ARGININE SUPPLEMENTATION FOR IMPROVED REPRODUCTIVE PERFORMANCE IN BEEF CATTLE

A Thesis

by

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ABSTRACT

The reproductive efficiency of beef cows has long been a major determinant of success for cattle producers nationwide, and any method of improvement to this efficiency will aid the industry in being more profitable. Arginine has been proven as an effective nutrient for enhancing embryonic survival in non-ruminant species. Arginine improves the intrauterine environment by increasing levels of nitric oxide (NO) and polyamines, which promote angiogenesis, increase the amounts of nutrients for the fetus, and enhance placental and fetal development. Therefore, its use in ruminant species should provide an effective means of improving pregnancy rates among cow-calf producers.

This study also establishes for the first time that two extracellular amino acids (AAs), glutamate and citrulline, may be able to bypass the rumen and its microbes without undergoing significant degradation. This was substantiated in two in-vivo studies with multiple ruminant species and in one in-vitro study. The experimental results go against the long-held notion that all AAs undergo extensive degradation when exposed to the rumen's microbial population. The fact that no degradation of extracellular citrulline occurs in the rumen allows for the provision of this AA to ruminants as an economically viable and easily implemented means for cow-calf producers to increase arginine concentrations in cows. Collectively, the results of this thesis research indicate that dietary supplementation with citrulline is a useful, cost-effective method for improving reproductive efficiency across the beef cattle industry.

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NOMENCLATURE

AA	Amino acid
Ala	Alanine
Arg	Arginine
Asp	Aspartate
Asn	Asparagine
BW	Body Weight
Cit	Citrulline
Cys	Cysteine
CP	Crude Protein
DIP	Digested Intake Protein
EAA	Essential Amino Acid
Gln	Glutamine
Glu	Glutamate
Gly	Glycine
GnRH	Gonadotropin Releasing Hormone
His	Histidine
HPLC	High-performance Liquid Chromatography
Ile	Isoleucine
IUGR	Intrauterine Growth Retarded
Leu	Leucine
Lys	Lysine
Met	Methionine

NEAA	Non-essential Amino Acid
NPN	Non-protein Nitrogen
No	Nitric oxide
Orn	Ornithine
Phe	Phenylalanine
Pro	Proline
RPAA	Rumen Protected Amino Acids
RUAA	Rumen Unprotected Amino Acids
RUP	Ruminally Undigested Protein
Ser	Serine
Thr	Threonine
Tyr	Tyrosine
UIP	Undigested Intake Protein
Val	Valine

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Human consumption of animal protein is projected to increase with the expected increase in the world population. Currently, on the global average, animal-derived protein is about 30% of the total protein consumption and this percentage is expected to increase (Wu et al. 2014) due to the growing middle class. To supply adequate amounts of animal protein, both increases in the number of animals and productivity across animal industries will be required. In terms of the beef cattle industry, the enhancement of reproductive efficiency is an attractive approach to increasing both beef cow inventory and the beef industry's overall productivity.

Among the most important determinants of a cow-calf enterprise's financial viability and sustainability is reproduction. Quantification of infertility is challenging when considering the numerous methods of management and potential environmental factors that can play a part in altering reproductive performance in beef cattle. Bellows et al. (2002) estimated that $441 to $502 million in losses of yearly income occurs in the beef cattle industry due to reproductive diseases and conditions. Three-fourths of this cost was associated with infertility of females and the inability to produce a healthy calf that survived over 24 h of life. This estimated loss was approximately 3.6% of the total value of production by the beef industry the same year. This reproductive failure creates losses from a decline in production stemming from delayed conception and increases in financial inputs in the form of treatment and preventative measurement costs (Bellows et. al, 2002). Due to reproduction being the primary factor influencing profitability, new strategies that enhance fertility and promote efficiency of reproduction within the beef cattle industry should be studied and implemented.

The objective of this chapter is to highlight the literature pertinent to the author's M.S. thesis, namely, reproductive efficiency in beef cattle, amino acid nutrition in ruminants, and the use of arginine to improve embryonic/fetal survival in mammals.

Reproductive Efficiency in Beef Cattle

In terms of increasing the net calf crop, the primary factor becomes the ability of the heifer or cow to become pregnant and maintain their pregnancy to term. Prior research has shown that embryonic death is the primary determinant of reproductive failure and ultimately the major source of inefficiency in livestock reproduction. Estimates have been reported that approximately 30% of all potential neonates are lost between the events of the initial cleavage stages of embryos and parturition (Bolet, 1986) and other researchers reported that embryonic death usually begin by d 16 of gestation (Maurer and Chenault, 1983). This was further confirmed by findings of higher embryonic loss (~30%) by d 7 of pregnancy in sub-fertile cows (e.g., repeat breeders) (Ayalon, 1978; Maurer and Chenault, 1983; Gustafsson, 1985), whereas in cows with improved fertility, embryonic losses (~40%) were observed to occur more gradually between d 8 to 17 of gestation (Ayalon, 1978; Diskin and Sreenan, 1980; Roche et al., 1981). Bellows et al. (1979) recorded the various factors accounting for reductions in net calf crops over 14 years in a naturally bred group of beef cattle. A calf crop of 71% was reported and this reduction resulted from females that failed to conceive or early embryonic death (17.4%), fetal deaths during gestation (2.9%), perinatal calf deaths (6.4%), and calves dying between birth and weaning (2.9%).

Several different factors can influence the prevalence and specific timing of embryonic death such as parity, nutritional status, semen quality of the bull, environmental factors, hormonal influences, genetics, and disease (Thatcher et al., 1994). An experiment exposing beef cows to

heat stress during early pregnancy showed potential negative impacts such as decreased pregnancy rates and lower conceptus weights (Biggers, 1987; Putney et al., 1989). Heat stress also results in an increase in uterine temperature, which is believed to alter the metabolic rate of the developing conceptus and create a sub-optimal environment for growth (Biggers, 1987). By changing the metabolic rate, the uptake of nutrients by the conceptus is also affected and was thought to be the primary factor behind decreased pregnancy rates and decreased conceptus weights (Biggers, 1987). Another potential reason for higher embryonic loss is the increase in temperature causing chromosomal abnormalities as the oocyte is released from its first meiotic arrest and resumes meiosis (Thatcher et al., 1994).

