows supplemented only with RP-Met or not supplemented suggesting a co-limitation in Lys and Met.

Several hypotheses could explain the lack of response to RP-Lys feeding especially in diets that were formulated to be lysine deficient. Among them, is the potential that a single level of Lys feeding may create an AA imbalance at the intestine to impair Lys absorption (Robinson et al., 2011). Other explanations include insufficient supply of a colimiting essential amino acid (Robinson, 2010; Mullins et al., 2013; Lee et al., 2019), or increased hepatic catabolism and loss of absorbed Lys to other tissues including the mammary gland (Doepel et al., 2004; Swanepoel et al., 2010; Nichols et al., 2019). Evaluating these hypotheses will require a more detailed understanding of metabolism of AA and the relationship to other nutrients including energy metabolism.

Although Lys and Met have been identified as potentially limiting in corn based diets, histidine is been identified as also colimiting amino acid in the corn based diets fed to dairy cattle in the US and as first limiting for cows feed with diets based on grass silage and a cereal-based supplement (Korhonen et al., 2000). Hadrová et al. (2012) evaluated

the effect of duodenal infusion of histidine on milk yield, milk composition, and plasma amino acids in lactating dairy cows, failed to observe any difference for milk production or composition despite of the increase in plasma His concentration.

Most of the beneficial effects of His supplementation have been observed when low protein diets were fed to dairy cows (Kim et al., 1999; Lee et al., 2012) and have led to the working hypothesis (Lee et al. 2012) that low CP diets increase the dependency on the microbial crude protein (**MCP**) to supply to meet MP supply and that MCP may be limiting in His. Despite recognition as one of the top three limiting amino acids for high yielding dairy cows, the benefits of postruminal supplementation of Met Lys and His combined has been small. A more thorough understanding of feeding strategies, evaluation of amino acid sources, and determination of cow requirements are necessary to improve response supplementation and to achieve the goal of improving performance and efficiency of dairy cows N use.

2.5. Postabsorptive Lys metabolism

Once absorbed by the intestine AA are first transported to liver through portal blood. Is well recognized that approximately 1/3 of the EAA amino acid pool that disappears from the lumen of the small intestine will be oxidized by the portal drained viscera, primarily enterocytes and another third by the hepatic tissue (Hanigan et al., 2004; Pacheco et al., 2006; Larsen et al., 2015). Removal of amino acids by the splanchnic tissues (intestine and liver) is not constant for all AA, and the composition of the post-splanchnic AA availability to other tissues is altered relative to absorbed AA profile (Hanigan and Cyriac, 2006). It is recognized that uptake and oxidation of amino acids is a mass action reaction and increases in of AA availability will result in higher oxidation by peripheral tissues and consequently in lower efficiency use (Hanigan et al., 1998). The AA removed from the circulation by intestine and liver can be used for several anabolic processes; for example, synthesis of export proteins (e.g., albumin, peptide hormones, and immune proteins) and constitutive proteins, as well as for catabolic processes such as gluconeogenesis and complete oxidation (Larsen et al., 2015).

Hepatic extraction of Lys is less than 10% of portal drained viscera Lys flux, therefore intestinal oxidation is mainly responsible for reducing the net flux of absorbed Lys to the mammary gland. The gut oxidation of Lys in sheep accounted for 29% of whole body Lys oxidation (Lapierre and Lobley, 2001) and this estimate did not account for oxidation of AA derived from the gut lumen (i.e. during absorption). Van Goudoever et

al. (2000) observed, in pig, a negligible oxidation of systemic (arterial) Lys, although the luminal-derived Lys catabolism represents 30% of whole-body Lys oxidation. These authors also showed that the source of Lys used by these tissues is sensitive to protein status, although Lys is catabolized by the intestines and accounts for 31% of whole-body Lys oxidation in high protein fed piglets, it is strongly suppressed when protein becomes the limiting nutrient. The data would suggest that intestinal lysine supply does not translate to a linear increase in Lys supply to other tissues therefore a linear response in milk production to increased postruminal Lys supply, if possible, would not be expected to be linear.

Liver represents only 2% of the body weight but is highly active and account for 25% of the whole-body oxygen use (Huntington and Reynolds, 1987). The high blood flow through the liver, that can reach 2000 l h^{-1}, and the fact that AA could be extracted in the first or second pass through this organ, results in an clearance capacity of up to two-thirds of circulating AA (Reynolds et al., 1988; Wray-Cahen et al., 1997; Hanigan et al., 2004). The AA clearance by the liver vary widely between AA. Bequette et al. (2003) indicated that although the regulation of hepatic removal and oxidation of AA is still not understood, there must be coordination between the demand by peripheral tissues and hepatic removal of AA which suggests that AA catabolism by liver is a potential point of regulation (Figure 2).

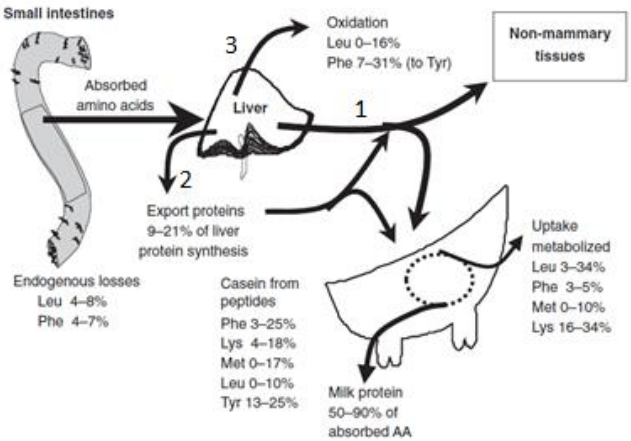

Figure 0-2 Summary of studies in lactating dairy cows and goats where the metabolic fates and partition of amino acids were monitored by tracer kinetics in vivo. There are represented the three options when AA are presented to the liver. 1 - AA may pass directly through and become available to peripheral tissues. 2 - AA may be extracted for synthesis of proteins, constitutive of liver matrix or exported as plasma proteins. 3- AA may be oxidized with the nitrogen potentially lost as urea. (Bequette et al. 2004).

Lys catabolism, that occurs mainly through the saccharopine-dependent pathway (Fig. 3), is coordinate by combined action of Lys ketoglutarate reductase (**LKR**; EC 1.5.1.8) and saccharopine dehydrogenase (**SDH**; EC 1.5.1.9) (Benevenga and Blemings, 2007). These enzymes reside on a bi-functional protein complex, aminoadipic semialdehyde synthase (**AASS)** (Markovitz et al., 1984), present in numerous tissues (Kiess et al., 2008; Gatrell et al., 2013).

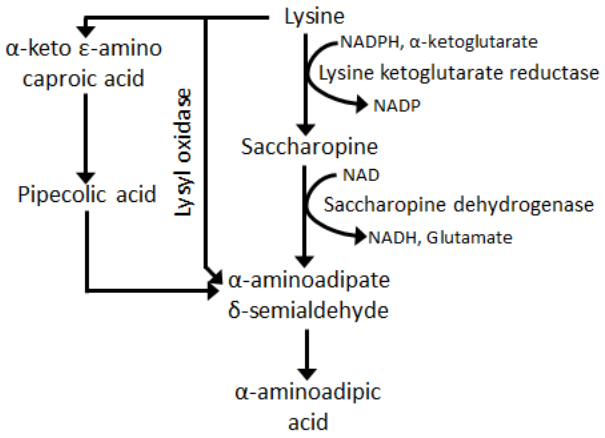

Figure 0-3 Lys catabolism in mammals. Involvement of Lys ketoglutarate reductase and saccharopine dehydrogenase in catabolism of Lys to α-aminoadipic acid constitutes the saccharopine-dependent pathway of Lys catabolism.

In liver Lys is metabolized via the saccharopine-dependent pathway to α-aminoadipic acid. Activities of both enzymes in the AASS protein have been shown to be sensitive to Lys supply (Muramatsu et al., 1984; Blemings et al., 1994) and dietary protein content (Kiess et al., 2008). Increase in the activity of those enzymes could result in a high activity of this complex and high oxidation rate of Lys when this AA is supplemented. Kiess et al., (2008) observed higher LKR activity when mice were fed with 46% casein diet compared with a 21% casein diet. However, the observed increases in AASS mRNA did not translate to an increase in AASS protein abundance (Kiess et al., 2008). Tucker et al., (2017) did not observe a change in AASS due to post abomasal Lys infusion in early lactation dairy cows, although there was an observed increase in plasma α-aminoadipic acid.

The reduction of Lys oxidation by saccharopine-dependent pathway could be an opportunity to increase Lys availability for peripherical tissues including mammary gland. Therefore, the fully understand the regulation of AASS expression and activity

may provide important strategies to reducing postabsorptive Lys catabolism and increasing Lys use for protein synthesis in mammary gland and other tissues.

2.6. Summary and conclusions

Lys is the first limiting amino acid for high producing lactating dairy cows when fed a corn-based diet. There are few sources of proteins that can meet the shortfall of Lys when corn-based diets are fed and protein sources that are available are often expensive and/or are inconsistent on the amount of Lys that is biologically available to the cow.

The use of rumen protected AA is a well-established method to augment the profile of AA needed to maintain high levels of milk and milk protein production and maximizing the N use efficiency. Products that provide rumen protected Lys must be evaluated for both rumen protection against microbial degradation and also evaluated for postruminal delivery of Lys that can be absorbed and utilized by the lactating dairy cow. Milk protein production and plasma amino acid concentrations are response variables that have been used previously to assess the efficacy of rumen protected Lys products in meeting the Lys needs of lactating dairy cows. Matching Lys supply to requirements depends complete knowledge of rumen dynamics and the impact on postruminal Lys supply and the post absorptive Lys metabolism. Identifying processes that are sensitive to altered AA supply may prove beneficial in determining the source of inefficient N utilization.

The guiding hypothesis explored in this dissertation is that value of RP Lys is a function of its stability when premixed in feeds, inertness in the rumen, post ruminal bioavailability, and impact on production parameters when fed to lactating dairy cows. A second hypothesis tested was that lysine and energy supply regulate genes that are critical for lysine catabolism in bovine. Taken together this dissertation explores important criteria necessary for the full evaluation of RP Lys for lactating dairy cows and subsequent use of absorbed lysine in dairy cattle.

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CHAPTER III– ARTICLE I

Evaluation of a novel rumen-protected lysine product on lysine retention after feed and ruminal exposure, bioavailability and effects on lactation performance of dairy cows

2.1. ABSTRACT

Lysine (**Lys**) is the first limiting amino acid (**AA**) when high producing dairy cows are fed corn-based diets. Consequently, rumen protected forms of Lys have been developed for use in diets for lactating dairy cows. Products purported to provide rumen-protected Lys (**RP-Lys**) must be evaluated for both rumen protection against microbial degradation and for postruminal delivery of Lys for absorption and metabolism. The objective of this study was to determine the N retained after feed exposure, ruminal protection, bioavailability and effects of feeding a novel RP-Lys product on feed intake, plasma amino acids, and milk yield and composition. To access the N retention after feed and ruminal exposure, three different batches of a prototype NutraPass RP-Lys products; RP-Lys 1, RP-Lys 2, RP-Lys 3 (Archer Daniels Midland Company, Decatur, IL), and one commercially available RP-Lys product, RP-Lys Control (AjiPro-L, Ajinomoto Animal Nutrition North America, Inc., Chicago, IL) were mixed to a concentrate and storage, exposure to corn silage or to TMR and then incubated for 16h in the rumen. Overall, the product retained 67, 78 and 69% of N after ruminal incubation and previous mixing and storage in concentrate or silage and TMR exposure, respectively. To evaluate the lactation performance, corn-based diets were formulated so that the control diet was Lys deficient but adequate in methionine (**Met**), energy and metabolizable protein content following the NRC (2001) requirements. Thirty-six lactating Holstein cows were fed either a control diet (**CON**) with no added RP-Lys, or diets containing 0.3% of RP-Lys (**0.3RP-Lys**), or 0.6% of RP-Lys (**0.6 RP-Lys)** for eight weeks. There were no differences in DMI (26.2 ± 3.3 kg DM, $P = 0.97$), milk yield (37.8 ± 5.5 kg/d; $P = 0.41$) and milk composition. Total essential amino acids (**EAA**) in plasma did not differ (916,

893, 862 ± 44.5 μM, for CON, 0.3RP-Lys, and 0.6 RP-Lys, respectively). Plasma histidine (**His**) was reduced in a linearly by Lys feeding *(P* < 0.05, with 45.68, 41.28, 32.80 ± 3.661 μM, for CON, 0.3RP-Lys and 0.6 RP-Lys, respectively). Plasma Lys and Met, as a % of EAA, increased linearly *(P* < 0.05) with RP-Lys supplementation. There was a trend ($P \leq 0.10$) for cows fed 0.6 RP-Lys to have higher plasma Lys as % EAA than cows fed CON (5.72, 5.94, and 6.46 ± 0.242 % EAA for CON, 0.3 RP-Lys and 0.6 RP-Lys, respectively). Three rumen-cannulated lactation cows were used in a 3 x 3 Latin square design study to assess the bioavailability of the Lys product. HCl-Lys, RP-Lys product and water were administered separately by postruminal infusion and Lys bioavailability accessed by the ratio of area under the complete curve of concentration x time for RP-Lys when compared with free Lys (HCl-Lys) that is 100% absorbed and discounted for the area under the curve for water infusion. RP-Lys was 22.7 % available. Cows supplemented with RP-Lys had similar DM intake and milk yield, despite elevated plasma Lys concentration. Rumen protection of RP-Lys and postruminal delivery of Lys from the RP-Lys product are confirmed despite the lack of effect on milk production or composition. The product was effective in delivering Lys postruminally.

Keywords: amino acids, protein, dairy cows.

2.2. INTRODUCTION

The ideal protein provides the perfect balance of biologically available amino acids relative to the physiological needs of the animal (Overton and Chase, 1999). The act of reducing this to practice in order to meet the needs of high producing dairy cows is complex and involves accurately predicting the contributions from rumen microbial protein and rumen undegraded protein to the absorbable amino acid pool in the small intestine and the postabsorptive tissue needs for amino acids. Greater precision in meeting the amino acids needs of the lactating dairy cows may increase productivity, as milk output or milk components output, could reduce N excretion to the environment or result in a combination of both factors (Castillo et al., 2000; Godden et al., 2001). Improved knowledge of protein and AA profiles of conventional feed ingredients, combined with an understanding of the value of AA supplements in current feeding systems, is necessary

in order to advance feeding management strategies toward greater precision in meeting amino requirements for lactating cows.

In many parts of US, rations for dairy cows contain corn and corn-based products. Corn milling by-products are an economical source of NE_L and MP, but can lead to limitations in Lys supply when these ingredients are included at high amounts in dairy rations (Mullins et al., 2013; Weiss, 2019) due to the predominance of zein protein, a poor source of Lys (Coleman and Larkins, 1999). Thus, dairy nutritionists have sought to formulate rations using rumen-protected sources of Lys in order to meet Lys requirements for high producing cows. Despite recognition that AA, particularly Lys, are likely limiting, there is a scarcity of research demonstrating consistent responses to Lys supplementation on milk and milk components (Robinson et al., 2010). Often times a small or nonsignificant response in milk production and milk protein composition and yield are observed with RP-Lys supplementation (Mullins et al., 2013; Giallongo et al., 2016; Weiss, 2019).

The success of Lys supplementation in RP form success is dependent on adequacy of protection against Lys breakdown by rumen microorganisms and availability for absorption into blood by the cow (Berthiaume et al., 2000). Amino acid bioavailability is the definition of the percentage of the amino acid which was digested and absorbed into the blood is available to be used by the tissues (ARC, 1981; Danfær and Fernández, 1999). Methods commonly used in the feed industry to protect AA from rumen degradation include: 1) feeding AA analogs, which are stable under ruminal conditions and are available to the cow; 2) coating AA with fat, mixtures of fats and proteins, proteins or calcium soaps of long chain fatty acids to protect them from attack by rumen microbes; 3) encapsulation with polymer compounds resistant to rumen degradation but are dissolved at the pH of the abomasum (Alves, 2004). Regardless of the method of protection all RP-AA should have the following attributes: a) ruminal stability, b) intestinal degestibility, and c) high bioavailability. Consequently, newly developed products purported to provide RP-Lys must be evaluated for both rumen protection against microbial degradation and for postruminal delivery of Lys that can be absorbed and utilized by the lactating dairy cow.

Hydrogenated fatty acids (**FA**) have been used as a coating strategy to deliver AA to the intestine. These coatings are effective partly because highly saturated FA are not subject to the biohydrogenation process in the rumen, remain insoluble at normal rumen pH, and can be digested readily in the intestine this releasing their AA payload. Moreover,

fats are included on 'generally regarded as safe' list and don't require special approval for safety considerations (Watanabe et al., 2006). However, fats, like all protective AA coatings may be subject to loss of effectiveness as a result of moisture, acidity and storage in the diet (Ji et al., 2016; Reiners et al., 2017). Therefore, to be considered a viable postruminal delivery strategy for AA the protection material and the delivery of AA must be validated in combination. Thus, the objectives of this study were (1) determine the stability accessed by the N retention in the RP-Lys products after ruminal incubation when previously mixed and storage in concentrate or exposed to the corn-silage or TMR, (2) determine the effects of feeding the RP-Lys product on lactation performance (3) determine the postruminal bioavailability of the RP-Lys product. We hypothesized that providing additional Lys through the feeding of RP-Lys source would increase milk yield and protein in lactating dairy cattle.

2.3. MATERIAL AND METHODS

The experiments were conducted at the Purdue University Dairy Research and Education Center following animal care and handling protocols that were approved by the Purdue University Animal Care and Use Committee.

2.3.1. Experiment I

The first experiment assessed the stability determined by the N retention in the RP-Lys product after 16h of ruminal incubation when previously mixed with concentrate and storage, or exposure to silage or TMR.

Three different batches of a prototype NutraPass RP-Lys products; RP-Lys 1, RP-Lys 2, RP-Lys 3 (Archer Daniels Midland Company, Decatur, IL), and one commercially available RP-Lys product, RP-Lys Control (AjiPro-L, Ajinomoto Animal Nutrition North America, Inc., Chicago, IL), were evaluated. Each combination was intended to assess the stability of the protected for RP-Lys product in delivery scenarios commonly observed in dairy feeding operations.