Dystocia poses a significant threat to cow calf producers, due to the potential loss of both cow and calf. This not only effects the likelihood of a successful birth, but the future production of both cow and calf (Bellows, 1988). The occurrence of dystocia-related fatalities was estimated to be 45.9% of all preweaning deaths (Patterson et. al, 1987). Laster and Gregory (1973) studied the factors related to early post-natal mortality over a 5-year period involving 5,064 cows where all parturitions were evaluated and scored on their difficulty. Calf mortality for parturitions experiencing dystocia was recorded at 20.4%, while only 5.0% mortality was seen in parturitions requiring no assistance (Laster and Gregory, 1973).

Nutritional strategies to enhance embryonic survival has drawn increased attention due to the livestock producers' ability to control nutritional inputs (Dunn and Moss, 1992). Oocyte development, ovulation, fertilization, embryonic survival, and establishing pregnancy are all directly influenced by nutrition through the supply of specific nutrients, whereas nutrition also indirectly effects fertility through circulating concentrations of hormones and metabolites in blood (Robinson et al., 2006). Diets that are lacking in either energy or protein put animals in a

negative energy balance and the animal's energy demands for maintenance, reproduction, and lactation exceed the energy intake. For this reason, body weight and body condition scoring have been used by producers and researchers as indicators of energy status in cattle and potential rebreeding performance post-calving (Randel, 1990). Body condition or body nutrient stores has a strong influence on pregnancy rates in beef cows and heifers. One potential mechanism whereby nutritional deficiencies can affect embryonic survival is impaired embryonic growth and development. Ewes that were fed 25% of their maintenance energy requirements showed no differences in embryonic survival, but restricted ewes from 11 to 21 d post-mating had fetuses with shorter crown-rump lengths and were less developed (Parr et al., 1982).

Quality of nutrition has also been found to alter normal hormonal cycles that accompany stages of the cow's normal estrous cycles. Increased sensitivity to the negative feedback effects of estradiol was observed in cows that were nutritionally deficient (Keisler and Lucy, 1996; Wettemann et al., 2003) and this resulted in the animal being acyclic for up to 100 days or longer (Williams, 1990; Hunter, 1991). This hypersensitivity works to prolong the postpartum anestrus period due to a decrease in release of GnRH and this delayed return to cyclicity will reduce a cow's reproductive efficiency. Poor nutrition was also shown to be correlated with higher levels of circulating progesterone. This relationship was reported to occur whether the progesterone source was endogenous (ovary) or from injections administered to ovariectomized ewes (Parr et al., 1982). Underfed ewes had concentrations of progesterone in blood that increased more rapidly between d 2 and d 6, when compared to ewes who consumed diets with adequate energy (Rhind et al., 1989).

Impacts of Protein and Amino Acid Supplementation

Continuous fermentation within the rumen environment is possible due to its wide array of bacteria, archaea, protozoa, and fungi (Mackie, 2011). The ruminant's utilization of both dietary protein and non-protein nitrogen (NPN) involves the rumen, abomasum, and small intestine (Wu, 2018). For the ruminant to achieve maximum feed intake, nutrient digestion, and ruminal health sufficient for rumen-degraded CP (RDP) must be provided (Wu, 2018). As research on protein nutrition has continued, more emphasis has been put on the AA profile that is absorbed after microbial and enzymatic digestion occurs. The breakdown of dietary protein and the resulting synthesis of microbial proteins in the rumen results in a lack of correlation between the AA profile of the diet and the AA profile that is found in the bloodstream (Weller, 1957).

The microbiome within the rumen allows ruminants to receive higher-quality microbial protein than the protein provided in lower quality feedstuffs. Research by Sok et al. (2017) attempted to show the different AA profiles that existed between fluid associated bacteria and particle-based bacteria within the rumen and how this could affect the AA composition of the microbial protein flow. Optimizing protein nutrition in ruminants has long been pursued by researchers as a strategy to increase overall production without sacrificing profitability. The pursuit of optimizing the ruminant's protein supply requires reliable estimates of quantities pertaining to 1) the AA profile of protein flow, 2) dietary AA entering and being absorbed by the small intestine, and 3) AA requirements for maintenance and production (Merchen and Titgemeyer, 1992).

The 20 proteinogenic AA in animal cells were traditionally classified as nutritionally essential (EAA) or non-essential AA (NEAA). In conjunction with this classification, arginine, cysteine, glutamine, glycine, proline, and tyrosine are now referred to as conditionally EAA (Nelson and Cox, 2008; Wu, 2013). Early studies involving isotopic tracers performed by Black

et al. (1957) and Downes (1961), which used dairy cattle and sheep, lead to the conclusion that the classification of EAA in ruminants was similar to EAA of non-ruminants.

AAs serve as precursors for protein synthesis and other nitrogen containing metabolites involved in gluconeogenesis, and as metabolic energy when they are oxidized to CO_2 (Wallace and Chesson, 1995). Rumen bacteria can synthesize all EAA, assuming supplies of ammonia, carbohydrates, and sulfur are readily available. Although the synthesis of these AA is ongoing, it may not be an adequate supply to meet the EAA requirement of a high-producing animal (Lapierre et al., 2006).

In non-ruminants, dietary intake reflects the supply of nutrients. This makes correcting dietary deficiencies relatively easy to manage. Deficiencies of AA can be corrected by simply adding the deficient AA directly into the diet. AA deficiencies in ruminants must be overcome using other strategies to fulfill AA requirements because of the lack of control of the microbes' metabolic pathways. Simply adding any AA into the diet is not an efficient option to increase AA flow within the duodenum in cattle (Lapierre, 2006). There is a limited amount of data regarding the AA content in rumen fluid, endogenous protein sources, and undegraded intake protein (UIP) fractions of consumed feedstuffs (Clark et al., 1992). This protein, once enzymatically digested, will be hydrolyzed by proteases in the small intestine to free AA, dipeptides, and tripeptides. Researchers have attempted to predict this supply of protein with estimates deriving from experiments with ruminants cannulated either at the abomasum or small intestine level (Hvelplund, 1986). Results from several experiments have been called into question because the location of the cannula in relation to the digestive tract can cause wide variations in results. The interpretation of the data becomes difficult because the non-ammonia nitrogen supply that reaches the small intestine consists of UIP, microbial protein, and

endogenous protein. This creates more difficulty in making inferences on any one of the individual sources of protein.

An assumption existed that the AA composition of the RUP was identical to that of the original feedstuff. Results from the work of W.G. Bergen and colleagues indicate that this assumption is not valid for plant-based diets (Bergen, 1979). Studies have shown that the AA profile of the undegradable fraction of protein leaving the rumen is different than the dietary AA profile following rumen fermentation (Puchala et al., 1991) and other studies have shown that the profiles of some AA in both the RUP and original dietary source are similar (Ganev et al., 1979). There have been attempts to use individual AA concentrations in a wide variety of feeds (Hvelplund, 1989) and silages (Von Keyserlinkgk, 1998) and create relationships between the flow of individual AA from feed to the duodenum, but no strong relationship could be established.

There is limited data on the contributions of the endogenous protein supply to total protein flow in the small intestine. Likewise, little is known about the factors that affect its overall impact. Most attention is directed towards the other two sources of protein, although estimates have varied from 16% of the total protein flow (Lammers-Weinhoven et al., 1998) to as high as 56% (Hannah et al., 1991). This endogenous source protein ultimately provides AA to the body although the protein is originally derived from multiple sources such as glycoproteins from mucus, epithelial cells that are shed, bile, and digestive enzymes released in the abomasum and duodenum (Larsen et al., 2010).

Of the sources of protein leaving the rumen, microbial protein has been estimated to be the largest contributor. Results from experiments estimate that 60 to 90% of the total AA that enter the small intestine of the ruminant are from microbial proteins (Butter and Folds, 1985;

Nocek, 1988). It is apparent that microbial protein has a large role in dictating the quality of protein entering the small intestine. Because rumen fermentation leads to production of microbial proteins, ruminants are not thought to have dietary requirements for EAA. Many of the biosynthetic pathways have been identified by in vitro experiments. It is difficult to ascertain the activity of these bacteria and their enzymes in vivo. Although rumen bacteria can synthesize all EAA as noted previously, the supply of microbial EAA may not be adequate to meet the EAA requirement of a high-producing animal (Lapierre et al., 2006).

Free AA are intermediate products as protein is broken down upon entry into the rumen and these AA can have multiple fates including degradation to ammonia by microbes, rumen microbe assimilation, absorption from the rumen, and becoming bound to microbial cells or feed particles (Chalupa, 1975). There is also limited research concerning the degradation rates of AA within the rumen. Low concentrations of these AA were assumed to be a result of proteolysis occurring more rapidly than the rate of uptake (Annison, 1956; Leibholz, 1969). Several factors affect the metabolic activity of microbes such as: pH, protein structure, and the predominant species of microbes in the rumen (Bach, 2005; Scheifinger, 1976). Conflicting views exist pertaining to the estimated degradation rates of individual AA undergoing rumen fermentation. Individual strains of microbes use the supply of AA differently and this is believed to cause the utilization rate for individual AA to be different (Scheifinger, 1976). Mixtures of AA were found to be degraded more rapidly than individual AA, although no explanation or mechanism was offered to explain this finding (Lewis, 1955). Continued research on this topic seemed to indicate that all EAA are degraded to the same extent (Macgregor et al., 1978), while the results from other studies indicated that this assumption may not be true. EAA have been found to be degraded more slowly compared to the NEAA fraction of proteins (Cozzi et al., 1995). It should

be noted that rates of degradation of individual EAA differ among different feedstuffs, including meat meal, herring meal, and corn gluten meal (Cozzi et al., 1995). Hydrophilic AA like arginine, histidine, lysine, and threonine may be degraded more rapidly when compared to degradation rates of hydrophobic AA, such as leucine, isoleucine, methionine, phenylalanine, tryptophan, and valine (Van Soest, 1994). Branched-chain AA (BCAA) also have slower rates of degradation in comparison with other AA (Varvikko, 1986).

In the rumen, only a small proportion of free AA are incorporated intact into microbial proteins; therefore, the microbial population's ability for *de novo* synthesis of AA is very important. This point is clearly shown in the case of both growing and lactating ruminants fed diets that provided either deficient or no AA (Loosli et al., 1949; Virtanen, 1966). Although the synthesis of these AA is ongoing, it may not be adequate to meet the EAA requirements of a high-producing animal (Lapierre et al., 2006), as noted previously. Improving ruminant protein nutrition has always centered around the optimization of efficient dietary nitrogen usage as a means of maximizing growth and milk production per unit of nitrogen consumed (Wallace and Chesson, 1995). This pursuit involves the provision of an adequate intake of digestible protein (DIP) to meet the microbial population's requirement for nitrogenous substrates and it involves an adequate provision of UIP with the correct AA balance that complements the microbial AA profile (Wallace and Chesson, 1995). For this reason, supplementing rumen-protected AA to ruminants was considered a viable option for meeting AA requirements and promoting optimal growth, reproduction, and lactation (Kung and Rode, 1996). For ruminants to efficiently use both sources of protein, rumen-protected protein and rumen-degradable protein must be supplied at their optimal ratio (NRC, 2001). In other terms, the provision of rumen-protected AA (RPAA) is thought to be necessary to ensure that the ruminant would have sufficient nutrition to support the

microbial population and allow for enzymatic digestion and absorption of the intended AA profile by the small intestine. The use of RPAA allows producers the opportunity to increase protein production in their livestock due to their ability to optimize the balance of AA absorbed by the small intestine and ultimately decrease the amount of UIP needed in the diet to satisfy requirements (Wallace and Chesson, 1995). There have been numerous methods to reduce the degradation of protein inside the rumen including: mild heating, chemical treatment, polyphenolic phytochemicals, and encapsulation (Wallace and Chesson, 1995; Wu, 2013).