The RP-Lys Control and RP-Lys 1, RP-Lys 2, RP-Lys 3, were mixed with ground concentrate (Table 1) to assess the effect of premixing and storage on RP-Lys stability. The concentrate was ground through a 1 mm mesh screen and mixed with each RP-Lys product in a ratio of 10% of RP-Lys to 90% of concentrate by weight. The resulting mixes

were stored, in duplicate, at room temperature (22ºC), for 14 and 28 days relative to the end of the test. Samples designated 'Time 0' were RP-Lys products that were not mixed with the supplement. At the conclusion of the storage period the RP-Lys products were recovered from the grain component of the mixes by dry sieving through a 1 mm wire mesh screen. Five grams of the recovered RP-Lys products were weighed and placed, in duplicate, into 10-cm × 20-cm Dacron bags, 50-µm porosity (Ankom Technology, Macedon, NY) and assess for rumen protection as described below.

To assess stability of RP-Lys in TMR and corn silage eight grams of each RP-Lys product was weighed into 10-cm × 20-cm dacron bags, 50-µm porosity (Ankom Technology, Macedon, NY). Duplicate bags containing RP-Lys were mixed with 20 kg of corn silage (32 % of DM) or a total mixed ration (TMR, 51.9 % of DM) and incubate at room temperature (22ºC) in an open top 51.1 L plastic container (61 x 41.9 and 28 cm L x W x H) for 12h or 24h. Samples designated 'Time 0' were RP-Lys product that were not mixed with the silage or TMR. Following incubation in TMR or corn-silage for the designated time the Dacron bags containing the RP-Lys products were recovered manually. The recovered RP-Lys products were weighed, dried in an air force oven at 45°C for 72 h for determination of dry matter content.

Ruminal protection of RP-Lys products was assessed by 16 h *in situ* ruminal incubation for each RP-Lys product, concentrate storage duration (14 or 28 days), and incubation with TMR or corn silage. Dacron bags were placed in a weighted mesh bag and submerged into the ventral sac of a rumen-cannulated Holstein cow. The cow was housed in a tie-stall pen, milk twice daily, and fed a corn silage-based diet once daily at 0600 h to ensure at least 10% feed refusals. Following incubation, bags were gently hand washed in cold tap water and placed in a forced air-drying oven at 45°C for 72 h to dry. Samples designated as 'Time 0' were not placed in the rumen but were placed in Dacron bags and exposed to the same handling and washing procedures as samples undergoing rumen incubation. The recovered RP-Lys products were weighed, dried in an air force oven at 45°C for 72 h for determination of dry matter content.

The N content in the samples was measured using infrared absorption and thermal conductivity using LECO apparatus (LECO Corporation, Saint-Joseph, MI, USA).

The N remained in the samples after storage or incubation, after washing and after ruminal incubation were determined as the ratio of the N of the sample after each step by

the initial N content of the product as following equation: $\%N_{remaining} = [N\ remaining \cdot (N_{total})^{-1}]$. Values are expressed as a fraction 1.0.

2.3.2. Experiment II

The second experiment assessed the response of milk production and components to feeding rumen protected Lys (**RP-Lys**) in a Lys deficient diet. Thirty-six Holstein dairy cows (6 primiparous and 30 multiparous), averaging 123 ± 32 DIM, 653 ± 67 kg BW, and 38.3 ± 5.0 kg of milk per day were stratified by milk production within parity and randomly assigned to one of the three treatment groups. Cows were moved to tie stalls and allowed to acclimate over a period of 7 days to the stalls and the experimental diets. Cows were fed once daily with a blend diet 75:25 (DM basis) of the main herd diet and the control diet.

During the treatment period, cows were fed the treatment diet presented as a TMR once daily in amounts that ensured *ad libitum* consumption with a target of 10% feed refusals and fresh water was freely provided. Cows were weighed weekly and body condition was scored by two independent scorers. Cows were fed one of three rations (Table 2) for 56-d as a TMR. The treatment diets consisted of a control diet (**CON**), a diet with inclusion of 0.3% of diet DM of RP-Lys (**0.3RP-Lys**) product, and a diet with inclusion of 0.6% of diet DM of RP-Lys (**0.6 RP-Lys)** product. The base diet was formulated to be Lys deficient (Table 3) while meeting the NE_L and CP to support 40 kg of milk (NRC, 2001). The Met content of the diet was adjusted to achieve 2.6% of MP through addition of Smartamine M (Adisseo, Anthony, France). Based on a DM intake that was predicted by the NRC (2001) the control was designed to be deficient for Lys and imbalanced with respect to Lys:Met ratio. The RP-Lys product used for the experiment were analyzed to determine how much Lys would be available to be absorbed in the intestine taking account the water solubility and the amount that scape from the rumen.

Cows were released from tie stalls twice daily and milked in a parlor. Milk yield was measured electronically at each milking. Milk samples were collected during two consecutive milking each week and assayed for milk fat, protein, lactose, SCC (B2000 Infrared Analyzer, Bentley Instruments, Chaska, MN) and MUN (Skalar, Norcross, GA) by Dairy One (Ithaca, NY).

Feed intake was determined daily by difference from feed offered and refused. Samples of TMR and individual feeds were collected weekly, dried in a forced-air oven

at 60°C, and ground to pass through a 2-mm screen. Weekly feed samples were analyzed for DM content and used to adjust the proportions of diet ingredients on an as-fed basis. Weekly TMR and feed samples were analyzed for DM, CP, ADF and minerals by a commercial lab (Dairy One; Ithaca, NY) using wet chemistry and following AOAC methods (AOAC, 2000). NDF was analyzed following the method of Goering and Van Soest (1994). Amino acid profile of the rations was analyzed at Missouri Agricultural Experiment Station Chemical Laboratories (ESCL), (Columbia, MO) following AOAC methods (AOAC, 2000, method 994.12).

2.3.3.1. Measurement of Blood Amino Acids and Metabolites

Blood samples were collected in week five by coccygeal venipuncture into vacutainers containing sodium heparin (BD Diagnostics, Franklin Lakes, NJ). Samples were inverted several times to ensure mixing of the anticoagulating agent and then kept on ice until centrifugation at 1,000 x g for 15 min. Centrifugation and plasma separation occurred within 30 minutes of sample collection. Harvested plasma was stored at -80℃ until analysis. Harvested plasma was stored at -80°C until analysis. Plasma samples were analyzed for amino acid concentration at the University of Missouri–Columbian Agricultural Experiment Station Chemical Laboratory (Columbia, MO; Deyl et al. 1986; Fekkes, 1990).

2.3.3.2. Calculations

Weekly BW and BCS for each cow were used to calculate the change in body weight and BCS during the study. Milk protein, fat, solids and lactose yield were calculated from milk volume and milk composition data. Energy corrected milk (kg/d) was calculated as ECM = kg of milk × [(38.3 × % fat × 10 + 24.2 × % true protein × 10 + 16.54 × % lactose × 10 + 20.7) ÷ 3,140] (Sjaunja et al., 1990). Nitrogen efficiency for was calculated as N yield in milk divided by N intake × 100 and milk N yield was calculated from the milk protein yield in kg divided by a factor of 6.38 (Giallongo et al., 2016; Ding et al., 2019).

Daily observations of DMI, milk production and milk composition were reduced to weekly averages within cow and data. Plasma AA concentrations as % of the EAA were calculated as following equation: Individual EAA as %EAA = [Individual EAA concentration / (total EAA concentration – Lys concentration)].

2.3.3. Experiment III

The third study assessed the intestinal bioavailability of the RP-Lys product in comparison to free Lys through the bolus-dose approach.

Three lactating rumen-cannulated multiparous Holstein dairy cows weighing 724 ± 28 kg producing 31.4 ± 5.1 kg/d of milk were used in a 3 x 3 Latin square design consisted of 3 periods of 7 d. Each period consisted of 6 d of wash out and 1 d of infusion and sampling. Cows were housed in tie-stalls, milked twice daily, and individually fed at 0600 h in amounts to ensure intake in ad libitum amounts. Milk production was recorded daily. Cows were weighed once each period after the morning milking on the day of Lys infusion. Body condition score was recorded by 2 trained individuals after the morning milking on the day of infusion.

Cows were fed a basal TMR through the entire experiment (Table 4). The diet was formulated to meet or exceed protein, Lys, and methionine requirements NRC (2001) to avoid adverse effects of AA deficiencies on plasma AA profile. Dry matter intake was assessed daily and TMR was fed to amounts to ensure 10% feed refusals.

Abomasal infusion lines were constructed following the procedure of Gressley et al. (2006). The treatments were infused through PVC tubing (Nalgene 980 braided clear PVC tubing; 19.05 mm i.d., 3.18 mm wall; Fisher Scientific, Hampton, NH) placed into the abomasum via the reticular omasal orifice. One hundred and fifty grams of RP-Lys product (39.3% of Lys, ± 0.9; NutraPass-50L, Archer Daniels Midland Company, Decatur, IL) were infused into the abomasum. The positive control was, 58.9 g of crystalline HCl-Lys diluted in 300 mL of water and the negative control was an abomasal infusion of 360 mL of water.

On day 7 of each period, approximately 1 hour prior the infusion, cows were fitted with indwelling catheters (16-gauge, Abbocath-TAL catheter; Abbott Laboratories, North Chicago, IL) in the left and right jugular veins for blood sample collection.

The intestinal bioavailability of free Lys and the RP-Lys product was assessed using changes in plasma Lys concentrations following a postruminal bolus dose. The rise in blood concentration was used to determine relative appearance of the RP-Lys product compared with HCl-Lys (positive control) and water (negative control). After the infusion, lines were flushed with an additional 1,500 mL of water to ensure complete delivery of the dose.

Blood samples were collected prior to (-15 min) and following the dose (30, 60, 90, 120, 240, 360 and 480 min) for each treatment. Blood samples were inverted several

times to ensure mixing of the anticoagulating agent and then kept on ice until centrifugation at 1,000 x g for 15 min. Centrifugation and plasma separation occurred within 30 minutes of sample collection. Harvested plasma was stored at -80°C until analysis. Plasma samples were analyzed for amino acid concentration at the University of Missouri–Columbian Agricultural Experiment Station Chemical Laboratory (Columbia, MO; Deyl et al. 1986; Fekkes, 1990).

Plasma Lys absolution absorption was estimated from the area under curves (**AUC**) with respect to time (Chiou, 1978). The AUC was numerically estimated using the trapezoidal rule (Cardoso et al., 2011). The relative bioavailability of intestinal absorption for RP-Lys products determined as the ratio of the RP-Lys AUC over the free HCl-Lys AUC after subtracting the AUC values of the water. The clearance rate (**CR**) and half-life of Lys were calculated using the kinetic analysis described previously for blood glucose (Pires et al., 2007).

2.3.4. Statistical Analysis

In the experiment I nitrogen remaining in the samples after pre mixing and storage or incubation in feed, washing and ruminal incubation were analyzed using the MIXED procedure of SAS (ver. 9.4, SAS Institute Inc., Cary, NC). The model included the random effect of sample, the fixed effect of product, time of storage or incubation in the feed and the interaction between time and product. Data are least squared means and standard errors unless otherwise indicated. Means were considered different when $P < 0.05$. Tukey-Kramer studentized adjustments were used for multiple comparisons.

In the experiment II weekly observations were analyzed using the MIXED procedure of SAS (ver. 9.4, SAS Institute Inc., Cary, NC). The model included the fixed effect of treatment, the random effect of cow within treatment, the fixed effect of week and the interaction between week and treatment. For the plasma AA data, the model included the random effect of cow and the fixed effect of diet. Linear and quadratic contrasts were used to determine effects of RP-Lys. Significance was declared when $P \leq 0.05$. Tukey-Kramer studentized adjustments were used for multiple comparisons.

In the experiment III data were analyzed using the Mixed Procedure of SAS (ver. 9.4, SAS Institute Inc., Cary, NC). The normality of data was determined using the UNIVARIATE procedure and the Shapiro-Wilk test. The model for milk production, DMI and plasma AA accounted for the effects of treatment, period, and the random effect of cow nested within treatment. The model for Lys kinetics data accounted for the effects

of treatment, period, and the random effect of cow. Means were considered different when $P < 0.05$. Tukey-Kramer studentized adjustments were used for multiple comparisons.

2.4. RESULTS

2.4.1. Experiment I

The CP content of the RP-Lys products was 48.91 (± 0.69), 48.86 (± 0.36), 48.45 (±0.17), and 49.43 (±0.21) % on a dry basis for RP-Lys Control, RP-Lys 1, RP-Lys 2 and RP-Lys 3 respectively. Because there was a significant difference ($P < 0.05$) in CP between the products further comparison with regard to stability are expressed as a fraction of the initial CP concentrations of the initial product. Data for each phase of the stability analysis is presented in Table 5 and time effects represented in Figures 1 through 3.

Premixing and storage with concentrate and recovery of the RP-Lys sources yielded in excess of 98% of the starting CP in all samples (Table 5). The RP-Lys products responded differently to premixing and storage with concentrate mix ($P < 0.05$) with RP-Lys Control and RP-Lys 1 showing similar recovery after premixing and storage with concentrate. Washing in water after premixing and storage, resulted in an overall loss of CP ($P < 0.05$). The samples with the least amount of loss were RP-Lys Control (AjiPro-L) and RP-Lys 1 being the next most resilient to the effects of storage with concentrate and washing. A similar separation of means was observed for CP recovered after premixing and storage with concentrate following by washing and incubation in the rumen. There was recovery of 81% of the N for RP-Lys Control with RP-Lys 2 and -3 yielding 68 and 70% respectively and RP-Lys 1 yielded the least amount of CP recovery through the preincubation, wash and rumen incubation sequence. There was a tendency ($P = 0.06$) for a time x treatment interaction of premixing and storage with concentrate on post washing CP recovery that is characterized by greater losses from RP-Lys 2 and 3 after 28 d premixing and storage with concentrate. There was a reduction in CP recovered from RP-Lys 1, -2, and -3 relative to RP-Lys Control with the least recovery occurring for RP-Lys 2. The maximal accumulation of CP losses appears to occur within 14 days for all products tested (time x products; $P < 0.01$). The CP recovery from products

following ruminal incubation ranged between 63 and 81% of available CP within samples placed in the rumen. There was a significant time x product effect for CP recovery with the additional losses observed for RP-Lys 3 when premixing and storage with concentrate mix was extended to 28 d.

Incubating the RP-Lys products with corn silage provides an assessment of stability upon contact with fermented feeds and indicated greater ($P < 0.05$) recovery of CP from RP-Lys Control relative to the other RP-Lys sources following preincubation, washing, and ruminal incubation. There was a significant effect of time of incubation with silage across RP-Lys products ($P < 0.01$) that is characterized by a reduction in CP for RP-Lys 1, 2, and 3 following 24 h of incubation with corn silage that is not observed for RP-Lys Control. There were no product x time interactions detected for CP recovery following washing and ruminal incubation.

Premixing with TMR did not change ($P = 0.13$) N retained in the samples regardless of incubation duration (12 and 24 h; time x product effect $P = 0.55$) although, the RP-Lys 1 product presented the lowest CP retained. There was a significant effect of washing on CP recovery and an effect ($P < 0.05$) of product x time where a further reduction in CP recovery was observed for RP-Lys 1 after 24 h but was not observed for the other products. Ruminal protection of the products was not changed due to the incubation in the TMR independent of the storage duration. There were no time x product differences ($P = 0.58$) for recovery of CP following ruminal incubation.

2.4.2. Experiment II

In this experiment we evaluated the effect an RP-Lys prototype product on DM intake, milk production and composition in a diet designed to be inadequate in Lys for mid lactation dairy cows. Diet ingredients and chemical composition are presented in the Tables 1 and 2. The initial Lys content in the RP-Lys product was 39.3 (± 0.9) g of Lys per 100 grams of product. The content of Lys that is released from the rumen and reach the intestine was 63.1 (± 5.5) % of the initial content. Therefore, 24.7 (±1.9) g of Lys per 100 g of RP-Lys product were available in the intestine. The feeding goal for the control group was to maintain a corn-based diet to create a MP and Lys deficiency. Therefore, the control diets had 29.5 % corn grain and corn byproduct feeds and 42.5 % corn silage on a DM basis. Diets supplemented with RP-Lys were formulated to contain similar ingredient levels as the control diet.

The observed DMI was greater than expected, based on the NRC intake model (NRC, 2001) for all cows in the experiment (Table 6) although there were no differences ($P > 0.05$) in DMI (26.2 ± 3.3 kg DM) between treatments. However, there was a tendency for an interaction effect for week and treatment (Figure 4) with cows receiving 0.6 RP-Lys displaying reduced intake during the first weeks of the trial. There were no differences ($P > 0.05$) between treatment for milk yield (37.8 ± 5.5 kg/d; $P = 0.41$), milk components and milk component yield, feed efficiency and milk N efficiency and BW and BSC (Table 6). Because observed intake was greater than expected the MP and Lys balance predicted (NRC, 2001) was greater than expected (Table 7). However, despite a greater than expected overall intake the cows fed the control diet had a Lys intake that was 10.3 % less than the NRC (2001) target of 6.6 % Lys as a percentage of digestible MP. This resulted in Lys supply for the 0.3 RP-Lys to be 2.3 % in excess of requirements and a 12.9% excess Lys for the 0.6 RP-Lys treatment group.

There were no treatment differences in plasma AA concentration (μmol/L) (Table 8) for all essential amino acids except for His that presented as a linear reduction as Lys inclusion increased. However, plasma Lys and Met, expressed as % of EAA (Table 9) increased linearly ($P \leq 0.05$) with RP-Lys supplementation. There was a tendency ($P < 0.10$) for cows in 0.6 RP-Lys to have greater plasma Lys concentrations (% of EAA) than CON cows.

2.4.3. Experiment III

The postruminal bolus infusion method of evaluating rumen protected AA (Fleming et al., 2019) was used to determine the bioavailability of RP-Lys product relative to free Lys. The treatments did not alter milk production, DMI, BCS and BW ($P > 0.05$) (Table 10). The Hcl-Lys infusion increased plasma Lys concentration ($P < 0.01$) in comparison to water and RP-Lys, increased EAA ($P < 0.05$) and tended to increase plasma Arg ($P \leq 0.09$), and plasma TAA ($P \leq 0.09$) in comparison to water infusion (Table 11). The infusion of RP-Lys decreased plasma Cit ($P < 0.05$) concentration in comparison to water. There was interaction between treatment and time for aminoadipic acid (**Aad**), hydroxylysine (**Hyl**), ornithine (**Orn**), Lys, 1-methyl histidine (**1MHis**), Arg, EAA, TAA and for Lys and Arg when evaluated as % of EAA (Table 12); Figure 6).

Postruminal delivery of free Lys ad RP-Lys products resulted in an increase in plasma Lys concentration, usually within 30 minutes of dosing, that exceeded pre-infusion concentrations (Figure 5). Plasma Lys concentration peaked between 90 to 120

minutes after the postruminal Lys dose and plasma Lys concentrations returned to baseline levels within 420 min after receiving the postruminal Lys dose. Postruminal dosing with free Lys resulted in a greater AUC. There is a trend for greater Lys half-life in the plasma of cows receiving the postruminal bolus dosing of RP-Lys relative to water. There was no significant effect of form of Lys (free or RP-Lys) on plasma Lys clearance rate (Table 13). The relative bioavailability of intestinal absorption for RP-Lys products determined as the ratio of the RP-Lys AUC over the free HCl-Lys AUC after subtracting the AUC values of the water infusion was 22.68 %.

2.5. DISCUSSION

Current RP-Lys technologies protect Lys from ruminal degradation by virtue of a physical barrier between Lys and the rumen environment. However just as the integrity of protection is subject to rumen conditions the diet composition, moisture, acidity and the time that RP-Lys product is in contact with feeds prior to consumption by the cows may impact the integrity of Lys protection in the lipid-coated products. The storage of RP-Lys products in the concentrate results in a small loss of N for both incubation times for all products tested. The loss of N in the rumen, after storage in the concentrate, increased the N losses when compared to samples that were not stored. It is recognized that small cracks or flaws in the RP AA coating would allow solubilization and utilization of the amino acids by the rumen microorganisms (Koenig and Rode, 2001). The loss of CP with exposure to concentrate feeds represents a combination of effects of the mixing process and prolonged physical contact with concentrate feeds and likely represents damage to the lipid coating encapsulation material. The damages caused in the lipid coating during prolonged exposure to concentrate feeds would reduce rumen integrity of the products leading to reduced effectiveness in postruminal Lys delivery. Data from the current study supports these conclusions in that the RP-Lys products with the lowest recoveries of CP after incubation with concentrate also had the lowest subsequent recovery after rumen incubation.

Similarly, losses in integrity of protection is observed during the mixing and storage of RP-Lys products with corn silage or TMR despite shorter exposure times. The effects of Corn silage and TMR to reduce CP recovery from all products is potentially compounded by the moisture content and acidity of corn silage and TMR. The

hydrolyzation of lipids can be increased when they are exposed to environments with acids and water (Le Chatelier, 1884), which may explain the CP losses in products incubated in the silage and TMR. Previous reports (Ji et al., 2016; Reiners et al., 2017) indicate that mixing with silage decreased the Lys retained by the rumen-protected Lys products as a result of the interaction between the coating and the chemical and physical compounds of the feed. Similar losses are observed in the current study. For some, but not all products tested in the current study, the loss of integrity protection continued to accumulate through 24h of exposure to both corn silage and TMR and appeared to be more pronounced with exposure to TMR.

Overall storage in the concentrate, silage and TMR in this present study resulted in remaining N values after ruminal incubation lower than 80, 87 and 86 %, respectively. The observed values indicated a significative loss of the Lys until the product reached the abomasum. Several studies have been showing high variability in the amount of the Lys retained in the ruminal protected products exposed or not to the feed after ruminal incubation (Reiners et al. 2018; Fleming et al. 2018 and Ji et al., 2016). These data highlight a critical element in evaluating rumen protected AA sources but, unfortunately, a standardized method for this evaluation has not yet been established which limits the direct comparison of results across experiments.

In testing the feeding level of RP-Lys we formulated diets to be negative in RDP balance as a strategy to change the AA profile of MP flowing from rumen to intestine. Reducing the predicted microbial protein production through limiting RDP led to a greater contribution of RUP to metabolizable protein and inclusion of corn-protein sources in the diet resulted in the Lys deficiency in the control diet. Although the severity of this deficiency was less than expected due to higher DMI. The DMI was 9% higher than predicted by NRC (2001) model, even if the negative RDP balance, and without differences between treatments. The higher DMI shows that the negative RDP balance probably did not compromise the rumen fiber digestibility and consequently the intake (Allen, 2000). In the present study the CP content of the diets were 15.9 (±0.75) %, in diets with same CP or even lower CP contents the reduction in dry matter intake were not observed (Olmos Colmenero and Broderick, 2006; Lee et al., 2012; Giallongo et al., 2016). The RP-Lys did not affect the DMI independent of the inclusion, similar to other studies using RP-Lys sources (Robinson et al., 1998; Swanepoel et al., 2010; Giallongo et al., 2016; Chen et al., 2018) although decreases in DMI had been reported by (Robinson, 2010) in a systematic review of the literature.

Yields of milk, ECM, protein, fat and lactose were not different between cows fed with the control diet or supplemented with RP-Lys. The milk composition as % of milk yield for all components tested presented no difference as well and were within the expected range for the average for US Holstein cows (Salfer et al., 2019). The lack of significant effects due to RP-Lys supplementation on milk components could be related to the fact that another AA or nutrient was co-limiting milk and milk component synthesis mainly because the margin between first, second and third limiting AA or nutrient is small (Schwab et al., 1976). The BW change was not different between the treatments. Cows lost BW during the study despite being, on average 123 DIM at the initiation of the feeding study, which may indicate that mid lactation cows could still have high demand for AA. The change in BW may contributed to the effectiveness of testing the impact of AA supply as dairy cows are able to mobilize the body AA reserves which limits the ability to detect effects of feeding AA on milk production (Patton et al., 2014). Given this response, there could be more than one limiting nutrient or AA exerting a lack of response in milk production when Lys is adequately supplied.

Further, to our knowledge, no previous research has examined RP-Lys supplementation in a corn-based Lys deficient diet that is adequate in methionine. Giallongo et al. (2016) observed no effect when supplementing RP-Lys in a MP and Lys deficient diet on milk yield, fat and lactose and protein yield and fat and lactose composition but protein composition. The negative MP balance observed in the above-mentioned study despite the less severe Lys deficiency may have exacerbate the negative effect of AA supply to mammary gland thus leading a significant reduction on milk protein composition.

There were no observed effects of either level of RP-Lys on ECM. Likewise, MUN did not differ between the treatments although the values were similar (Giallongo et al., 2016) or lower (Swanepoel et al., 2010; Mullins et al., 2013; Weiss, 2019) than the values observed in other studies suggesting N supply that was adequate but not excessive or severely imbalanced.

There was no effect of treatment on efficiency parameters. However, values of feed efficiency and N use efficiency obtained were similar than observed by Huhtanen and Hristov (2009). These authors showed mean values for feed efficiency in the USA and Europe of 1.42 and 1.41 kg of milk per kg of DM consumed, respectively. For N use efficiency the values were 24.7% for the US cows with 31.4 kg milk/d and dietary crude

protein content of 17.8% in DM and 27.7% for European data with 25.4 kg milk/d dietary protein content of 16.5%.

Due to supplementation with RP-Lys an increase in plasma Lys concentration was expected since the Lys requirements are met. However, the Lys uptake by the mammary gland exceeds the secretion into milk protein and this excess increases with the increased supply (Lapierre et al., 2012). In addition, the hepatic removal of AA appears to be mainly a mass action function of total liver inflow and higher removal is expected with increased Lys intestinal absorption (Lapierre et al., 2005) that might explain the lack of differences in Lys plasmatic total concentration due to supplementation.

There was no effect of treatment on total essential amino acids in plasma. But, when considered individually, plasma His concentration was altered ($P < 0.05$) by Lys feeding which presented as a linear reduction. However, when His is evaluated as % EAA the effect of treatment is not observed. This result may have happened because there was a no significant reduction in the concentration of the EAA which kept the His proportion the same. When Lys is supplemented in deficient diets plasma Lys concentration should not increase because it will be used by the tissues. His could be co-limiting in this situation. Histidine is recognized as the third limiting amino acid in the corn-based diets and because Lys and Met requirements were met in this study, then His might be the first limiting. Weekes et al. (2006) suggested that reduction in His not accompanied by the positive response in milk production or milk components yield could be explained by the more efficient catabolism or deposition of AA into body protein.

The linear effect of RP-Lys on plasma Lys and Met as % EAA and a tendency to increase in Lys indicates that Lys was delivered to small intestine, absorbed and available to be used by the mammary gland. Previous data suggest that when postruminal Lys is provided in excess of tissue needs that an AA imbalance may result which negatively impacts animal production and performance (Robinson et al., 2000). This does not appear to be the case in the present study despite a predicted Lys balance of 18 g/d for the 0.6%RP-Lys group, or 109% of their calculated intestinally absorbed Lys requirement.

Given the lack of effects of RP-Lys supplementation on milk production and composition, the lack of negative consequences of Lys supplementation on feed intake or BW loss, and associated changes in EAA with RP-Lys feeding that feeding RP-Lys has a physiological impact in mid-lactation dairy cows. Presently however, the physiological targets for increased postruminal Lys supply and impact on health and productivity remain elusive.

A primary goal in feeding rumen protected AA is to correct deficiencies or imbalance of the specific AA through the release of this amino acid in the intestine. Therefore, assessing the changes in plasma AA concentration in response to acute changes in postruminal AA supply is a strategy that has been used to test the ability of RP-Lys products deliver the AA that can be absorbed from the small intestine (Blum et al., 1999). This approach based on the assumption that a pulse dose of AA delivered postruminally, can be detected as a change in plasma AA concentration over time. Furthermore, that the area under the excursion curve for Lys concentration x time provides an assessment of the relative capacity of a RP-Lys product to deliver Lys when compared with free Lys (HCl-Lys).

Plasma AA concentrations reflect the balance between AA supply and utilization. In the present study, the Lys provided by the RP-Lys product increased Lys plasma concentration and induced changes in the proportion of Lys as % of EAA. This observation showed the possibility of manipulating the ratio of Lys related to other EAA using the RP-Lys product aimed to achieve the desirable EAA profile for milk protein synthesis and improve the N use efficiency (Arriola Apelo et al., 2014b). The lower plasma cit observed in RP-Lys treatment suggests a reduction in the urea synthesis, since it's an intermediate in the urea cycle (Wu, 2013), although, plasma urea concentrations were similar. The increase in the EAA concentration in the HCl-Lys treatment, was expected due to the greatest Lys concentration due to the infusion. The plasma concentration of α-aminoadipic acid did not differ between treatments. Changes in α-aminoadipic acid were expected as the primary breakdown product of Lys and effects of prolonged Lys infusion to increase circulating α-aminoadipic acid concentrations (Tucker et al., 2017). Although there was no difference between the treatment groups due to the high variability, the plasma α-aminoadipic acid concentration increased 101% peaking at 120 min post infusion in animals receiving HCl-Lys and 31% peaking at 240 min post infusion in animals receiving RP-Lys.

The combined results of the postruminal infusion experiment show that the RP-Lys source tested was able to deliver Lys to the intestine for absorption. This statement can be made based on the difference between plasma concentrations before infusion and also when comparing plasma concentrations of the group receiving water pulse dose. However, when comparing the AUC of the RP-Lys infusion and the water infusion there is no difference. Intestinal availability of amino acids is related to the ruminal protection providing that the protective coating can release the AA in the abomasum and duodenum.

In cases where there is inconsistency in the postruminal degradation of the protective coating there is a failure in AA delivery for absorption (Robinson, 2010; Koenig and Rode, 2001). The lack of increase in plasma Lys with the RP-Lys source tested here may be a consequence of impaired postruminal release of the AA payload. However, a drawback of the method used in the present experiment is the lack of previous exposure to the feed, mastication and eating dynamics, exposure to the rumen environment, or a combination of these effects. Furthermore, the dose used in the pulse dose infusion represents a large portion of both the Lys and the coating material of the product, in this case saturated fat. A bolus dose of the product has exceeded the ability of the abomasum and intestine to digest the lipid coating in the time it takes the product to leave the intestine (Koenig and Rode, 2001). Data is lacking on the digestibility of these products in the small intestine, but hydrogenated fatty acids have lower digestibility in the small intestine compared with unsaturated fatty acids (Wu et al. 2012; Harvatine and Allen, 2006.) which could affect the ability of the coated product be released in the intestine. Furthermore, the small damages caused in the RP-Lys products during the feed mixing, ingestion, mastication and rumen exposure could help the degradation of the lipid capsule without releasing the Lys from the core. This could allow for better emulsification in the intestine and release of the Lys to be absorbed (Fleming et al. 2019).

The estimated bioavailability observed in this study (22.68 %) is lower than observed by Fleming et al. (2019) that presented value of to the observed Lys bioavailability of 42.1%. The results of the present study are similar to the results presented by Reiners et al. (2017) that observed bioavailability of 15 and 23 % for ruminal protected Lys products. There is a lack of studies evaluating Lys bioavailability in RP products and there is no standardized technique for this evaluation, so comparing experiments should be done with caution. Despite this, Whitehouse et al. (2017) studied the dose-response technique for determining the relative bioavailability and showed a wide range (5 to 85% in the bioavailability between 27 products tested in 13 different experiments using the free AA dose technique. The high variability observed could be related to the difficulties faced by the industry in the last 40 years to effectively create a coating process that protects Lys, and other AA, from ruminal degradation while allowing Lys to release in the intestine (Robinson, 2010).

The bioavailability in this study was accessed without prior exposure of the RP-Lys product to the base diet, feeding process, act of eating and ruminal exposition, thus the calculated bioavailability could be lower or greater depending on the effects of each

one of those factors and the interaction among them.

2.6. CONCLUSIONS

The storage in the feed altered the stability for all Lys prototypes resulting in a protection from ruminal degradation as low as 56 % with high variation depending of the duration of the incubation and the feed matrix. Although supplemental RP-Lys increased estimated digestible Lys supply and increased plasma Lys the results observed did not support the hypothesis that increasing the Lys supply would increase production and milk composition of cows during a Lys deficient diet. The results of the bioavailability study (Experiment III) indicated that the RP-Lys product was able to deliver Lys and be absorbed in the small intestine based on the difference in the pre and post infusion plasma Lys concentration. Overall, summarizing all those studies we conclude that association of the storage practices before feeding RP-Lys and the low bioavailability observed in the bioavailability trial might result in a lower delivery than estimated of the digestible Lys in the small intestine resulting in a lack of response in the lactation trial. Multiple AA may have been limiting when all dietary protein was derived from corn products which could tamper the expected increase in plasma Lys and increase of milk yield and milk components.

2.7. ACKNOWLEDGEMENTS

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Table 0-1 Ingredients composition of experimental concentrate used to mix with the RP-Lys products to evaluate the stability of the products during different storage times (% of diet DM)

Item	% by weight
Corn grain, ground	30
Soybean meal	30
Dried corn distillers grain	30
Salt	5
Vitamin/trace mineral mix[1]	5

[1] Vitamin/trace mineral Mix : Purdue Dairy TMV premix- Calcium, Minimum 5.00%; Calcium, Maximum 7.00%; Copper, Minimum 12,000ppm; Selenium, Minimum 260 ppm; Zinc Minimum 54,500 ppm; Manganese Minimum 44,500 ppm; Iodine Minimum 440 ppm; Cobalt Minimum 1,580 ppm; Vitamin A Minimum 3,000,000 IU/lb; Vitamin D Minimum 750,000 IU/lb; Vitamin E Minimum 14,000 IU/lb.

Table 0-2 Ingredients composition of experimental diet fed during Experiment II (% of diet DM)

Composition, % DM	Treatments [1]		
	CON	0.3RP-Lys	0.6RP-Lys
Corn silage	42.5	42.5	42.4
Haylage	12.7	12.7	12.7
Rye hay	1.9	1.9	1.9
Corn grain ground	6.4	6.4	6.3
Soybean hulls	7.2	7.2	7.2
Dried corn distillers grain	7.6	7.6	7.6
Corn Gluten meal, dried	2.8	2.8	2.8
High moisture corn	12.7	12.7	12.7
Soybean meal	2.3	2.3	2.2
Calcium carbonate	0.85	0.85	0.84
Sodium bicarbonate	0.85	0.85	0.84
Fat [2]	0.50	0.50	0.51
Urea	0.51	0.51	0.51
Salt	0.30	0.30	0.29
Calcium phosphate	0.21	0.20	0.21
Magnesium oxide	0.17	0.17	0.17
Mycotoxin binder[3]	0.04	0.04	0.04
Vitamin/trace mineral Mix[4]	0.09	0.08	0.08
Monensin[5]	0.01	0.01	0.01
Rp-Lys[6]	0.00	0.30	0.59
Hydrogenated fat[6]	0.30	0.15	0.00
Rumen protect Methionine[7]	0.13	0.13	0.13

[1] Control diet –no RP-LYS; 0.3 RP-Lys- supplemented with 0.3 % of diet DM of RP-Lys; 0.6 RP-Lys- supplemented with 0.6 % of diet DM of RP-Lys;

[2]Enertia® rumen bypass fat from palm oil (ADM Animal Nutrition, Decatur,IL)

[3] ETX-5® (Feedworks USA Ltd. Cincinnati, OH)

[4] Purdue Dairy TMV premix: Calcium, Minimum 5.00%; Calcium, Maximum 7.00%; Copper, Minimum 12,000ppm; Selenium, Minimum 260 ppm; Zinc Minimum 54,500 ppm; Manganese Minimum 44,500 ppm; Iodine Minimum 440 ppm; Cobalt Minimum 1,580 ppm; Vitamin A Minimum 3,000,000 INT UNITS/Lb; Vitamin D Minimum 750,000 INT UNITS/Lb; Vitamin E Minimum 14,000 INT UNITS/Lb.

[5] Rumensin® (Elanco, Greenfield, IN)

[6] Hydrogenated fat (Dritex X®- ADM Animal Nutrition, Decatur,IL)

[7] RP-Lys (ADM Animal Nutrition, Decatur,IL)

[8] Smartamine M (Adisseo Inc., Alpharetta, GA)

Table 0-3 Nutrient composition of diets fed during Experiment II

Composition, % DM	Treatments [1]		
	CON	0.3RP-Lys	0.6 RP-Lys
DM	45.3	45.4	45.3
CP	16.0	15.7	16.2
NDF	29.2	30.0	28.9
Crude fat	4.3	4.2	4.1
NFC	44.0	43.8	44.3
Ash	6.5	6.3	6.5
NE_L, (Mcal/kg)[2]	1.74	1.73	1.75
Ca	0.62	0.56	0.63
P	0.38	0.38	0.38
Mg	0.28	0.27	0.26
K	1.08	1.06	1.05
Na	0.42	0.39	0.39
Amino acid composition, g/kg of DM			
Ala	10.15	10.15	10.15
Arg	4.65	4.65	4.65
Asp	10.40	10.40	10.40
Cys	2.15	2.15	2.15
Glu	19.10	19.10	19.10
Gly	5.85	5.85	5.85
His	3.00	3.00	3.00
Iso	5.80	5.80	5.80
Leu	14.20	14.20	14.20
Lys	5.65	6.82	8.00
Met	3.50	3.50	3.50
Phe	6.90	6.90	6.90
Pro	9.45	9.45	9.45
Ser	5.25	5.25	5.25
Thr	4.95	4.95	4.95
Trp	0.95	0.95	0.95
Tyr	3.40	3.40	3.40
Val	7.15	7.15	7.15

[1] Control diet –no RP-LYS; 0.3 RP-Lys- supplemented with 0.3 % of diet DM of RP-Lys; 0.6 RP-Lys- supplemented with 0.6 % of diet DM of RP-Lys;

[2] Calculated (NRC, 2001) using treatment average DMI, weight, milk production and composition, and assayed feed composition.