Prior studies involving supplementing RPAA mainly provided lysine (Lys) and methionine (Met). This is because the direct evidence from abomasal or duodenal infusion studies showed that Lys and Met are often the most limiting AA involved with growing ruminants (Merchen and Titgemeyer, 1992) and lactating dairy cows (Schwab et al., 1993). A limiting AA is defined as an AA, which is in the shortest supply from the diet relative to its maintenance and growth requirements (Wu, 2018). In terms of nitrogen retention, Met and Lys are also the first and second limiting AA within microbial proteins in growing sheep (Nimrick et al., 1970, Storm and Ørskov, 1984) and growing cattle (Richardson and Hatfield, 1978). There are a few reports that claim that arginine (Arg) and histidine (His) could be limiting depending upon the growth stage and the diet of the animal. Veira et al. (1988) showed theoretical calculations and recorded changes in concentrations of AA in plasma when a combination of fish meal and silage were fed to growing steers and suggested that both Arg and His were limiting in growing steers fed grass silage.

Studies involving beef cattle have observed increased average daily gains with supplementation of RPAA. Veira et al. (1991) observed that growing steers fed grass silage gained weight when supplemented with small quantities of RPAA that were thought to be

limiting (Lys and Met). This weight gain was thought to result from the RPAA sufficiently meeting the AA requirements for both maintenance and growth (Veira et al., 1991). Mowat and Deelstra (1972) supplemented encapsulated Met to lambs consuming a basal corn-alfalfa diet. They observed that Met had no effect on gains or feed efficiency, when lambs were supplemented with soybean meal in addition to their basal diet. However, the authors did observe an increase in gains (11%) and feed efficiency (9%) when the basal diet was supplemented with corn-urea or corn-blood meal. A metabolism trial was then conducted, and the supplemented encapsulated methionine was reported to increase nitrogen digestibility, nitrogen retention, and energy digestibility (Mowat and Deelstra, 1972). Davenport et al. (1995) supplemented growing lambs with rumen-protected arginine or ornithine due to their actions on stimulating the secretion of somatotropin and determined its effect on growth. The supplementation of arginine and ornithine both increased circulating concentrations of both somatotropin and insulin-like growth factor, but failed to improve the performance of growing lambs (Davenport, 1995). Although results of experiments observing growth responses of beef cattle and other ruminants are inconsistent (Kung and Rode, 1996), this inconsistency is thought to be the result of some AA being co-limiting (Merchen and Titgemeyer, 1992) or AA having metabolic roles other than for synthesis of proteins.

Researchers studying the effects of supplementation of rumen-protected Met to dairy cattle observed mixed results, but there were beneficial effects such as enhanced milk protein synthesis (Pisulewski et al., 1996; Armentano et al., 1997; Dinn et al., 1998), milk yield and milk protein synthesis (Illg et al., 1987) and yield of fat corrected milk and milk fat (Overton et al., 1996). Other studies have supplemented rumen-protected Met and found no effects on milk production (Papas et al., 1984; Overton et al., 1998). Izumi et al. (2000) found that

supplementing rumen-protected Met over a 22-week period resulted in a significant, but temporary, increase in milk yield. This temporary effect was significant only through the peak to middle periods of lactation, but over the complete lactation period there was no significant effect on milk yield, milk fat, or milk protein synthesis (Izumi et al., 2000). Many studies have tested the hypothesis that there is more than one co-limiting AA for lactating animals.

Supplementation with certain RPAA has also been found to overcome the decrease in milk protein synthesis observed in dairy cattle consuming rations with higher fat content (Canale et al., 1990). In a study that observed the effects of RPAA on cow and calf production, primiparous beef cows supplemented with increasing levels of rumen-protected Lys and Met had increased milk production (Hess et al., 1998). This increase in milk yield was also paired with decreasing body weight gain after parturition (Hess et al., 1998). Rode et al. (1993) supplemented RPAA as a replacement for 0.5 kg of soy/blood meal and found that cows consumed less protein and increased their forage consumption, when compared to their non-supplemented counterparts.

Benefits of Arginine Supplementation to Gestating Mammals

Arg catabolism involves multiple pathways and is capable of providing a variety of useful products to the body including ornithine, polyamines, proline, glutamate, agmatine, creatine, and NO (Wu, 2013). The wide array of products allows Arg supplementation to improve cardiovascular function, immunity, neurological function, wound healing, fertility in both genders, absorption of nutrients, and insulin sensitivity (Wu, 2013). These benefits allow the potential use of Arg supplementation to also reduce hyperglycemia, dyslipidemia, obesity, high blood pressure, atherosclerosis, infections, embryonic and fetal death, and diarrhea (Wu, 2013).

NO and polyamine products serve vital roles in placental angiogenesis and growth in mammals (Hosomi et al., 1987). Arg has the potential to be a vital nutrient for both dam and fetus during pregnancy (Wu et al., 2013). Arg is also part of the urea cycle and because of this role it is essential for ammonia detoxification via the urea cycle (Leonard, 2001). During normal conditions Arg is considered unessential for protein metabolism, but during periods of higher production or stress (pregnancy, rapid growth, lactation, or disease) Arg is considered a conditionally essential AA (Hou and Wu, 2017; Visek, 1985).

Zeng et al. (2008) conducted four separate experiments testing the effects of Arg supplementation on embryonic survival in Sprague-Dawley rats. In rats supplemented with arginine throughout their pregnancy, litter size was increased by 3.2 pups per dam (14.5 ± 0.062 vs. 11.3 ± 0.61). An increase in litter size was also observed in rats supplemented Arg between d 1 and 7 of pregnancy (14.7 ± 0.39 vs. 11.4 ± 0.66). The Arg treatment also led to an increase in embryo survival on d 7 of pregnancy and this was thought to be the primary factor behind the increased litter size.

Arg supplementation shows promise for enhancing swine production. Results of a study in which gilts were supplemented with 1% L-Arg-HC between d 30 and 114 of gestation showed a 22% increase in the live litter birth weight and a 24% increase in the number of pigs born alive (Mateo *et al.*, 2007). This enhancement was thought to occur through an increase in placental angiogenesis and growth during early- to mid-gestation. This would help improve the intrauterine environment throughout pregnancy (Wu *et al.*, 2004). Another possible conclusion was that the uptake of Arg by the uterus was insufficient and failed to meet growth requirements during late gestation (Wu *et al.*, 1999). The increased concentrations of Arg in plasma would

correct the deficiency and the fetus would be able to have optimal metabolism and growth during late gestation.