Table 0-4 Ingredient composition of the diets fed to rumen-cannulated lactation dairy cows treated with an abomasal bolus of the rumen-protected RP-Lys, HCl-Lys or water

Item	% DM
Corn silage	31.78
High moisture corn	13.31
Alfafa haylage	9.44
Alfafa hay	9.08
Corn grain, ground	9.05
Soybean meal	6.18
Wheat straw	5.85
Soyplus	4.86
TMR blend[2]	3.45
Palmitic acid [3]	1.82
Calcium carbonate	1.13
Sodium bicarbonate	0.86
Blood meal	0.69
Salt	0.43
Calcium phosphate	0.35
DCAD plus [4]	0.34
Omnigen AF[5]	0.21
Magnesium oxide	0.21
Diamond V XP [6]	0.20
Calcium sulfate	0.19
Fat yellow grease	0.19
Vitamin/trace mineral Mix[7]	0.11
Mycotoxin binder [8]	0.06
Select biocycle [9]	0.06
Smartamine M [10]	0.06
Ajipro L [11]	0.03
Vitamin E	0.02
Rumensin [12]	0.01
Chemical composition	
DM	45.70
CP	15.20
NDF	30.20
Crude fat	5.30
NFC	40.90
Ash	8.40
NEL (Mcal/kg)[13]	1.74
Ca	1.10
P	0.45
Mg	0.38
K	1.59
Na	0.50

[1] Landus Cooperative, Ralston, IA.
[2] QLF Dairy SUGR 38 (QLF- Quality liquid feed, Dodgeville, WI).
[3] Rumen protected palmitic acid
[4] DCAD Plus (Church & Dwight Co., Inc, Ewing Township, NJ, Us).
[5] OmniGen-AF (Phibro Animal Health, Teaneck, NJ).
[6] XP Yeast Culture (Diamond V, Cedar Rapids, IA).
[7] Purdue Dairy TMV premix: Calcium, Minimum 5.00%; Calcium, Maximum 7.00%; Copper, Minimum 12, 000ppm; Selenium, Minimum 260 ppm; Zinc Minimum 54,500 ppm; Manganese Minimum 44,500 ppm; Iodine Minimum 440 ppm; Cobalt Minimum

1,580 ppm; Vitamin A Minimum 3,000,000 INT UNITS/Lb; Vitamin D Minimum 750,000 INT UNITS/Lb; Vitamin E Minimum 14,000 INT UNITS/Lb.

[8] ETX-5® (Feedworks USA Ltd. Cincinnati, OH).

[9] Select BioCycle (Agrarian Solutions West Wayne St. Middlebury, IN).

[10] Smartamine M (Adisseo Inc., Alpharetta, GA).

[11] Ajipro L (Ajinomoto Animal Nutrition North America, Inc., Chicago, IL).

[12] Rumensin® (Elanco, Greenfield, IN).

[13] Calculated (NRC, 2001) using treatment average DMI, weight, milk production and composition, and assayed feed composition.

Table 0-5 Crude protein remaining (g/g) in rumen-protected lysine products after pre-mixing and storage in concentrate, or incubation in corn silage or TMR, washing and rumen incubation for 16 hours

Pre-mixing and storage with concentrate														
CP remaining[1]	RP-Lys Control			RP-Lys 1			RP-Lys 2			RP-Lys 3			SEM	*Prod*
Premixing and storage	0.99[a]			0.99[ab]			0.98[bc]			0.98[c]			0.001	<0.01
Washing	0.98[a]			0.96[b]			0.93[d]			0.94[c]			0.001	<0.01
Rumen	0.80[a]			0.63[c]			0.67[b]			0.70[b]			0.009	<0.01
	0	14 d	28 d	0	14 d	28 d	0	14 d	28 d	0	14 d	28 d		Prod x time
Premixing and storage	1.00[a]	0.99[ab]	0.99[ab]	1.00[a]	0.98[bc]	0.98[bc]	1.00[a]	0.98[bc]	0.97[bc]	1.00[a]	0.97[bc]	0.97[c]	0.002	0.06
Washing	0.98[a]	0.98[a]	0.98[a]	0.97[a]	0.95[b]	0.95[b]	0.98[a]	0.91[c]	0.89[d]	0.99[a]	0.93[c]	0.91[c]	0.003	<0.01
Rumen	0.86[a]	0.79[ab]	0.76[b]	0.73[bc]	0.59[de]	0.56[e]	0.85[a]	0.60[de]	0.56[e]	0.82[ba]	0.66[dc]	0.62[de]	0.015	<0.01
Preincubation with corn silage														
CP remaining[1]	RP-Lys Control			RP-Lys 1			RP-Lys 2			RP-Lys 3			SEM	*Prod*
Incubation	0.98[a]			0.91[c]			0.94[b]			0.95[b]			0.017	<0.01
Washing	0.97[a]			0.84[a]			0.93[a]			0.93[a]			0.017	<0.01
Rumen	0.85[a]			0.71[b]			0.84[a]			0.83[a]			0.01	<0.01
	0	12 h	24 h	0	12 h	24 h	0	12 h	24 h	0	12 h	24 h		Prod x time
Incubation	1.00[a]	0.99[a]	0.97[b]	1.00[a]	0.89[d]	0.83[e]	1.00[a]	0.94[c]	0.89[d]	1.00[a]	0.94[c]	0.91[d]	0.299	<0.01
Washing	0.98[a]	0.97[a]	0.96[a]	0.87[ba]	0.87[ba]	0.78[b]	0.98[a]	0.93[ab]	0.88[ab]	0.98[a]	0.92[ab]	0.89[ab]	0.299	0.71
Rumen	0.87[a]	0.86[ab]	0.83[ab]	0.76[ba]	0.71[cd]	0.65[d]	0.86[ab]	0.86[ab]	0.81[abc]	0.84[ab]	0.84[ab]	0.81[ab]	0.017	0.36
Preincubation with TMR														
CP remaining[1]	RP-Lys Control			RP-Lys 1			RP-Lys 2			RP-Lys 3			SEM	*Prod*
Incubation	0.97			0.96			0.935			0.904			0.02	0.13
Washing	0.96[a]			0.733[b]			0.924[a]			0.932[a]			0.029	<0.01
Rumen	0.82[a]			0.57[b]			0.764[ab]			0.802[ab]			0.053	<0.02
	0	12 h	24 h	0	12 h	24 h	0	12 h	24 h	0	12 h	24 h		Prod x time
Incubation	1.00	0.98	0.94	1.00	0.90	0.81	1.00	0.96	0.84	1.00	0.95	0.92	0.036	0.55
Washing	0.99[a]	0.96[a]	0.93[a]	0.98[a]	0.79[a]	0.42[b]	0.98[a]	0.94[a]	0.83[a]	0.98[a]	0.92[a]	0.88[a]	0.0520	<0.01
Rumen	0.88[a]	0.81[ab]	0.78[ab]	0.75[ab]	0.57[ab]	0.39[b]	0.85[ab]	0.85[ab]	0.58[ab]	0.84[ab]	0.85[ab]	0.70[ab]	0.080	0.58

. [1.] CP remaining (g/g) after each test compared with initial CP content of the products. [2.] Means within row with different superscripts differ P < 0.05)

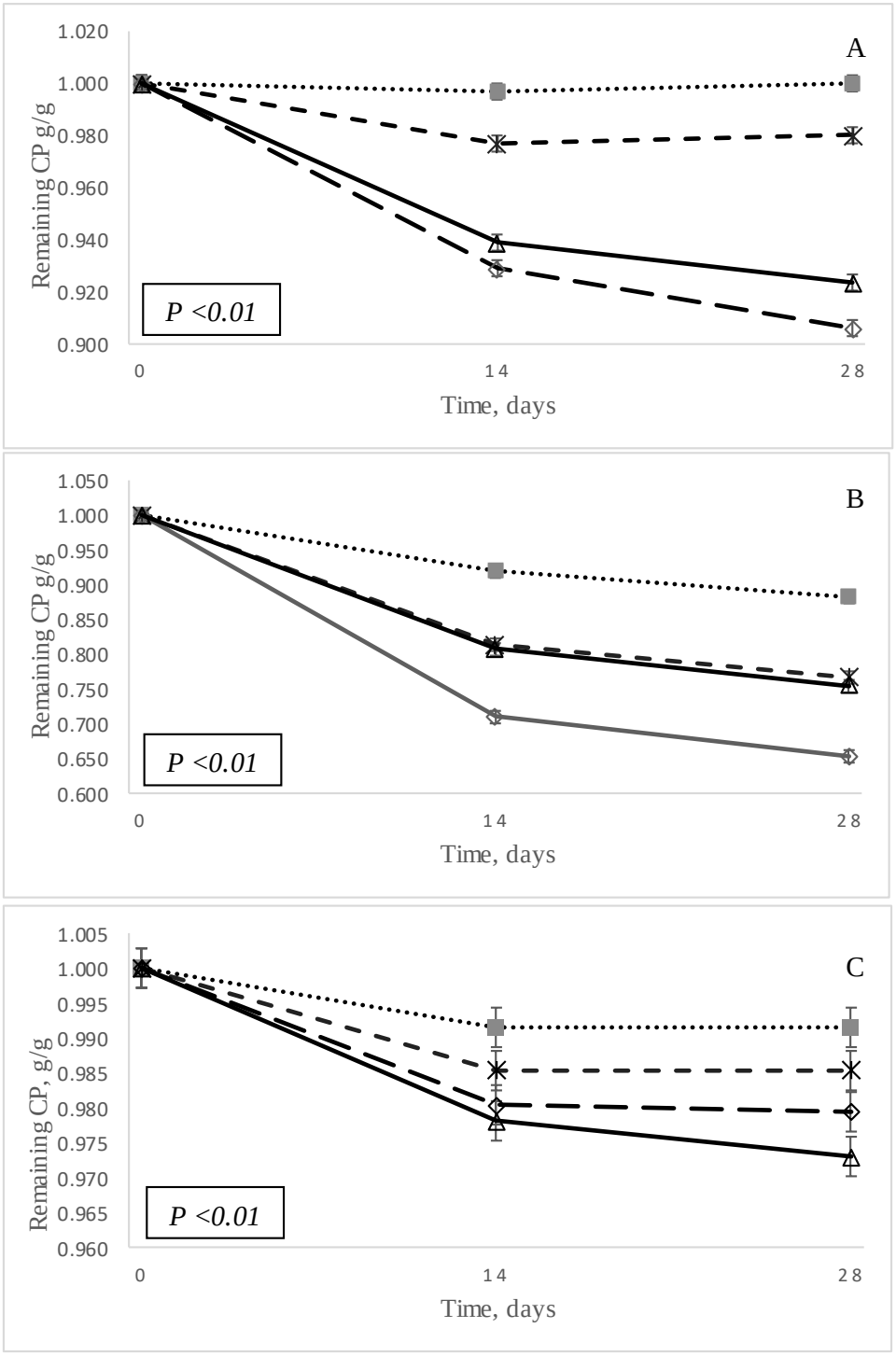

Figure 0-1 Remaining N content in the rumen protected Lys products, (■) RP-Lys Control, (×) RP-Lys 1, (◊) RP-Lys 2, (△) RP-Lys 3, with different exposures time to the concentrate. After exposure to concentrate (A panel) After washing (B panel) and after 16 h of ruminal incubation (C panel). The N remaining in the samples was calculated as ratio of the N in the initial product and the N content of the sample after each procedure.

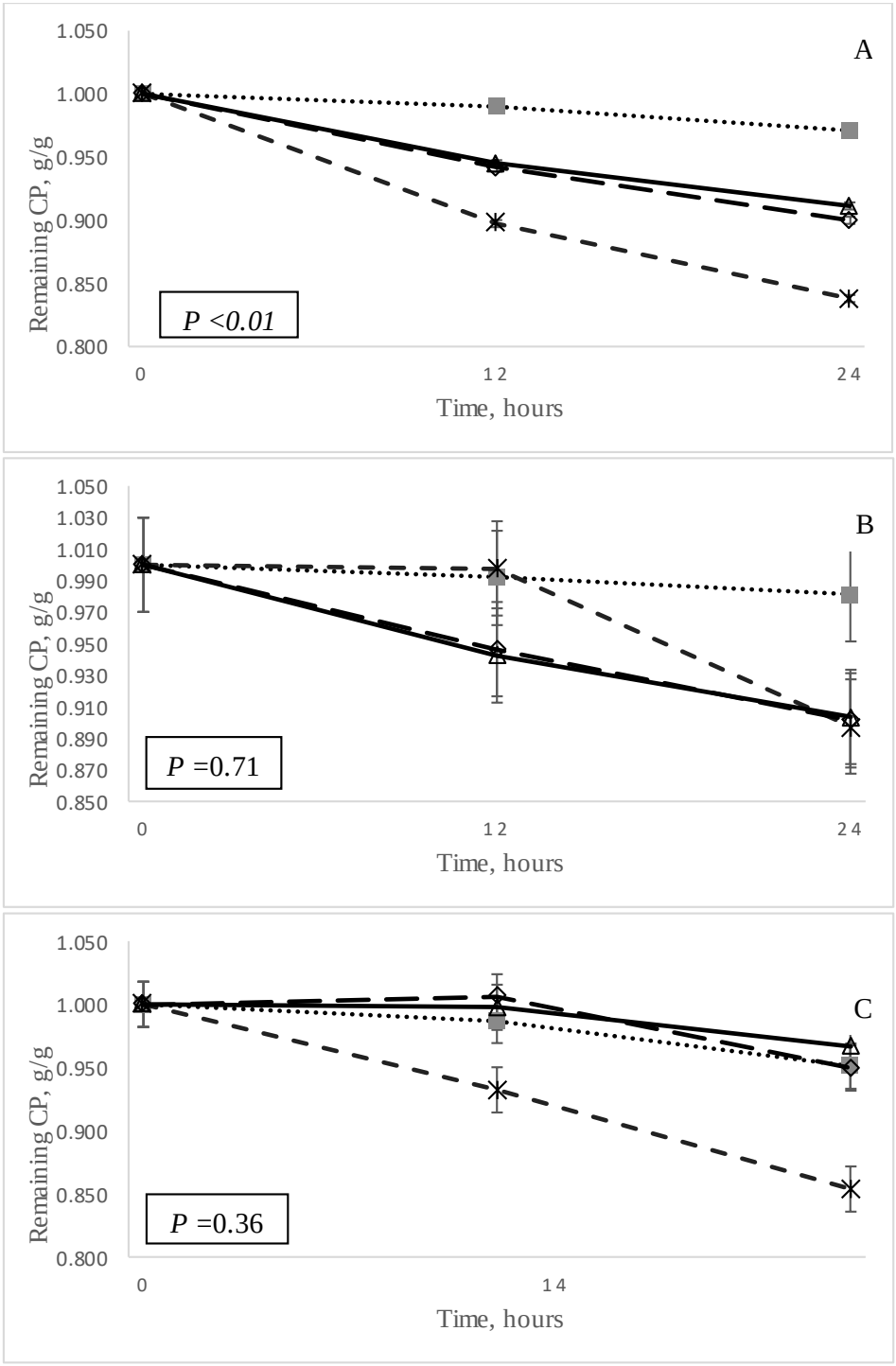

Figure 0-2 Remaining N content in the rumen protected Lys products, (■) RP-Lys Control, (×) RP-Lys 1, (◊) RP-Lys 2, (△) RP-Lys 3, with different exposures time to the silage. After exposure to silage (A panel) After washing (B panel) and after 16 h of ruminal incubation (C panel). The N remaining in the samples was calculated as ratio of the N in the initial product and the N content of the sample after each procedure.

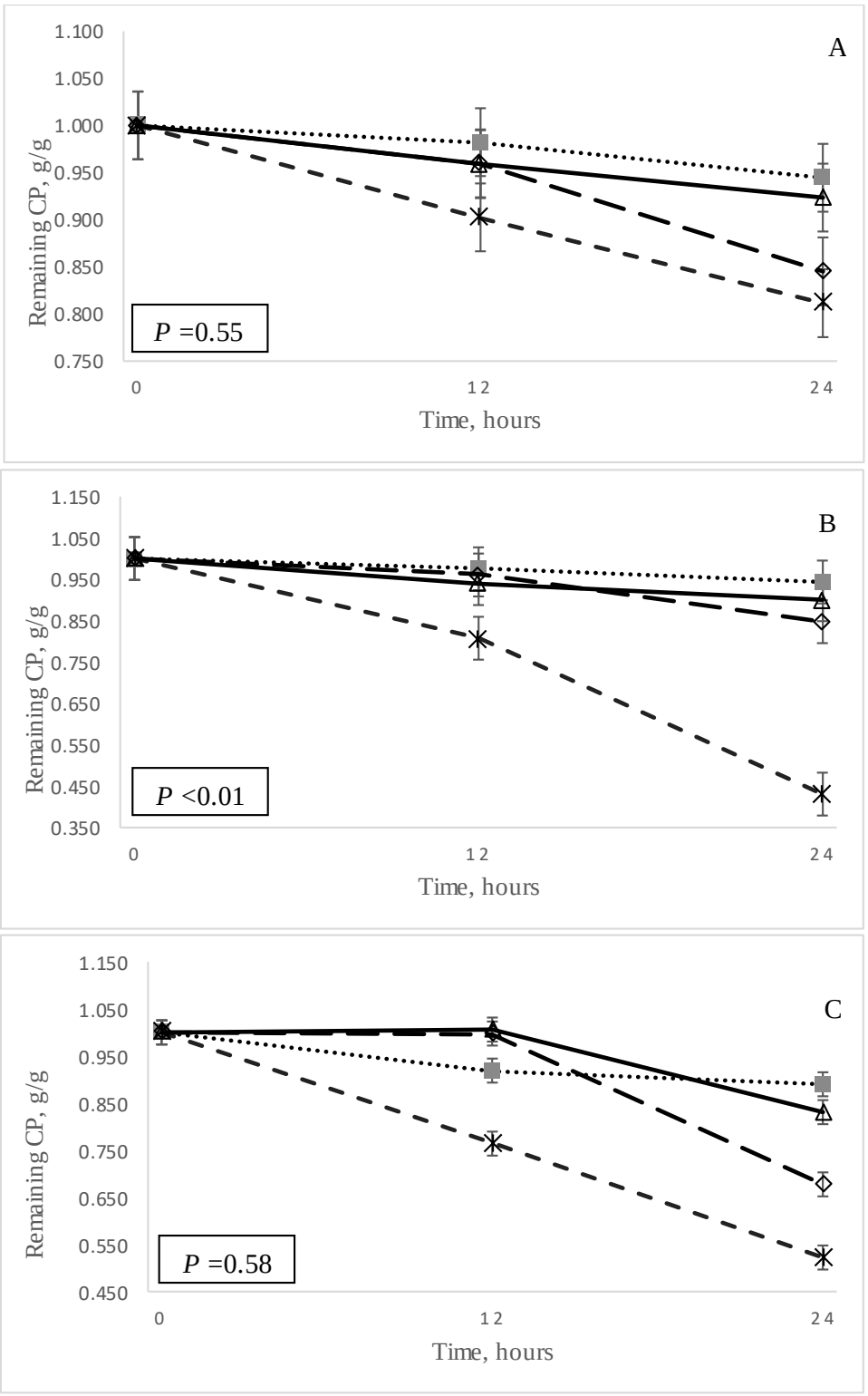

Figure 0-3 Remaining N content in the rumen protected Lys products, (■) RP-Lys Control, (×) RP-Lys 1, (◊) RP-Lys 2, (△) RP-Lys 3, with different exposures time to the TMR. After exposure to TMR (A panel) After washing (B panel) and after 16 h of ruminal incubation (C panel). The N remaining in the samples was calculated as ratio of the N in the initial product and the N content of the sample after each procedure.

Table 0-6 Performance data of dairy cows fed with Lys deficient diet and supplemented or not with rumen protected Lys (RP-LYS)

Item	Treatments[1]			SEM	*P value*		
	CON	0.3RP-Lys	0.6RP-Lys		Trt[2]	WK[3]	Trt x WK[4]
BW, kg	674	651	668	20.8	0.73	< 0.01	0.23
BSC	3.08	3.07	2.89	0.069	0.38	< 0.01	0.23
DMI, kg/d	26.2	26.1	26.0	0.78	0.97	< 0.01	0.06
Milk, kg/d	39.1	37.6	36.8	1.21	0.41	< 0.01	0.64
ECM, kg/d[5]	34.2	33.0	33.3	0.97	0.66	< 0.01	0.89
Protein, %	2.97	3.02	3.09	0.055	0.30	< 0.01	0.57
Protein, kg/d	1.12	1.10	1.11	0.028	0.80	< 0.01	0.41
Fat, %	3.31	3.34	3.48	0.153	0.70	< 0.01	0.99
Fat, kg/d	1.26	1.21	1.25	0.052	0.77	< 0.01	0.98
Lactose %	4.87	4.88	4.83	0.042	0.68	< 0.01	0.99
Lactose, kg/d	1.85	1.79	1.74	0.066	0.46	< 0.01	0.34
Solids, %	12.10	12.20	12.36	0.172	0.55	< 0.01	0.99
Solids, kg/d	4.61	4.45	4.45	0.129	0.59	< 0.01	0.68
SCC x 1,000 cells/mL	83	55	51	1.3	0.45	0.09	0.15
MUN, mg/dL	9.66	9.49	9.90	0.513	0.85	< 0.01	0.29
DMI as %BW	3.90	4.00	3.90	0.08	0.45	< 0.01	0.26
Feed Efficiency[6], kg/kg	1.31	1.26	1.28	0.048	0.78	0.01	0.92
Milk N efficiency[7], %	26.52	26.31	26.09	0.869	0.93	<0.01	0.10
△ Body weight, kg/week	-3.21	-2.81	-3.70	1.22	0.88	<0.01	0.27
△ BSC, points/week	-0.05	-0.03	-0.02	0.019	0.58	<0.01	0.21

[1] Control diet –no RP-LYS; 0.3 RP-Lys- supplemented with 0.3 % of diet DM of RP-Lys; 0.6 RP-Lys- supplemented with 0.6 % of diet DM of RP-Lys;
[2] Main effect of treatments.
[3] Week of lactation effect
[4] Treatment x Week effect
[5] ECM (kg/d) = kg of milk × [(38.3 × % fat × 10 + 24.2 × % true protein × 10 + 16.54 × % lactose × 10 + 20.7) ÷ 3,140] (Sjaunja et al., 1990).
[6] ECM/DMI
[7] Milk N yield/N intake × 100.

Table 0-7 Protein fractions and AA balance[1] in dairy cows fed with Lys deficient diet and supplemented or not with rumen protected Lys (RP-LYS)

	Diets		
Item	Control	RP-Lys 0.3	RP-Lys 0.6
Protein fraction balance g/d			
MP			
Requirements	2658	2613	2615
Supply	2828	2829	2845
Balance	170	216	230
MP allowable milk, kg/d	42.9	42.4	41.8
RDP and RUP			
RDP supply	2454	2431	2441
RDP balance	-128	-126	-123
RUP supply	1723	1740	1754
RUP balance	214	272	289
AA balance g/d			
dLys			
Requirements	175	172	173
Supply from the diet	156	155	155
Supply from RP-Lys	0	20	39
Balance	-19	3	21
dMet			
Requirements	58	57	58
Supply	75	71	72
Balance	17	14	14
dHis			
Requirements	58	57	58
Supply	59	58	58
Balance	1	1	0

[1] All values were estimated using NRC (2001) based on actual averaged DMI, milk yield and composition, and BW of individual cows during 8 wk of data collection.

[2] Control diet –no RP-LYS; 0.3 RP-Lys- supplemented with 0.3 % of diet DM of RP-Lys; 0.6 RP-Lys- supplemented with 0.6 % of diet DM of RP-Lys;

[3] Due to rounding, balance may not exactly match requirements and supply.

[4] Requirements of dMet, dLys, and dHis were calculated as 2.2, 6.6, and 2.2%, respectively, of MP requirements; dMet, dLys, and dHis = digestible Met, Lys, and His, respectively.

[5] Supply of dLys was estimated from Lys content and rumen escape data provided by the manufacturer: 39.3 % Lys and 63.1% rumen escape.

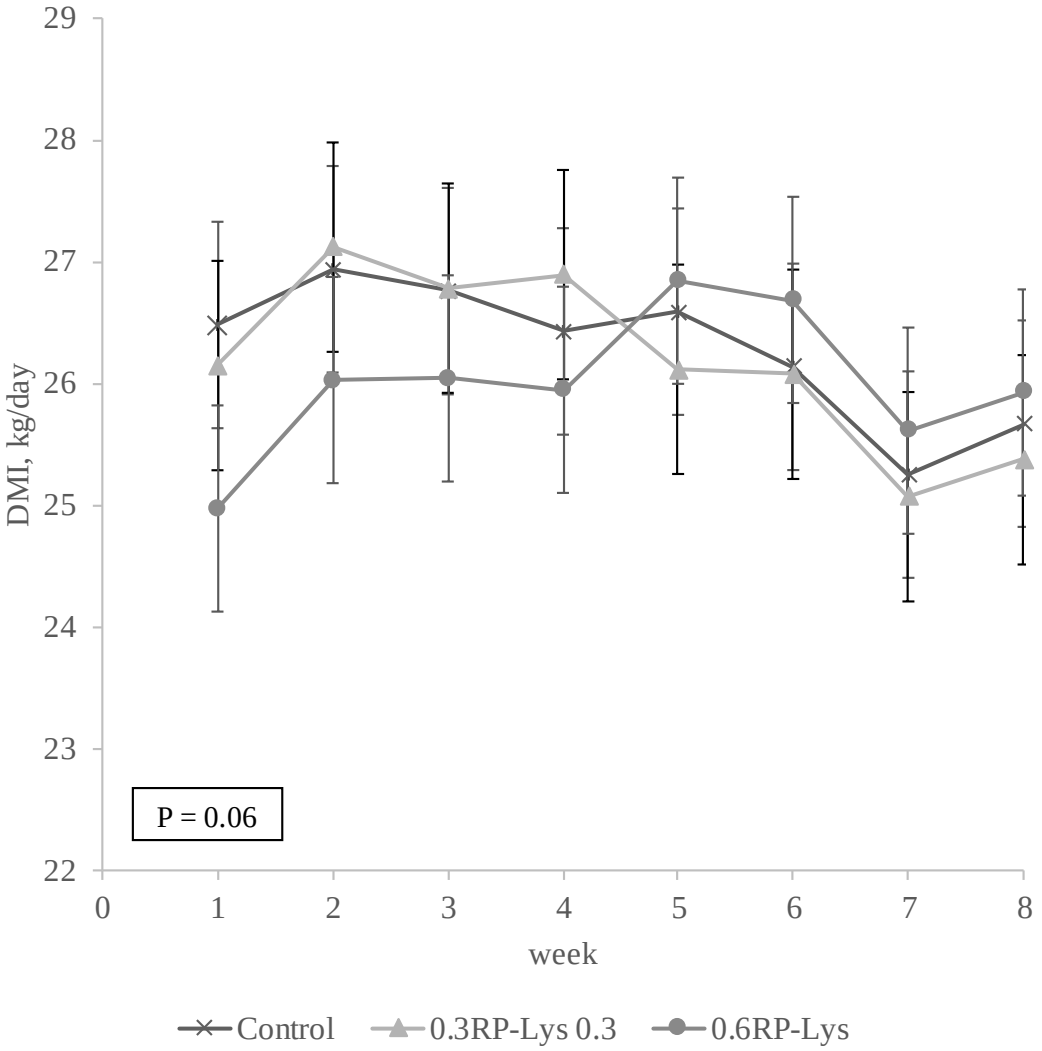

Figure 0-4 Dry matter intake of mid-lactation Holstein dairy cows fed with corn-based Lys deficient diet – control (×) or supplemented with 0.3 % of diet DM of rumen protected Lys (▲) or with 0.6 % of diet DM of rumen protected Lys (●).

Table 0-8 Plasma amino acid concentration (μM) of dairy cows fed with Lys deficient control diet and supplemented with rumen protected Lys (RP-Lys) at 0.3 or 0.6 % of DM

Item	Treatments[1]			SEM	*P value*		
	Control	0.3 RP-Lys	0.6 RP-Lys		Trt[2]	Linear[3]	Quadractic[4]
Arg	59.6	63.7	59.9	4.20	0.73	0.96	0.41
His	45.6[a]	41.2[ab]	32.8[b]	3.66	0.05	0.01	0.65
Ile	111.9	101.9	100.4	6.56	0.41	0.22	0.60
Leu	218.0	203.7	191.7	11.82	0.30	0.12	0.93
Lys	52.5	53.2	56.0	3.70	0.77	0.50	0.81
Met	47.0	47.8	51.0	3.56	0.70	0.43	0.79
Phe	48.1	46.8	43.7	1.96	0.29	0.12	0.71
Thr	88.3	82.7	82.7	5.04	0.66	0.44	0.65
Trp	26.9	25.2	24.4	1.22	0.34	0.15	0.77
Val	241.4	236.1	219.0	12.84	0.44	0.22	0.70
EAA[5]	915.9	893.6	862.2	44.51	0.68	0.39	0.93
Ala	237.7	244.8	240.3	12.75	0.92	0.88	0.71
Asn	46.9	45.2	45.6	2.54	0.88	0.70	0.73
Asp	4.0	4.3	4.1	0.38	0.90	0.94	0.66
Gln	308.7	355.7	331.4	15.16	0.10	0.29	0.06
Glu	44.7	46.1	44.4	1.63	0.74	0.88	0.45
Gly	304.1	301.2	306.3	19.53	0.98	0.93	0.86
Pro	92.8	94.2	90.0	4.56	0.80	0.67	0.62
Ser	79.9	76.2	80.2	3.97	0.73	0.95	0.43
Tyr	55.0	55.0	57.2	3.09	0.86	0.97	0.58
NEAA[6]	1176.2	1222.9	1191.8	40.08	0.71	0.68	0.48
TAA[7]	2285.6	2334.0	2259.1	81.91	0.80	0.81	0.54
Urea	4620.5	4766.7	4726.7	294.8	0.87	0.72	0.71
Aad[8]	14.1	11.7	11.6	1.48	0.41	0.24	0.52
Cit	91.2[ab]	99.9[a]	81.5[b]	4.50	0.02	0.13	0.02
Orn[9]	34.4	33.6	35.7	2.50	0.85	0.73	0.65
Hyl[10]	1.4	1.4	1.6	0.08	0.27	0.19	0.34
1MHis[11]	10.4	9.6	9.7	0.62	0.60	0.41	0.55

[1] Control diet –no RP-LYS; 0.3 RP-Lys- supplemented with 0.3 % of diet DM of RP-Lys; 0.6 RP-Lys- supplemented with 0.6 % of diet DM of RP-Lys;
[2] Main effect of treatments.
[3] Linear effect
[4] Quadractic effect
[5] Essential amino acids
[6] Non-essential amino acids
[7] Total amino acids
[8] Aminoadipic acid
[9] Ornitine
[10] hydroxylysine
[11] 1- methyl histidine

Table 0-9 Plasma amino acid concentration as % of EAA of dairy cows fed with Lys deficient control diet and supplemented with rumen protected Lys (RP-Lys) at 0.3 or 0.6 % of DM

Item	Treatment[1]			SEM	*P value*		
	Control	0.3 RP-Lys	0.6 RP-Lys		Trt[2]	Lin[3]	Quad[4]
Ala	25.8	27.1	28.0	1.20	0.41	0.19	0.86
Arg	6.2	6.8	6.9	0.28	0.16	0.08	0.36
His	4.8	4.4	3.8	0.40	0.25	0.10	0.82
Ile	11.8	11.1	11.5	0.25	0.16	0.36	0.08
Leu	23.1	22.5	22.1	0.46	0.28	0.11	0.84
Lys	5.7	5.9	6.4	0.24	0.09	0.04	0.61
Met	5.0	5.4	5.9	0.31	0.15	0.05	0.99
Phe	5.2	5.2	5.0	0.21	0.76	0.68	0.55
Thr	9.4	9.4	9.7	0.60	0.92	0.73	0.87
Trp	2.8	2.8	2.8	0.12	0.94	0.94	0.73
Val	25.6	25.9	25.4	0.59	0.84	0.84	0.59

[1] Control diet –no RP-LYS; 0.3 RP-Lys- supplemented with 0.3 % of diet DM of RP-Lys; 0.6 RP-Lys- supplemented with 0.6 % of diet DM of RP-Lys;
[2] Main effect of treatments.
[3] Linear effect
[4] Quadractic effect

Table 0-10 Performance data of rumen-cannulated lactation dairy cows treated with postruminal bolus infusion of HCl-Lys, RP-Lys or water

Item	Treatments			SEM	*P* value
	HCl-Lys	RP-Lys	Water		
DMI, kg/d	22.4	23.0	23.0	1.98	0.96
Milk, kg/d	31.0	32.2	31.2	4.90	0.98
BW, kg	719.1	726.6	728.6	22.70	0.95
BCS	3.08	3.25	3.16	0.276	0.91

Table 0-11 Amino acid and metabolites concentrations (μM) in plasma from rumen-cannulated lactating dairy cows treated with postruminal bolus infusion of HCl-Lys, RP-Lys or water

	Treatments			SEM	*P* value		
Item	HCl-Lys	RP-Lys	Water		Trt[1]	Time[2]	Trt x time[3]
Arg	91.8	72.3	69.6	5.60	0.09	< 0.01	< 0.01
His	55.5	53.8	53.8	9.03	0.99	0.62	0.64
Ile	86.1	90.3	86.9	6.12	0.88	< 0.01	0.30
Leu	116.6	119.8	107.7	15.00	0.85	0.59	0.23
Lys	353.9[a]	123.6[b]	68.6[b]	15.35	< 0.01	< 0.01	< 0.01
Met	22.8	22.7	21.4	1.95	0.86	0.54	0.16
Phe	33.9	35.2	31.0	3.03	0.65	0.67	0.33
Thr	87.0	85.4	84.2	5.82	0.95	0.90	0.26
Trp	36.8	36.5	35.4	2.40	0.92	< 0.01	0.37
Val	206.8	209.6	204.1	18.41	0.98	0.06	0.18
EAA[4]	1091.2[a]	849.3[ab]	762.9[b]	63.50	0.046	< 0.01	< 0.01
Ala	283.4	298.0	283.8	22.94	0.88	0.12	0.06
Asn	44.0	44.8	45.2	3.05	0.96	0.90	0.45
Asp	5.8	5.8	4.6	0.73	0.452	0.34	0.53
Gln	278.7	268.9	270.2	14.70	0.88	0.23	0.68
Glu	50.0	55.2	35.6	5.37	0.13	0.05	0.43
Gly	350.1	322.3	368.3	27.41	0.543	0.07	0.62
Pro	79.4	80.0	81.2	7.02	0.984	0.98	0.46
Sep	8.4	7.7	7.8	0.69	0.745	< 0.01	0.86
Ser	82.8	81.7	76.6	3.76	0.513	0.57	0.45
NEAA[5]	1410	1379	1405	32.4	0.781	0.59	0.20
TAA[6]	2501	2228	2168	80.8	0.087	0.01	< 0.01
Urea	4248	4264	4373	3178.5	0.956	< 0.01	1.00
Aad[7]	13.0	10.5	8.3	3.57	0.677	< 0.01	< 0.01
Cit	84.4[ab]	76.1[b]	92.9[a]	2.87	0.036	< 0.01	0.79
Hyl[8]	0.7	5.3	4.9	2.14	0.347	< 0.01	0.05
Orn[9]	49.2	45.2	42.9	4.28	0.607	0.06	< 0.01
1mhis[10]	18.4	18.4	18.3	4.47	1	0.05	< 0.01

[1] Main effect of treatment.
[2] Main effect of sampling time.
[3] Treatment x sampling time effect
[4] Essential amino acids
[5] Non-essential amino acids
[6] Total amino acids
[7] Aminoadipic acid
[8] Ornitine
[9] hydroxylysine
[10] 1- methyl histidine

Table 0-12 Effect of postruminal bolus infusion on EAA concentrations (% EAA) in plasma from lactating dairy cows

Item	Treatments			SEM	*P* value		
	HCl-Lys	RP-Lys	Water		Trt[1]	Time[2]	Trt x time[3]
Arg	12.4	9.9	10.0	0.58	0.06	<0.01	<0.01
His	7.5	7.4	7.8	0.89	0.94	0.04	0.11
Ile	11.5	12.4	12.4	0.89	0.74	<0.01	0.85
Leu	15.5	16.2	15.4	0.98	0.81	0.96	0.72
Lys	27.7[a]	14.3[b]	8.9[c]	1.00	< 0.01	< 0.01	< 0.01
Met	3.0	3.1	3.0	0.17	0.98	< 0.01	0.82
Phe	4.5	4.8	4.4	0.23	0.58	0.82	0.80
Thr	11.9	11.8	12.1	1.15	0.97	< 0.01	0.12
Trp	5.0	5.1	5.1	0.28	0.99	<0.01	0.07
Val	28.1	29.0	29.4	0.90	0.62	0.07	0.11

[1] Main effect of treatment.
[2] Main effect of sampling time.
[3] Treatment x sampling time effect.