A similar experiment by Thureen *et al.* (2002) in which pregnant ewes were given arginine to further study how maternal supplementation of Arg could potentially affect maternal, uteroplacental, and fetal metabolism revealed that an increase in concentrations of Arg in plasma of ewes increased uterine, uteroplacental, and fetal uptake of Arg. Also, the increase in maternal and fetal concentrations of Arg in blood were associated with increases in maternal and fetal concentrations of insulin in blood. The results from the study seemed to indicate that Arg supplementation has profound effects on the maternal, placental, and fetal Arg flux and metabolism (Thureen *et al.*, 2002).

A separate study focused on effects of Arg supplementation on IUGR fetuses showed that increasing circulating concentrations of Arg increased fetal protein accretion by decreasing protein turnover, synthesis, and breakdown (De Boo *et al.*, 2005). Increasing concentrations of Arg in blood was also thought to result in an increase in vasodilation of blood vessels in the uterus and placenta through NO production, which helped to distribute a greater amount of blood towards the fetus (De Boo et al., 2005). Arg's ability to inhibit whole-body protein metabolism was also observed in a study pertaining to sepsis in pigs (Bruins *et al.*, 2002).

Saevre et al. (2010) supplemented rumen-protected Arg to ewes to determine if they might exhibit an enhancement in reproductive efficiency previously seen in monogastric animals supplemented with Arg. It was found that the rumen-protected Arg product increased circulating levels of Arg and increase ovarian blood flow in ewes fed the Arg supplement over a 5-d period. de Chávez et al. (2015) reported that dietary supplementation with rumen-protected Arg [7.8 g Arg (as arginine-HCl)] to sheep (45 kg BW) between the onset of estrus and Day 25 after

breeding enhanced embryonic and fetal survival during early pregnancy. Likewise, Zhang et al. (2016) demonstrated that dietary supplementation with rumen-protected Arg (10 g/day) to underfed ewes (40 kg BW; 50% of NRC-recommended nutrient requirements) between d 35 and 110 of gestation enhanced fetal weight by 18%. These results show the promise of Arg in improving fertility and fetal growth in sheep under production conditions.

Reducing embryonic loss in beef cattle would have beneficial impacts on the profitability and efficiency of the cattle industry. After reviewing previously conducted research involving Arg supplementation in other species (Zeng et al., 2008; Wu et al., 2007), the author proposes that providing rumen protected precursors of Arg could have the potential of being an economical and effective strategy to minimize embryonic loss and increase the reproductive efficiency of beef cows.

CHAPTER II

DEGRADABILITY OF CITRULLINE AND GLUTAMINE BY RUMEN MICROORGANISMS

Abstract: The microbial population within the rumen has long been considered to have the capability of extensively degrading all dietary amino acids (AA). Results from our feeding trial showed that this view might not be accurate. In vivo and in vitro studies were conducted to test the hypothesis that certain AA undergo little degradation by ruminal microbes. In the in vivo experiment, at 0.5 h before and 0, 0.5, 1, 2, and 4 h after cannulated adult steers (~500 kg, n = 8) consumed 0.56 kg dried-distillers grain mixed with 70 g L-glutamine plus 70 g L-citrulline, samples of rumen fluid and plasma were collected for AA analysis using HPLC. Results showed that the concentrations of glutamine in the rumen fluid declined rapidly, but that of glutamate (a product of glutamine catabolism) increased gradually between 0.5 and 4 h after consumption of the meal. In contrast, the concentration of citrulline in the rumen fluid was constant through the 4-h period after steers consuming the meal. The concentrations of L-citrulline and L-arginine, but not L-glutamine or L-glutamate, were greater in the steers at 1 and 2 h after the meal, respectively, when compared with the values at 0 h. Results of in vivo studies involving adult sheep indicated that oral administration of L-citrulline increased its concentration in plasma, suggesting the bypass of this amino acid through the rumen. In the in vitro experiments, whole rumen fluid (3 mL) from the steers was incubated at 37ºC with 5 mM L-glutamine, 5 mM L-glutamate, or 5 mM L-citrulline for 0.5, 1, 2 or 4 h, and 20 µl samples were collected at the predetermined time points for AA analyses. Results showed extensive hydrolysis of L-glutamine into L-glutamate, but little degradation of either L-glutamate or L-citrulline by rumen microbes during the 4-h period of incubation. Collectively, these in vivo and in vitro results indicate that

rumen microbes of adult steers extensively degrade extracellular L-glutamine, but not extracellular L-glutamate or L-citrulline. We suggest that L-citrulline, without any encapsulation or protection from rumen microbes, can be effectively supplemented to the diets of ruminants to increase its concentrations and concentrations of L-arginine in plasma for utilization and metabolism by various organs and tissues.

Introduction

In ruminants, dietary protein is hydrolyzed by bacterial proteases and peptidases into small peptides and amino acids (AA) in the rumen, whereas free AA are further degraded to ammonia and their carbon skeletons by a number of bacterial enzymes, including deaminases, transaminases, hydrolases, and decarboxylases (Wu, 2013). In the presence of α-ketoacids (e.g., pyruvate, oxaloacetate, and α-ketoglutarate which are products of carbohydrate metabolism) and sulfur, ammonia is utilized by ruminal bacteria for the synthesis of new AA and proteins (Wu, 2018). The rates of AA catabolism are high in the rumen, such that all AAs studied to date (Ala, Arg, Asn, Asp, Cys, Gln, Gly, His, Ile, Leu, Lys, Met, Orn, Phe, Pro, Ser, Thr, Tyr, Val) do not escape the rumen. Thus, the microbial population within the rumen has long been considered to have the capability of extensively degrading all dietary AAs (Chalupa 1976; Lewis and Emery 1962; Scheifinger et al. 1976). However, we are not aware of studies regarding the degradability of extracellular citrulline (Cit, the immediate precursor of arginine) and Glu by ruminal bacteria. Therefore, the objective of the present study was to determine whether or not these two AA are catabolized by ruminal bacteria. Towards this goal, the author conducted a series of three experiments with steers involving: (1) utilization of Cit, Gln and glutamate (Glu) by microbes in whole rumen fluid; (2) changes in the concentrations of AA in the plasma and rumen fluid of steers receiving oral administration of Cit plus glutamine (Gln, a positive control); and (3)

changes in the concentrations of AA in plasma of adult sheep receiving oral administration of Cit, Gln or urea (isonitrogenous control).