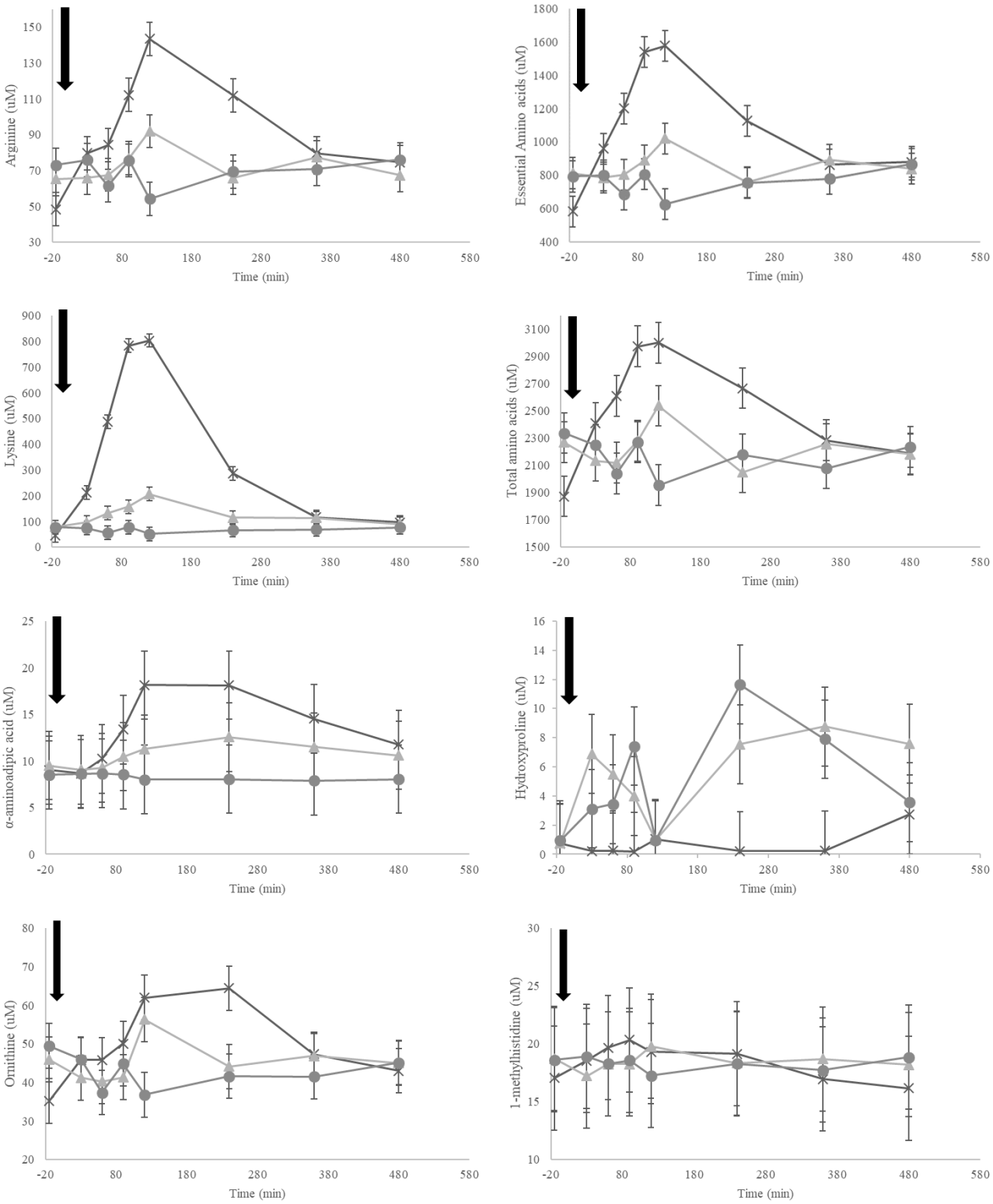

Figure 0-5 Observed amino acid concentrations (μM) following a postruminal bolus infusion of the rumen-protected RP-Lys (▲), HCl-Lys (×) or water (●) in lactation dairy cows, the infusion time (time 0 min) is indicated in the graphics (🡻).

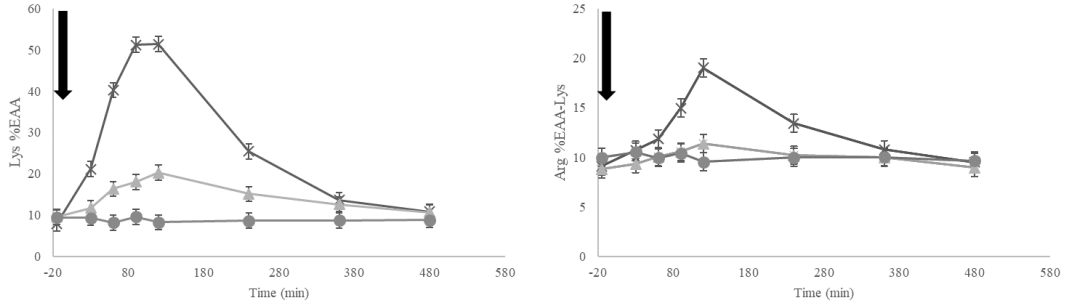

Figure 0-6 Observed amino acid concentrations as % of EAA following a postruminal bolus infusion of the rumen-protected RP-Lys (▲), HCl-Lys (×) or water (●) in lactation dairy cows, the infusion time (time 0 min) is indicated in the graphics (🡻).

Table 0-13 Effect of abomasal bolus of the rumen-protected RP-Lys, HCl-Lys or water in lactation dairy cows on plasma Lys kinetics

Item	HCl-Lys	RP-Lys	Water	SEM	*P* Value
AUC[1]	159621[a]	60989[b]	32064[b]	7638.9	< 0.01
Half Life (min)	546.9	853.0	-886.5	424.18	0.08
Clearance rate (%min)	0.16	-0.01	0.00	0.076	0.29

[1] Area under the curve during the 450 min [(uM)×60 min];

CHAPTER IV– ARTICLE II

Short communication: Increase in Lysine concentration and additional energy did not alter AASS gene expression in MDBK cells

2.1. ABSTRACT

The objective for this study was to determine the effects of extracellular Lys levels and energy supply on AASS mRNA expression. MDBK cells at 80% confluence were cultured in DMEM and exposed for 24 h to 0.5 % bovine serum albumin DMEM with antibiotic-antimycotic solution. The basal DMEM contained 0.8 mM Lysine. Cells received one of the 12 treatments as follows: basal DMEM media no additional Lys or energy source (Control), addition of 1mM of propionate (BLPr); addition of 1mM of lactate to the control media (BLL); addition of 1mM of alanine to the control media (BLA); addition of 0.8 mM of Lys to the control media (2XLB); addition of 0.8 mM Lys and 1mM of propionate to the control media (2XLPR); addition of 0.8 mM Lys and 1mM of lactate to the control media (2XLL); addition of 0.8 mM Lys and 1mM of alanine to the control media (2XLA); addition of 1.6 mM of Lys to the control media (3XLB); addition of 1.6 mM Lys and 1mM of propionate to the control media (3XLPR); addition of 1.6 mM Lys and 1mM of lactate to the control media (3XLL); addition of 1.6 mM Lys and 1mM of alanine to the control media (3XLA). Treatments were applied in duplicate to 3 separate MDBK cell replicates. Abundance of cDNA for each sample was quantified using real-time PCR. The 22DDCT method was used to calculate relative changes in gene expression determined from real-time quantitative PCR. There was no overall effect of treatment on AASS expression ($P = 0.16$), however, increasing Lys concentration in the media reduced AASS gene expression in a linear fashion ($P < 0.01$). Furthermore, the additional of energy to the cultures, regardless of source, did not alter the relative expression of AASS ($P=0.59$). We conclude that although MDBK cells have utility in the study of cellular metabolism and control of gene expression and display expression of AASS that additional information is necessary.

Keywords: amino acids, aminoadipic semialdehyde synthase, protein

2.2. SHORT COMMUNICATION

The high price of protein sources (Moraes et al., 2018) associated with the inefficiency of N use (Spek et al., 2013) and the impacts of N excretion on the environment (Arriola Apelo et al., 2014a) have necessitated a move towards formulating diets for dairy cattle with low crude protein (**CP**) content (St-Pierre and Sylvester, 2005). However, diets formulated with low CP content are typically associated with reductions in milk volume and milk component yield, mostly because of the reduction in the AA profile of metabolizable protein leading to a deficiency of essential amino acids (Arriola Apelo et al., 2014b). One strategy to avoid the drop in performance includes supplementation with rumen-protected essential amino acids (Hristov and Giallongo, 2014).

Lysine (**Lys**) is considered the first limiting amino acid for growth and milk synthesis in dairy cows' fed corn-based diets (NRC, 2001b). However, Lys supplementation does not always translate into an increase in milk production or milk protein yield (Robinson, 2010). The lack of effect on production may be related to an inability to model the impact of a postruminal increase in Lys supply on Lys metabolism. Specifically an increase in supply may increase Lys catabolism, in liver and other tissues, which potentially diverts Lys through the oxidative pathway for energy production and away from protein synthesis (Papes et al., 1999).

Lysine catabolism, in mammals, occurs mainly through the saccharopine-dependent pathway, which is coordinated by the combined actions of lysine ketoglutarate reductase (LKR; EC 1.5.1.8) and saccharopine dehydrogenase (SDH; EC 1.5.1.9) (Benevenga and Blemings, 2007). These enzymes are present in several tissues and reside within aminoadipic semialdehyde synthase (AASS), a bi-functional protein complex (Markovitz et al., 1984; Kiess et al., 2008; Gatrell et al., 2013). Lysine is catabolized to acetoacetyl-CoA and is further converted to ketone body acetoacetate, by acetoacetyl-CoA hydrolase (Voet et al., 2016), that can be used as energy source by extrahepatic tissues.

The activities of both enzymes in the AASS have been shown to be sensitive to Lys supply (Muramatsu et al., 1984; Blemings et al., 1994) and dietary protein content (Kiess et al., 2008). In the liver, LKR and SDH could also participate in the control of the N balance through Lys degradation into α-aminoadipic semialdehyde.

Lysine oxidation increases when intracellular Lys and saccharopine increases through allosteric activation of LKR and SDH by phosphorylation (Foster et al., 1993; Papes et al.,

1999). An increase in AASS activity due to dietary Lys supply could reflect a portion of the observed inefficiency in milk production and milk composition response to increased postruminal lysine as a result of rumen protected lysine supplementation. Conversely a reduction in AASS would be expected to increase Lys availably to extrahepatic tissue and promote mammary protein synthesis when lysine is limiting. Previous studies have shown that reductions in AASS mRNA abundance in the liver could represent 50 % of reduction in the LKR activity and reduced Lys catabolism (Cleveland et al., 2008).

The reduction of lysine oxidation by saccharopine-dependent pathway may be an opportunity to increase Lys availability to peripherical tissues where the need for AA for protein synthesis is great, particularly lactating mammary gland. Therefore, to fully understand Lys use efficiency it is critical to understand the regulation of AASS expression and activity and role in Lys catabolism in dairy cows. Previous studies have shown that postruminal lysine infusion and postruminal protein infusions fail to induce AASS mRNA in lactating dairy cows (Tucker, 2013; Tucker et al., 2017) despite an increase in oxidation of lysine with increasing supply of postruminal protein (Tucker, 2013). Therefore, the role of AASS in Lys catabolism in lactating dairy cows is presently unclear. To address this knowledge gap, we explored the effects of Lys to induce AASS in a bovine cell culture model of Lys metabolism. We hypothesized that (1) increasing Lys would increase AASS mRNA expression due to increased substrate concentration and (2) that energy supplied to cells could alter the catabolism of Lys. Therefore, the objective for this experiment was to determine the effects of extracellular Lys levels and energy supply on AASS mRNA expression as an indicator of a gene specific to Lys catabolism.

For this study Madin-Darby bovine kidney epithelial (MDBK) cells (NBL-1 ATCC CCL-22) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were propagated in sterile polystyrene 182-cm^2 canted-neck, 0.2 µm vent cap flasks (DOT Scientific, Burton, MI) in Dulbecco's Modified Eagle's Medium (DMEM) containing 0.80 mM L-Lysine Hydrochloride, 5.5 mM D-glucose, and 1mM sodium pyruvate (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich Corp., St. Louis, MO), 100 units/mL penicillin, 100 µg/mL streptomycin, and 250 ng/mL amphotericin B (Antibiotic-Antimycotic Solution; Sigma-Aldrich Corp., St. Louis, MO) at 37°C in 5% CO_2 and 95% air. Media was refreshed every two to three days. Cells were dislodged from the plates using trypsin-EDTA (Sigma-Aldrich Corp., St. Louis, MO) and subcultured in 35 mm 6-well plates (Corning, Glendale, AZ) at a density of 5×10^5 cells well and incubated in 10 % FBS DMEM with antibiotic-antimycotic, as indicated above, to achieve approximately 80% confluence before exposure to treatments.

The MDBK cells at 80% confluence were cultured in DMEM as previously described (White et al., 2012) and exposed for 24 h to 0.5 % bovine serum albumin (BSA) (Probumin; Millipore, Billerica, MA) DMEM with antibiotic-antimycotic solution. The basal DMEM contained 0.8 mM Lysine (Sigma-Aldrich Corp., St. Louis, MO). Cells received one of the 12 treatments as follows: basal DMEM media no additional Lys or energy source (Control), addition of 1mM of propionate (BLPr); addition of 1mM of lactate to the control media (BLL); addition of 1mM of alanine to the control media (BLA); addition of 0.8 mM of Lys to the control media (2XLB); addition of 0.8 mM Lys and 1mM of propionate to the control media (2XLPR); addition of 0.8 mM Lys and 1mM of lactate to the control media (2XLL); addition of 0.8 mM Lys and 1mM of alanine to the control media (2XLA); addition of 1.6 mM of Lys to the control media (3XLB); addition of 1.6 mM Lys and 1mM of propionate to the control media (3XLPR); addition of 1.6 mM Lys and 1mM of lactate to the control media (3XLL); addition of 1.6 mM Lys and 1mM of alanine to the control media (3XLA). Treatments were applied in duplicate to three separate MDBK cell replicates.

After exposure to treatments for 24 h, media was removed and cells were disrupted and lysed by additional of 175 µL Buffer RLT (RNeasy Mini Kit, Qiagen Inc., Germantown, MD). Cell lysates from two wells (duplicate) were combined to a 1.5 mL microcentrifuge tube and stored at -80°C pending subsequent RNA isolation.

Cell lysates were homogenized by vortexing and processed using the RNeasy Mini Kit and RNase-Free DNase Set according to manufacturer directions (Qiagen Inc., Germantown, MD). Purified RNA was quantified for each sample by absorbance at 260 nm using a ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE) and sample purity was determined by the ratio of absorbance at 260 nm to 280 nm. Only samples with a ratio of 260:280 greater than or equal to 1.8 were used for further analysis. A 1 µg aliquot of purified RNA from each sample was reverse transcribed to cDNA using an Omniscript RT Kit (Qiagen Inc., Germantown, MD), random decamers (Invitrogen by Thermo Fisher Scientific Corp, Carlsbad, CA) and oligo-dT (Qiagen Inc., Germantown, MD). Abundance of cDNA for each sample was quantified using real-time PCR, Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent Technologies, Inc., Santa Clara, CA) and primers described below. A no-reverse-transcriptase template control was formed by combining equal quantities of purified RNA from each sample. A cDNA pool was formed from equal quantities of cDNA from each sample and then diluted with DNase-free water in a 1:3 dilution series to generate a standard curve. DNase-free water served as the no-template control. The abundance of AASS (EC 1.5.1.8 and EC 1.5.1.9) mRNA was determined using quantitative real-time PCR. Primers were as follows:

bovine AASS, AACAGAGATGCGTTTCCTGCCCTT (forward), AGGAAAGTTTCACGGCCAGATGCT (reverse); and bovine GAPDH, CATGTTTGTGATGGGCGTGAACCA (forward), TGATGGCGTGGACAGTGGTCATAA (reverse). Reactions were as follows: 1 cycle at 95°C for 3 min; 40 cycles of 95°C for 10 s, 60°C for 20 s; and 1 cycle of 95°C for 1 min, 55°C for 30 s, and 95°C for 30 s.

The 22DDCT method was used to calculate relative changes in gene expression determined from real-time quantitative PCR (Livak and Schmittgen, 2001). All samples, standards, and controls were analyzed in triplicate and mean values normalized to GAPDH abundance within each sample. Data are expressed as arbitrary units of mRNA relative to GAPDH abundance within sample. The resulting data were statistically analyzed using SAS software (version 9.3, SAS Institute Inc., Cary, NC). The data are reported as least squares means ± standard error of the mean. The MIXED procedure of SAS was used to analyze the effects of treatments on the AASS gene expression. Analyses of variance was performed using the Proc Mixed procedure of SAS. The model accounted for the fixed effects of treatment. Preplanned were built using coefficients for equally spaced treatment and used to determine the linear and quadratic effects of increasing Lys concentration in the media on the AASS gene expression. The addition of an extra energy source in the AASS gene expression was determined using preplanned comparisons. Means were considered different when $P < 0.05$ and tended to differ if $P \leq 0.05$. Tukey-Kramer studentized adjustments were used for multiple comparisons to separate treatment means.

There was no overall effect of treatment on AASS expression ($P = 0.16$), however, increasing Lys concentration in the media reduced AASS gene expression in a linear fashion ($P < 0.01$). Furthermore, the additional of energy to the cultures, regardless of source, did not alter the relative expression of AASS (P=0.59).

Liver, kidney and intestine are known to have higher AASS activity and therefore greater capacity for lysine catabolism and therefore may modulate Lys availability to other tissues such as the mammary gland. (Gatrell et al., 2013). We utilized MDBK cells for these experiments because they have been shown to display metabolism that resembles bovine liver and have been used previously as a model for study of functional control of metabolism and gene expression (Bionaz et al., 2008; Elgendy et al., 2017).

There is evidence that lysine catabolism catalyzed by the AASS in the liver tissue is an obligatory or inevitable process, and suppression of this catabolism would decrease the Lys requirement by 26%, which would indirectly increase Lys supply to extrahepatic tissues. (Klasing, 2008). We hypothesized in the present study that the increase of available Lys

concentrations in the media would result in increase in AASS expression and sought to make this determination as a first step in studying the control of AASS in bovine. From the data collected we reject our initial hypothesis and conclude that AASS in not upregulated in response to increasing Lys supply.

Concentrations of lysine tested in the current experiment represent 1X, 2X and 3X the concentration found in DMEM where 1X is 0.8 mmol/L. These qualities are 30 to 50X the concentration in portal and arterial blood (Blouin et al., 2002; Chapter 3). Supraphysiological lysine, such as the concentrations used here, can lead to erroneous conclusions when assessing changes in AA metabolism (Cantor et al., 2017) including the inability of cells to utilize additional Lys from the media due to saturation of transport mechanisms or reduction in the mitochondrial lysine uptake and consequently lysine oxidation (Benevenga and Blemings, 2007). The activity of LKR in the liver is sensitive to Lys concentration and concentrations exceeding 15 mmol/L resulted in decreased enzyme activity, eventually reaching complete inhibition when media lysine concentrations reached 75 mmol/L (Pink et al., 2011). A 50% reduction in LKR activity has been observed in murine hepatic cells line correlated to a 20% decrease in AASS mRNA abundance (Cleveland et al., 2008). Information of LKR inhibition in MBDK cells with increasing lysine is not available but our data would suggest a similar response to excessive lysine that is controlled at the transcript level.

Because lysine metabolism may be influenced by the supply of energy (Papes et al., 1999) we explored the effects of increasing Lys in the presence of several cellular energy sources, however the addition of energy source in the media had not effect on AASS gene expression. In the absence of any measures that would indicate energy insufficiencies or energetic challenges to cellular metabolism it is unlikely that the MDBK cells used in the present experiment were metabolically stressed, consequently the provision of additional energy, regardless of the substrate, was without effect.

We conclude that although MDBK cells have utility in the study of cellular metabolism and control of gene expression and display expression of AASS that additional information is necessary, relative to effect of limiting lysine concentrations, before this cell model can be used to better understand control of lysine catabolism. In summary, the Lys concentration and an additional energy source did not affect the AASS gene expression. The increase in the Lys concentration shown is the reduction in the AASS gene expression. Better understanding of Lys metabolism and AASS involvement is still needed to fully employ MDBK cells as a model system for understanding lysine catabolism in bovine.

2.3. ACKNOWLEDGEMENTS

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Table 0-1 Amino acid concentrations (mM) of the extracellular media provided to MDBK cells

Components	Treatments											
	Control	BLPr	BLL	BLA	2xL	2xLPr	2xLL	2xLA	3xL	3xLPr	3xLL	3xLA
Amino Acids												
Glyc	0.400	0.400	0.400	0.400	0.400	0.400	0.400	0.400	0.400	0.400	0.400	0.400
Arg	0.398	0.398	0.398	0.398	0.398	0.398	0.398	0.398	0.398	0.398	0.398	0.398
Cys	0.201	0.201	0.201	0.201	0.201	0.201	0.201	0.201	0.201	0.201	0.201	0.201
Glu	4.000	4.000	4.000	4.000	4.000	4.000	4.000	4.000	4.000	4.000	4.000	4.000
His	0.200	0.200	0.200	0.200	0.200	0.200	0.200	0.200	0.200	0.200	0.200	0.200
Iso	0.802	0.802	0.802	0.802	0.802	0.802	0.802	0.802	0.802	0.802	0.802	0.802
Leu	0.802	0.802	0.802	0.802	0.802	0.802	0.802	0.802	0.802	0.802	0.802	0.802
Lys	0.798	0.798	0.798	0.798	1.596	1.596	1.596	1.596	2.393	2.393	2.393	2.393
Met	0.201	0.201	0.201	0.201	0.201	0.201	0.201	0.201	0.201	0.201	0.201	0.201
Phe	0.400	0.400	0.400	0.400	0.400	0.400	0.400	0.400	0.400	0.400	0.400	0.400
Ser	0.400	0.400	0.400	0.400	0.400	0.400	0.400	0.400	0.400	0.400	0.400	0.400
Thr	0.798	0.798	0.798	0.798	0.798	0.798	0.798	0.798	0.798	0.798	0.798	0.798
Try	0.078	0.078	0.078	0.078	0.078	0.078	0.078	0.078	0.078	0.078	0.078	0.078
Tyr	0.398	0.398	0.398	0.398	0.398	0.398	0.398	0.398	0.398	0.398	0.398	0.398
Val	0.803	0.803	0.803	0.803	0.803	0.803	0.803	0.803	0.803	0.803	0.803	0.803
Energy source												
D-Glucose (Dextrose)	5.556	5.556	5.556	5.556	5.556	5.556	5.556	5.556	5.556	5.556	5.556	5.556
Sodium Pyruvate	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Propionate		1.000				1.000				1.000		
Lactate			1.000				1.000				1.000	
Ala				1.000				1.000				1.000

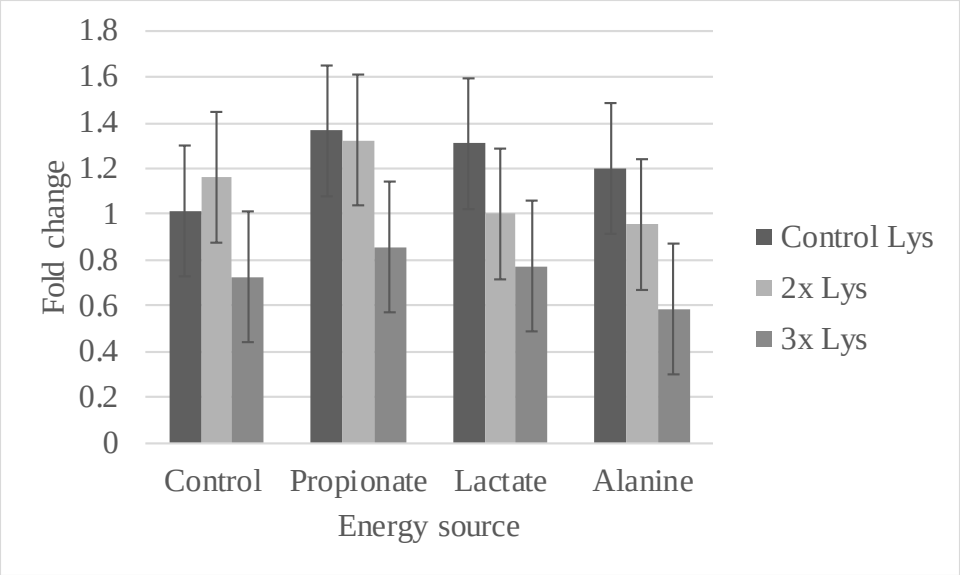

Figure 0-1 Expression of aminoadipate semialdehyde synthase (AASS) in MDBK cells after 24h of exposure to a media with different Lys concentrations (control, 2x Lys, 3x Lys) and energy source additional to media (propionate, lactate or alanine). Cells were cultured in media for 24 h, RNA was extracted and analyzed for AASS and GAPDH mRNA. Data are LSmeans and standard errors for 3 independent cell preparations.

APPENDIX

The appendix presented in this thesis is part of the first work in which I was involved as a member of Dr. Shawn S Donkin's laboratory. The data and sample collection were made by the other students and workers from Dr. Donkin's lab. I was responsible for extracting the cow performance data from Purdue Dairy files, preparing and organizing the blood analysis data in the laboratory, performing the statistical analysis, preparing the tables and drafting the first version of the manuscript. This study was important in training and developing my skills to work with data sets and statistical analysis and develop the skills needed for the main studies of this thesis. In the IVGTT study, data analysis tools and methodologies were used, such as the calculation of the area under the curve, clearance rate and half-life, which were used in the development and analysis of data from the lysine infusion study.

Short communication: Effect of glucose infusion dose and stage of lactation on glucose tolerance test kinetics in lactating dairy cows.

ABSTRACT

The objective for this study was to determine the effects of glucose dose, and stage of lactation during an intravenous glucose tolerance test (IVGTT) on plasma glucose, insulin, and NEFA kinetics in lactating dairy cattle. Six lactating Holstein dairy cows, (3 primiparous and 3 multiparous) were assigned to 2 squares and to receive 0.092, 0.15, or 0.3 g glucose/kg BW during IVGTT during early- (74 ± 8.5 DIM) and mid-lactation (221±8.5 DIM) in a replicated Latin Square design. Treatments were applied in a Latin square design using 21-d periods at each stage of lactation. Periods within stage of lactation were seven days in duration. Milk production and DM intake were determined daily during the first 6 d of each period and the last day of each period was

used for IVGTT. For IVGTT, cows were prepared with indwelling catheters in each jugular vein and blood samples were collected at -15, -10, 5, 10, 15, 20, 30, 45, 60, 90, and 120 min relative to the glucose infusion and analyzed for plasma glucose, serum insulin, and plasma NEFA. Increasing the glucose dose during IVGTT increased ($P < 0.05$) plasma glucose area under the curve (AUC), decreased glucose half-life, and increased maximal glucose concentrations in plasma during IVGTT. Greater glucose dose during IVGTT elevated serum insulin AUC and increased nadir NEFA concentrations. Maximal glucose during IVGTT was lower whereas maximum NEFA, NEFA AUC, and NEFA clearance rate were greater during early lactation compared with mid-lactation. Only glucose half-life was responsive ($P<0.05$) to stage of lactation × glucose dose effects during IVGTT and the decrease in glucose half-life with increasing glucose dose was greater during early lactation compared to mid-lactation. Glucose AUC was greater and NEFA AUC was lower ($P <0.05$) during mid-lactation compared to early-lactation. A glucose dose in excess of 0.092 g/kg BW resulted in peak blood glucose concentration that exceeded the previously reported renal glucose threshold of 8.3 mmol /L. Based on maximal peak glucose concentrations, insulin response criteria and effects on glucose half-life we identify 0.092 g/kg BW (0.46 g/kg $BW^{0.75}$) as a preferred glucose dose for IVGTT in early- and mid-lactation dairy cows.
Keywords: Glucose tolerance, insulin, dose, clearance, IVGTT.

SHORT COMMUNICATION

The insulin resistance is a natural homeorhetic adaptation that occurs in dairy cows and other mammals during the late gestation and persists into early lactation. During this period, tissues that are normally insulin responsive acquire reduced sensitivity to the effects of insulin to promote glucose clearance from blood. This adaptation serves to make more glucose available for non-insulin responsive glucose uptake by other tissues including the growing fetus and mammary gland (Bell and Bauman, 1997). Although the metabolic adaptations to divert glucose to fetal and mammary tissue are important during late gestation, the lack of appropriate timing and degree of adaptation may be associated with metabolic diseases like ketosis, fatty liver, and may be associated with a reduction in milk production potential (Hayirli, 2006).

Intravenous glucose tolerance test (IVGTT) is frequently used to assess systemic glucose metabolism and insulin sensitivity in dairy cattle (De Koster and Opsomer, 2013) and humans as an indicator of health (Singh and Saxena, 2010). Plasma glucose concentration is measured during the IVGTT at predetermined intervals prior to, and following, the intravenous infusion of a bolus dose of glucose. Glucose and insulin kinetics in blood are used to assess physiological status and insulin resistance. A rise in blood glucose in response to intravenous glucose infusion results in a commensurate release of insulin from the pancreas, which in turn acts to clear glucose by insulin sensitive tissues to preinfusion levels. The clearance rate of glucose from blood is dependent on the amount of insulin released from the pancreas and the peripheral response to insulin. Although the capacity for insulin release and responsiveness are confounded during the test, the technique provides a technically simple and inexpensive assessment of insulin responsiveness compared with other techniques (Defronzo et al., 1979). Despite its shortcomings the IVGTT remains as valuable clinical assessment of insulin resistance. In addition, insulin–inhibited adipose lipolysis is also a key feature of IVGTT that can be used to assess insulin responsiveness (Boston et al., 2008). Although the technique has been standardized for other species (Ayala et al., 2010) similar standardization does not yet exist for cattle.

Despite frequent use, there is little standardization of the IVGTT in characterizing glucose kinetics in dairy cattle. Glucose doses of 0.15 (Holtenius et al., 2003), 0.25 (Wheelock et al., 2010), 0.30 (Subiyatno et al., 1996) and 0.50 (Bigner et al. 1996) g/kg BW have all been reported in the literature. It has been suggested that an insufficient dose of glucose during IVGTT may fail to elicit maximal pancreatic insulin release whereas a dose that is too high may overwhelm the ability of the kidney to resorb glucose and result in excretion of glucose in urine (González-Grajales et al., 2018). Both scenarios could result in an inaccurate evaluation of glucose kinetics and estimate of insulin resistance. A direct comparison of glucose dose during IVGTT in lactating dairy cattle is lacking and evaluation of the impact of dose and physiological status is needed to establish a standardized method.

We hypothesize that there is an optimal glucose dose for IVGTT in lactating dairy cattle and that the parameter estimates of insulin, glucose and NEFA kinetics determined from IVGTT are responsive to the dose of glucose administered during the test. Furthermore we hypothesized that the effect of glucose dose during IVGTT would differ with stage of lactation. Therefore, the objective for this experiment was to determine the effect of dosing either 0.092, 0.15, or 0.3 g

glucose/kg BW during IVGTT on glucose, insulin and NEFA kinetics during early- and mid-lactation. The doses selected were based on previous work using 0.15 (Holtenius et al., 2003) and 0.30 (Subiyatno et al., 1996) with inclusion of a lower dose of glucose of 0.092 glucose /kg BW. The doses selected also represent approximately one-half and one-third of the values, per metabolic body weight of those explored to optimize IVGTT in rats (Frangioudakis et al., 2008).

Six lactating Holstein dairy cows, 3 primiparous and 3 multiparous, weighing 640 kg (SD = 116 kg) were blocked and assigned by parity to one of two 3x3 Latin squares. Cows selected for the experiment were housed in a freestall facility and managed as a group and were relocated to a tie-stall facility for two 21-d intensive data collection intervals during early- (74 DIM, SD = 8.5) and mid-lactation (221 DIM, SD = 8.5 DIM). Cows were fed a corn silage based diet throughout the experiment. Feed was delivered once daily as a TMR fed to meet or exceed all nutrient requirements according to NRC (2001). Cows had access to feed at all times including the period prior to and during IVGTT. All animal care and handling protocols were approved by the Purdue University Animal Care and Use Committee.

Each intensive data collection interval was of 21-d in duration and consisted of 3 periods of 7 d each. Each period included 6 d of adjustment and 1 d of IVGTT. Body weight was measured at the beginning of each 21-d interval. The BW for cows during early- late mid-lactation was 618 and 626 kg respectively with a standard error of least squares means for stage of lactation of 59.7 kg. Individual feed intake and milk production was determined for days 1 through 6 of each period and milk composition was determined on day 3 of each period. Milk samples, collected from consecutive milkings, were analyzed for fat, protein, lactose, total solids, MUN, and somatic cells by Dairy One (Ithaca, NY).

Cows were assigned within square to receive either 0.092, 0.15, or 0.3 g glucose/kg body weight (BW) during the IVGTT. On day 7 of each period, approximately 1 hour prior initiation of IVGTT, cows were fitted with indwelling catheters (16-gauge, Abbocath-TAL catheter; Abbott Laboratories, North Chicago, IL) in the left and right jugular veins. One vein was used for infusion and the other for sample collection. Cows were given a sterile 50% glucose solution (Huvepharma, Inc, St Joseph, MO) to deliver the assigned dose of glucose. Infusions were timed and all were completed with 2 minutes of initiation. Following glucose infusions and sample collection catheters were flushed and blocked with heparinized saline (2 I.U./ml).

Blood samples were collected from indwelling catheters using 12 cc syringes at -15, -10, 5, 10, 15, 20, 30, 45, 60, 90, and 120 min relative to the glucose infusion. Samples (10 ml) for glucose analysis were collected into BD Vacutainer tubes (Franklin Lakes, NJ) containing sodium fluoride and potassium oxalate, samples for NEFA contained K_3EDTA. Samples (10 ml) for insulin analysis did not contain any additive and were allowed to clot at room temperature for 30 minutes. Within 30 minutes of collection, plasma and serum were separated by centrifugation at 1,000 x g for 15 min and stored at -20º C or 1-3 months pending analysis.

Plasma glucose concentration was determined by an enzymatic method using commercially available kit (Autokit Glucose, Catalog nr 997-03001, Wako Diagnostics™, Richmond, VA). Plasma NEFA concentration was determined using a commercially available kit (HR Series NEFA-HR, Catalog nr 999-34691, Wako Diagnostics™, Richmond, VA). Serum insulin concentration was determined using a commercially available ELISA kit (ALPCO, Salem, NH). Intra-assay and interassay CV for insulin analysis was 4.71 % and 6.50%, respectively and was less than 5% for all other blood analytes. A single baseline value each insulin, glucose and NEFA excursion curve was calculated as the average of samples collected at -15 and -10 min relative to glucose infusion. The AUC of glucose, NEFA, and insulin were calculated using the trapezoidal rule (Cardoso et al., 2011) and ratios of glucose to insulin, glucose to NEFA, and insulin to NEFA ratios were calculated to estimate systemic insulin sensitivity (Hosseini et al., 2015) . The clearance rate (CR) and half-life of glucose, NEFA, and insulin were calculated as described by Pires et al. (2007).