Materials and Methods

Experiments involving the utilization of Cit, Gln and Glu by microbes in rumen fluid of steers

Catabolism of Gln and Cit, and treatments consisting of both AA supplements (rumen-protected and rumen-unprotected) was determined using an *in-vitro* procedure. For comparison, Glu was also included in the incubations with rumen fluid from steers. Fresh rumen-fluid was collected from six cannulated steers (n=6) that had been deprived of food for 16 h. Rumen fluid (20 ml) was taken from each steer and gently vortexed. The sample was then aliquoted in increments of 3 mL into six separate polypropylene tubes, which contained one of the following:

(1) No added AA;
(2) 2.2 mg L-glutamine (final concentration = 5 mM);
(3) 2.7 mg L-citrulline (final concentration = 5 mM);
(4) 10.6 mg rumen-unprotected AA (final concentration of Cit = 5 mM; final concentration of Gln = 6 mM);
(5) 10.6 mg rumen-protected AA (final concentration of Cit = 5 mM; final concentration of Gln = 6 mM);
(6)2.2 mg L-glutamate (final concentration = 5 mM).

No carbohydrate or sulfur compounds were added to the incubation medium. The tubes were vortexed gently, sealed with rubber stoppers, gassed with CO_2 for 20 seconds, and then placed in a shaking 37^{o}C water bath (70 oscillations/min). After 0, 0.5, 1, 2 or 4 h of incubation, 20 µl samples were collected from each tube. An aliquot (20 µl) of 1.5 M $HClO_4$ was added to the collected sample, followed by the addition of 10 µl of 2 M K_2CO_3 for neutralization and then 150 µl of HPLC-grade water. The extracts were placed at -20^{o}C until analyzed for AA by HPLC (Wu and Meininger 2008).

In a separate experiment (n = 4 steers), rumen fluid was incubated as described above, except that the medium contained 5 mM L-[U-^{14}C]Glu or L-[ureido-^{14}C]Cit (100 dpm/nmol) for

4 h. Blanks, in which Krebs-bicarbonate buffer replaced rumen fluid, were included for parallel incubation. At the end of the incubation period, $^{14}CO_2$ was collected into Soluene, as we described (Li et al. 2016). Thereafter, the acidified medium was neutralized with 2 M K_2CO_3 as described above, and 100 µl of the whole extract was loaded into the column of HPLC for separation and collection of AA fractions, as described previously (Wu et al. 2000). Incorporation of ^{14}C-AA into microbial protein was determined by measuring the radioactivity of ^{14}C-labeled proteins, as we described (Dai et al. 2010).

Data were analyzed by one-way analysis of variance (Assaad et al. 2014). Log transformation of variables was performed when the variance of data were not homogenous, as assessed by the Levene's test (Wei et al. 2012). Differences among treatment means were determined by the Student-Newman-Keuls multiple comparison test. P-values ≤ 0.05 were taken to indicate statistical significance.

Experiments involving changes in the concentrations of AA in rumen fluid and plasma of steers

A second experiment involving cannulated steers was conducted to confirm the previous findings in an in-vivo model. Sixteen ruminally cannulated Angus × Hereford steers (538.2 ± 4.1 kg BW) were used in a randomized complete block experiment. Steers (n=8/treatment group) were stratified by initial BW and assigned to either rumen-protected AA [25% Gln + 25% Cit encapsulated with 50% binder] or unprotected AA [25% Gln + 25% Cit + 50% free binder]. To facilitate consumption, an AA supplement was mixed with dried distillers' grains at a 1:2 ratio. An AA supplement was fed at 2% of daily dietary feed intake (0.5% Gln + 0.5% Cit in the diet). Each steer consumed 0.56 kg dried-distillers grain plus 0.28 kg of an AA supplement that provided 70 g Gln plus 70 g Cit. Distillers' grains were selected as a supplement due to availability, common usage in beef cattle operations, and palatability by all classes of cattle.

Throughout the duration of the project, steers were offered bermudagrass hay at 130% of their previous 5-d average of daily feed intake to ensure that access to feed would not be limited. All steers had *ad libitum* access to a trace mineral salt block and water for the duration of the project. Steers were individually housed for the duration of the project to facilitate determination of individual intake. Steers were adapted to their respective AA treatment for 10 d before sampling began, their daily feed intake was determined, and blood was sampled on d 15, the final day of the project.

At 0600, orts were collected and weighed. Steers received their supplements at 0630 and the time required to completely consume the supplement was recorded each day, any refusals were collected and weighed after the steers consumed the supplement for 30 min. Bermudagrass hay was fed at 0700. Steers were checked each afternoon between 1600 and 1800 to ensure that they had adequate bermudagrass hay remaining in their bunks. During the final 4 d, samples of supplements, hay, and orts were retained for nutrient analysis. Pens were scraped clean every evening starting on d 10 to ensure no cross-contamination of fecal samples. Fecal samples were collected during the final 4 days, every 12 h during the sampling period, with the sampling time advancing by 3 h each day. On d 15, a final BW was recorded, and blood (2 ml) and rumen fluid (20 ml) were collected from each steer at 0, 0.5, 1, 2, 4 and 6 h after feeding. Blood and rumen fluid samples were centrifuged at 600 g for 15 min to collect the supernatant fluid (plasma or microbe-free rumen fluid). An aliquot (100 µl) of the supernatant fluid was acidified with an equal volume of 1.5 M $HClO_4$, followed by the addition of 50 µl of 2 M K_2CO_3 for neutralization and then 2.25 ml of HPLC-grade water. The extracts were placed at -20^{o}C until analyzed for AA by HPLC (Wu and Meininger 2008). Ammonia, urea and glucose were analyzed using enzymatic methods, as we described previously (Satterfield et al. 2012, 2013).