Data were analyzed using the Mixed Procedure of SAS 9.4 with random effects of the cow nested within treatment by stage of lactation. The normality of data was determined using the UNIVARIATE procedure in SAS and the Shapiro-Wilk test. The model for milk production, DMI, milk composition, blood metabolites, and glucose, NEFA and insulin kinetics accounted for the effects of glucose dose, square, period, and the random effect of cow nested within square. Effects of parity were not tested due to confounding within square. Data are LSmeans and standard errors unless otherwise indicated. Means were considered different when $P < 0.05$ and tended to differ if $0.05 \leq P \leq 0.10$. Tukey-Kramer studentized adjustments were used for multiple comparisons.

Data for BW, DMI, milk production and composition are shown in Table 1. As expected milk production was greater in early lactation and milk fat and milk protein percentages were greater in mid-lactation. There were no other differences in milk composition or body weight

between the two stages of lactation. There were no effects of dose of glucose during IVGTT observed for milk protein, fat, and lactose. And no glucose dose x stage of lactation effects for milk production or composition.

Concentrations of glucose, insulin, and NEFA (prior to IVGTT) did not differ between assigned glucose infusion dose (Table 2). Basal NEFA tended ($P = 0.09$) to be lower for cows in mid-lactation but there were no glucose dose x stage of lactation effects for basal insulin, glucose, or NEFA. There was a significant effect of glucose dose during IVGTT on glucose, insulin and NEFA kinetics (Figure 1; Table 2). Maximal glucose achieved during IVGTT was increased with increasing dose of glucose with greater maximal concentrations observed for mid-lactation cows however there were no stage of lactation by dose of glucose effects for maximal glucose concentration. Glucose AUC increased with increasing quantity of glucose and was greater for mid-lactation cows. There were no stage of lactation × glucose dose effects during IVGTT on glucose AUC. The clearance rate of glucose was increased ($P<0.05$) and half-life was decreased ($P<0.05$) with increasing glucose dose and there was a tendency ($P=0.06$) for decreased glucose half-life for mid-lactation cows. There was a significant ($P<0.05$) stage of lactation × dose of glucose during IVGTT effect for glucose half-life. The longest half-life was observed for the 0.092 g/kg BW glucose dose during early lactation and increasing the glucose dose had a more pronounced effect to reduce glucose half-life during early lactation compared with mid-lactation.

There was no effect of dose of glucose given during IVGTT on peak insulin concentrations or insulin clearance rate but insulin half-life tended ($P=0.06$) to increase with glucose dose. The insulin AUC increased with increasing glucose dose during IVGTT. There was a tendency for greater insulin half-life for mid lactation cows. There were no dose of glucose during IVGTT × stage of lactation effects for any insulin kinetics.

All glucose doses selected for our study (0.092, 0.15, or 0.3 g glucose/kg BW) increased blood glucose concentrations and serum insulin in both lactation phases (Figure 1). In all cases glucose and insulin concentration returned to the baseline after 60 minutes and there was no stage of lactation effect. Others have reported when using 0.3 g/kg BW of glucose in lactating dairy cows that 160 min were required for plasma glucose to return to baseline concentration (Sano et al., 1993; Roche et al., 2008; Oliveira et al., 2016). Reasons for these differences in return to baseline between studies are not apparent but may warrant further investigation in assessing insulin resistance.

Plasma NEFA responded to IVGTT with a biphasic response characterized by decreasing concentration from 5 to 30 minutes post infusion and increasing concentrations from 30 through 120 minutes post infusion (Figure 1). There was a greater depression in plasma NEFA with increasing glucose dose that was not affected by stage of lactation (Table 2). Cows in early lactation displayed a greater increase in NEFA from nadir through 120 min post infusion resulting in a stage of lactation effect for NEFA AUC for 30 to 120 min post glucose infusion. There were no interaction effects of glucose dose during IVGTT or stage of lactation effects for NEFA kinetics.

Because of the physiological relationships between NEFA, insulin, and glucose we evaluated the effect of glucose dose during IVGTT and stage of lactation on the ratio of their AUCs. The AUC for glucose to AUC for NEFA through 60 minutes post glucose infusion increased ($P<0.05$) with increasing glucose dose and was greater ($P<0.05$) for cows in mid-lactation. There was no effect of glucose dose or stage of lactation on ratio of AUC for glucose AUC to insulin or insulin to NEFA.

Despite being a widely utilized assessment tool for glucose metabolism and insulin resistance the IVGTT test for dairy cattle has not been extensively evaluated and standardized. Previous reports of the dose of glucose used during IVGTT in cattle indicate a range of levels across age, physiological status, and BW (González-Grajales et al., 2018). Experiments in growing heifers using doses ranging from 0.5 to 3 kg glucose/kg $BW^{0.75}$ indicate an optimal dose for dairy heifers between 0.5 to 1 g of glucose/kg of $BW^{0.75}$ for IVGTT in order to maximize insulin response and avoid plasma glucose concentrations that approach the renal threshold of 5.5 to 8.3 mmol /L (Blum et al., 1999; Stämpfli and Oliver-Espinosa, 2015; González-Grajales et al., 2018). Based on the average BW for animals used in the present experiment the equivalent glucose doses were 0.46, 0.75 and 1.5 g/kg $BW^{0.75}$. Contrary to work in dairy heifers (González-Grajales et al., 2018) our lowest dose of glucose (0.092 g/kg BW, 0.46 g/kg $BW^{0.75}$) was enough to trigger a maximal response in insulin concentrations in our lactating dairy cows and there was no effect ($P>0.05$) of dose on maximum insulin concentration during IVGTT. In addition, all doses except 0.092 g/kg (0.46 g/kg $BW^{0.75}$) resulted in blood glucose concentrations in excess of the upper reported values for renal threshold of 8.3 mM (Blum et al., 1999). Furthermore a dose of 0.092 g/kg permitted observation of differences in glucose half-life between early and mid-lactation dairy cows, and observation that is obscured with glucose doses of 0.15 and 0.30 0.092 g/kg. Based

on maximal peak glucose concentrations and insulin response criteria and lack of dose × stage of lactation effects with IVGTT we identify 0.092 g/kg BW (0.46 g/kg $BW^{0.75}$) as the preferred target glucose dose for IVGTT in early- and mid-lactation dairy cows.

Cows in this study produced more milk during early lactation and had reduced maximal glucose, glucose AUC. It is generally accepted that dairy cows are insulin resistant in early lactation (De Koster and Opsomer, 2013) which helps to prioritize glucose supply for lactating mammary gland. Because glucose uptake by the mammary gland is insulin-independent (Laarveld et al., 1981) the level of milk production relative to dose of glucose during IVGTT may have been a factor in our experiments. The observed effect of glucose dose on glucose half-life during IVGTT is greater during early lactation which suggests an effect of level of milk production on glucose clearance rate. Greater milk production potentially provides for greater insulin independent mammary uptake and metabolism and may result in more rapid glucose clearance. However, the lack of effect of stage of lactation on the ratio of AUC glucose to AUC insulin would suggest the proportions of insulin and noninsulin mediated glucose uptake are similar between these stages of production. These findings parallel previous observations that the capacity of insulin to clear glucose is similar between physiological states in dairy cattle (dry versus lactating) and that the primary observed differences in glucose metabolism are due to ability to release insulin in response to changes in blood glucose (Sano et al., 1993) and that although the response of insulin-sensitive tissues may change with stage of lactation, whole body responsiveness to insulin does not appear to be altered (Marrett et al., 2015).

Because plasma NEFA is sensitive to changes in blood insulin through the inhibition of hormone-sensitive lipase by insulin (Ferrannini et al., 1997) the use of IVGTT has been explored as a tool to characterize the sensitivity of adipose tissue to changes in insulin concentrations (Boston et al., 2008). Current models represent the characteristic initial down-slope of NEFA concentrations in response to IVGTT, a nadir, and then rebound of NEFA to exceed pre IVGTT test levels (Boston et al., 2008). Our data follow this pattern and indicate a change in NEFA with glucose dose and stage of lactation. This is consistent with the greatest AUC for glucose at the highest glucose dose and greater AUC with advancing lactation. Consequently, there is a significant effect of glucose dose and stage of lactation on the ratio of glucose AUC to NEFA AUC however these effects are not mirrored by the ratio of AUC for insulin to AUC for NEFA. The latter suggest a codependency of insulin and NEFA that is not shared by glucose which may

represent an effect of glucose clearance that is dose dependent but not associated with insulin mediated clearance and may include renal clearance.

Our results reinforce the importance of standardization of IVGTT and justification of dose of glucose infused. For many IVGTT parameters, the dose and stage of lactation impact glucose, insulin and NEFA kinetics but only glucose half-life was responsive to both factors. Given the physiological connection between changes in insulin, glucose and NEFA it is important to minimize a bias in data resulting from excessive glucose dosing during IVGTT and the resulting impact of renal clearance on glucose kinetics. A glucose dose that exceeds the correct target dose for IVGTT may result in an overestimate of glucose clearance rate and inability to detect insulin resistance. Although we did not directly evaluate the glucose urinary excretion it appears that a dose of 0.092 g/kg BW is a desired target for IVGTT in early- and mid-lactation dairy cows based on ability to evoke an increase in plasma glucose that is below renal threshold, effects to stimulate an increase plasma insulin and NEFA concentrations and effect to reveal differences is glucose half-life that would be occurred with a higher glucose dose.

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Table 0-1 Effect of stage of lactation and glucose load during intravenous glucose tolerance test on mean DMI, body weight, daily milk yield, and milk composition

	Treatment[1] Glucose dose g/kg BW									
	0.092	0.15	0.3	0.092	0.15	0.3		P Value		
Item	Early lactation			Mid lactation			SEM	Dose[2]	Stage[3]	Stage x Dose[4]
DMI, kg/d	22.2	22.9	21.9	21.5	22.0	22.0	2.03	0.95	0.77	0.96
Milk yield, kg/d	39.5	39.4	40.3	32.4	36.23	33.6	3.18	0.83	<0.05	0.8
Fat, %	3.17	2.80	3.09	3.84	3.85	3.82	0.319	0.83	<0.05	0.8
Fat yield, kg/d	1.31	1.07	1.27	1.28	1.36	1.35	0.192	0.85	0.42	0.68
Protein, %	2.51	2.30	2.49	2.92	2.87	2.96	0.210	0.74	<0.05	0.91
Protein, kg/d	1.04	0.88	1.02	0.98	1.00	1.05	0.157	0.8	0.79	0.82
Lactose, %	4.45	4.10	4.42	4.87	4.78	4.82	0.321	0.72	0.05	0.86
Lactose, kg/d	1.84	1.55	1.81	1.64	1.67	1.72	0.268	0.81	0.77	0.81

[1]Treatments: Glucose dose of 0.092, 0.15, or 0.3 g glucose/kg body weight during IVGTT during early and mid-lactation.
[2]Main effect of glucose dose.
[3]Stage of lactation effect
[4]Stage of lactation x treatment effect
5

Table 0-2 Effect of stage of lactation and glucose dose during intravenous glucose tolerance test on plasma glucose, insulin, and nonesterified fatty acid kinetics

	Treatment[1] Glucose dose g/kg BW									
	0.092	0.15	0.3	0.092	0.15	0.3		P Value		
Item	Early lactation			Mid lactation			SEM	Dose[2]	Stage[3]	Stage x Dose[4]
Glucose										
Basal (mM)	3.90	3.86	3.66	3.74	3.48	3.83	0.16	0.67	0.35	0.27
Maximum (mM)	6.20	8.31	12.57	7.64	9.44	13.87	0.49	<0.05	<0.05	0.95
CR 5 to 60 (%/min)	0.95	1.40	2.19	1.25	1.61	2.07	2.19	< 0.05	0.15	0.14
Half life 5 to 60	74.35[a]	50.32[a]	31.87[a]	57.49[b]	44.22[bc]	36.04[c]	3.82	<0.05	0.06	<0.05
AUC 0 to 60 (mM/min)	84.66	102.54	143.49	94.90	114.28	158.52	4.63	< 0.05	<0.05	0.87
Insulin										
Basal (ng/mL)	0.13	0.07	0.10	0.13	0.05	0.11	0.02	0.07	0.80	0.87
Maximum (ng/mL)	1.38	1.78	2.11	1.60	1.34	1.86	0.28	0.20	0.52	0.50
CR 5 to 60 (%/min)	6.07	4.97	4.25	4.92	3.83	2.87	0.98	0.17	0.14	0.99
Half life, 5 to 60 min	16.15	15.76	18.20	14.66	21.84	31.62	3.76	0.06	0.06	0.16
AUC 0 to 60 (ng/min)	28.04	42.23	67.27	31.60	37.17	67.98	8.96	<0.05	0.97	0.88
NEFA										
Basal (mM)	0.27	0.22	0.27	0.21	0.18	0.20	0.27	0.49	0.09	0.91
Minimum (mM)	0.14	0.13	0.10	0.12	0.10	0.09	0.01	<0.05	0.12	0.79
Maximum (mM)	0.49	0.44	0.35	0.27	0.24	0.24	0.06	0.45	<0.05	0.67
AUC, 0 to 60 (mM/min)	4.41	3.56	4.25	3.15	3.02	2.92	0.57	0.69	<0.05	0.74
AUC, 0 to 120 (mM/min)	34.84	32.63	24.28	25.05	20.05	18.71	3.79	0.11	<0.05	0.65
CR, 5 to 30 (%/min)	1.89	2.06	3.52	2.38	2.06	2.18	0.49	0.06	0.14	0.41
CR, 30 to 120 (%/min)	-0.90	-1.16	-1.19	-0.73	-0.54	-0.82	0.18	0.53	<0.05	0.46
Half life, 5 to 30 min	86.02	34.77	37.66	74.18	101.81	39.86	32.18	0.43	0.19	0.57
Half life, 30 to 120 min	-22.51	-61.76	-65.65	-105.69	-58.30	-122.56	41.37	0.67	0.19	0.57
Ratio of AUC 0 to 60 min										
Glucose : Insulin	3.09	2.73	2.27	3.77	3.30	2.71	0.47	0.15	0.15	0.96
Glucose : NEFA	22.86	29.23	37.43	35.54	41.78	58.51	4.85	<0.05	<0.05	0.61
Insulin : NEFA	7.84	12.64	19.24	12.31	13.63	28.53	5.49	0.26	0.58	0.75

[1]Treatments: Glucose dose of 0.092, 0.15, or 0.3 g glucose/kg body weight during IVGTT during early and mid-lactation.

[2]Main effect of glucose dose.

[3]Stage of lactation effect

[4]Stage of lactation x treatment effect

[a,] Means with different superscripts differ $P < 0.05$

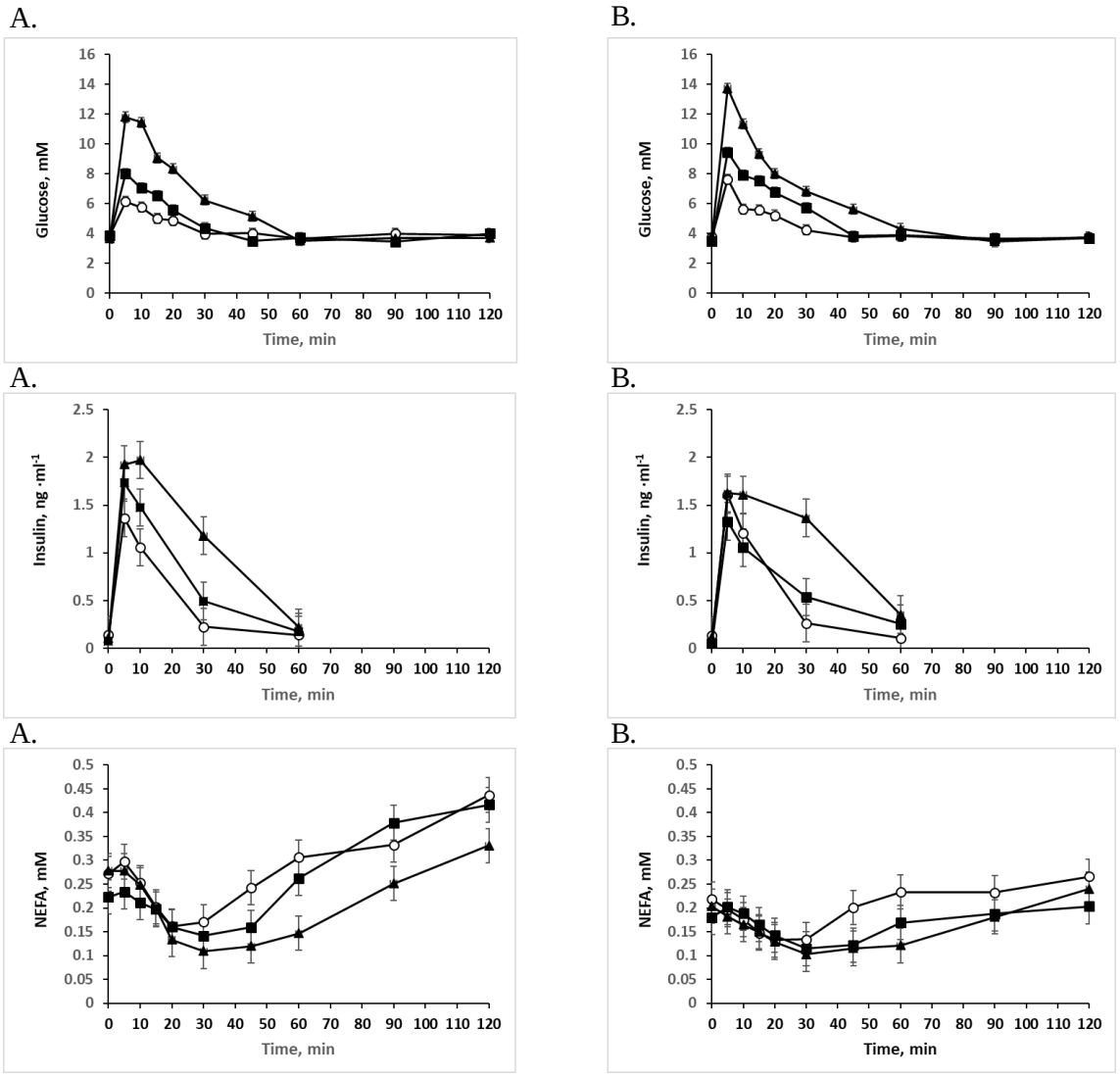

Figure 0-1 Plasma glucose, insulin and nonesterified fatty acids in response to glucose dose of 0.092 g glucose/kg BW (○), 0.15 g glucose/kg BW (■) or 0.3 g glucose/kg BW (▲) during IVGTT during early (A panels) and mid-lactation (B panels). Data are least-squares means and standard errors.