Data were analyzed by two-way analysis of variance, with AA and incubation time as two independent variables (Assaad et al. 2015). Log transformation of variables was performed when the variance of data were not homogenous, as assessed by the Levene's test (Wei et al. 2012). Differences among treatment means were determined by the Student-Newman-Keuls multiple comparison test. P-values ≤ 0.05 were taken to indicate statistical significance.

Experiments involving adult sheep

A third experiment was conducted to verify the results found in the cannulated steer trial. Five sheep were individually fed three separate supplements (Gln, Cit, or Urea) on three separate days. Each supplement (8 g) was offered along with 800 g of regular feed to each sheep. Blood (2 ml) was sampled from the jugular vein prior to feeding (time 0) and 0.5, 1, 2, and 4 h after consuming the supplement. We chose a 4-h period of blood sampling based on the results of our previous experiment that the concentrations of Arg, Cit, and Gln did not differ ($P > 0.05$) between 0 and 6 h after oral administration of 8 g Arg, Cit, or Gln to adult sheep. Blood samples (1 ml) were centrifuged immediately to obtain plasma. An aliquot (100 µl) of plasma was acidified with an equal volume of 1.5 M $HClO_4$, followed by the addition of 50 µl of 2 M K_2CO_3 for neutralization and then 2.25 ml of HPLC-grade water. The extracts were placed at -20^{o}C until analyzed for AA by HPLC (Wu and Meininger 2008). Ammonia, urea and glucose were analyzed by using enzymatic methods, as we described previously (Satterfield et al. 2012, 2013). Data were analyzed by one-way analysis of variance and the Student-Newman-Keuls multiple comparison test, as described for Experiment 1.

Results and Discussion

Experiments involving the utilization of Cit, Gln and Glu by microbes in rumen fluid from steers.

Results from the in-vitro experiment involving rumen fluid incubated in the presence of various AA showed the rapid degradation of Gln to Glu and ammonia in a time-dependent manner (Table 2.1). In addition, rumen microbes extensively catabolized Arg to form Orn, proline, and ammonia in a time-dependent manner. The concentrations of proline in the medium were 19.3 ± 1.7, 316 ± 25, 632 ± 59, and 994 ± 82 nmol/ml rumen fluid (mean ± SEM, n = 6), respectively, at the end of the 0, 0.5, 2 and 4 h of incubation ($P < 0.01$). In contrast, there was no detectable disappearance of extracellular unprotected Cit and Glu from the rumen fluid or detectable formation of products from these two AA during a 4-h period of incubation (Table 2.1). Consistently, the rates of formation of $^{14}CO_2$, ^{14}C-Gln, ^{14}C-Asp, ^{14}C-Ala and ^{14}C-Glu-protein from ^{14}C-Glu were 3.0 ± 0.16, 1.2 ± 0.11, 3.6 ± 0.29, 4.5 ± 0.44 and 14.7 ± 1.2 nmol Gln/ml rumen fluid per 4 h; mean ± SEM, n = 4). The sum of these Glu products represented only 0.6% of Glu present in the incubation medium (5000 nmol Glu/ml rumen fluid) and 1.2% of the rate of Gln hydrolysis into Glu (2206 nmol Gln/ml rumen fluid per 4 h; mean ± SEM, n = 6). There was no formation of any other ^{14}C-labeled AA (including Asn, Ser, Gly, Tau, Arg, Phe, Leu, Ile, Val, Orn, Lys or Pro) from ^{14}C-Glu detected during a 4-h period of incubation. Likewise, there was no detectable formation of $^{14}CO_2$ or any ^{14}C-labeled AA (including Asp, Asn, Ser, Gln, Gly, Tau, Arg, Ala, Phe, Leu, Ile, Val, Orn, Lys or Pro) from extracellular ^{14}C-Cit during a 4-h period of incubation. Collectively, these results indicate that extracellular Cit and Glu are not degraded significantly by microbes in the rumen of steers. It is possible that they do not take up extracellular Cit due to the lack of a transporter. In support of this view, Stalon and Merceniner (1984) reported that few bacteria utilize extracellular Cit as a nitrogen or carbon source. Furthermore, the possibility that extracellular Glu is not degraded by bacteria in the rumen due to

a limited availability of intracellular α-ketoacids for transamination reactions cannot be excluded.

To our knowledge, this is the first study on the degradability of extracellular Cit and Glu by microbes in the rumen of any species. However, Chalupa (1976) reported that bovine rumen fluid incubated in the presence of 2% starch plus 8 mM urea utilized extracellular Glu based on the disappearance of Glu from the medium over a 6-h period. Those results are cautiously interpreted as the "apparent degradation" of Glu by microbes in the rumen, due to the lack of any evidence for its true degradation such as the formation of metabolites from Glu. In the study of Chalupa (1976) that provided urea (a ready precursor of ammonia) and starch (a ready source of carbon skeletons including α-ketoacids) to support synthetic processes, the disappearance of Glu from the incubation medium may have resulted primarily from the utilization of extracellular Glu for protein or peptide production by microbes in the rumen, rather than degradation of extracellular Glu.

Table 2.1 Amino Acid Profile in the Incubated Steer Rumen Fluid After Addition of Amino Acid Supplement

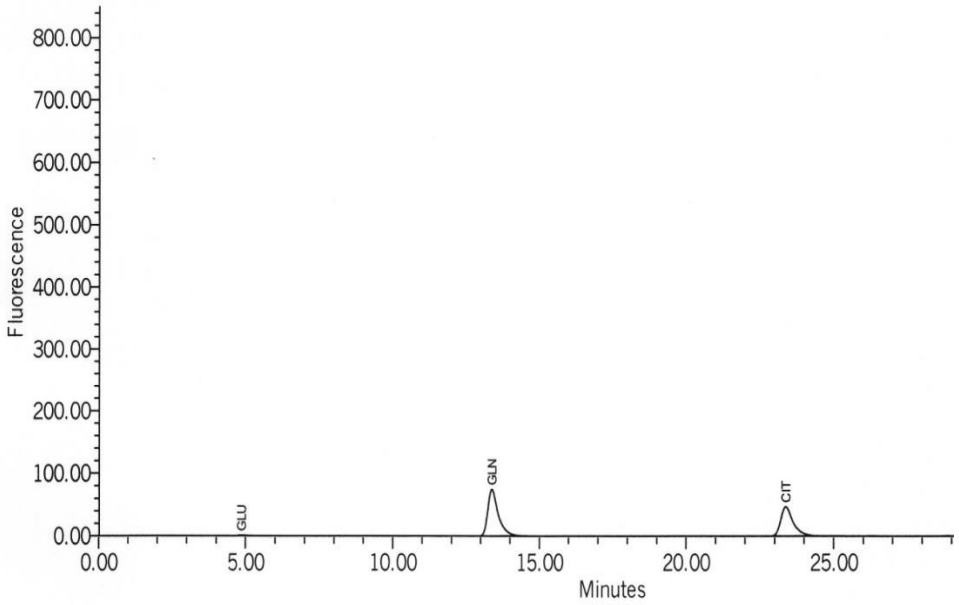
Addition to medium	Hour	Asp	Glu	Gln	Cit	Arg	Orn	NH_3
None	0	14.3 ± 3.1	46.0 ± 1.8	32.5 ± 7.5	5.7 ± 1.8	18.2 ± 5.0	19.2 ± 2.4	0.72 ± 0.08
	0.5	18.7 ± 4.4	64.5 ± 8.8	29.0 ± 4.8	6.4 ± 0.8	16.8 ± 5.4	22.3 ± 3.4	0.86 ± 0.15
	2	24.8 ± 5.3	76.0 ± 9.4	20.2 ± 3.7	6.5 ± 1.7	16.7 ± 5.1	24.2 ± 3.9	0.90 ± 0.15
	4	31.5 ± 6.8	83.0 ± 12.4	13.0 ± 2.5	7.3 ± 1.3	19.2 ± 4.9	27.1 ± 5.8	0.70 ± 0.14
	P value	0.164	0.542	0.025	0.440	0.674	0.096	0.646
5 mM Gln	0	14.3 ± 3.1	46.0 ± 1.8	5023 ± 9.0	5.7 ±1.8	18.2 ± 5.0	19.2 ± 2.4	0.72 ± 0.08
	0.5	18.9 ± 4.0	726 ± 33.8	4218 ± 11.0	6.1 ± 0.9	17.5 ± 5.6	25.1 ± 2.5	1.37 ± 0.08
	2	25.9 ± 5.2	1439 ± 47.0	3447 ± 44.0	7.3 ± 1.3	20.2 ± 4.4	26.1 ± 5.2	1.82 ± 0.06
	4	29.1 ± 4.9	2205 ± 105	2652 ± 87.0	9.5 ± 1.1	21.5 ± 3.6	31.4 ± 7.3	2.12 ± 0.06
	P value	0.240	0.068	0.733	0.202	0.454	0.010	<0.001
5 mM Cit	0	14.3 ± 3.1	46.0 ± 1.8	32.5 ± 7.5	5000 ± 7	18.2 ± 5.0	19.2 ± 2.4	0.72 ± 0.08
	0.5	25.0 ± 8.3	52.6 ± 8.4	32.5 ± 4.0	4990 ± 12	17.3 ± 4.9	24.3 ± 2.8	0.71 ± 0.08
	2	27.2 ± 7.0	55.1 ± 7.3	26.2 ± 5.1	4982 ± 13	19.3 ± 5.4	24.7 ± 3.7	0.84 ± 0.06
	4	25.2 ± 6.1	48.2 ± 10.0	26.4 ± 4.8	4978 ± 25	18.7 ± 4.3	30.1 ± 5.3	0.75 ± 0.12
	P value	0.263	0.507	0.998	0.899	0.845	0.001	0.710
Unprotected amino acid	0	14.3 ± 3.1	46.0 ± 1.8	6007 ± 14	5021 ± 6	18.2 ± 5.0	19.2 ± 2.4	0.72 ± 0.08
	0.5	21.8 ± 4.6	826 ± 45	5030 ± 39	5021 ± 22	20.8 ± 4.1	23.9 ± 3.5	1.09 ± 0.12
	2	22.4 ± 4.7	1450 ± 63	3970 ± 171	5031 ± 24	20.0 ± 5.4	30.9 ± 5.0	1.40 ± 0.11
	4	31.9 ± 5.6	2600 ± 178	2882 ± 233	5014 ± 10	23.2 ± 3.2	32.9 ± 5.0	1.75 ± 0.13
	P value	0.402	0.731	0.003	<0.001	0.309	0.628	0.003
Protected amino acid	0	14.3 ± 3.1	46.0 ± 1.8	1468 ± 91	1318 ± 98	18.2 ± 5.0	19.2 ± 2.4	0.72 ± 0.08
	0.5	19.1 ± 5.5	475 ± 85.4	984 ± 56	1303 ± 99	19.2 ± 4.8	28.7 ± 2.5	0.89 ± 0.17
	2	25.2 ± 4.7	709 ± 101	723 ± 39	1288 ± 92	19.8 ± 3.7	21.0 ± 2.9	1.0 ± 0.10
	4	27.9 ± 5.8	985 ± 112	498 ± 47	1296 ± 94	20.8 ± 3.7	25.1 ± 5.1	0.71 ± 0.17
	P value	0.146	0.220	0.033	0.293	0.712	0.182	0.388
5 mM Glu	0	14.3 ± 3.1	5012 ± 31	32.5 ± 7.5	5.7 ± 1.8	18.2 ± 5.0	19.2 ± 2.4	0.72 ± 0.08
	0.5	14.5 ± 3.2	4989 ± 16	30.5 ± 7.5	6.2 ± 1.4	18.0 ± 4.7	20.3 ± 2.7	0.72 ± 0.07
	2	16.3 ± 3.4	5007 ± 8	26.2 ± 6.2	7.3 ± 2.4	18.2 ± 5.2	24.3 ± 2.6	0.88 ± 0.09
	4	19.0 ± 3.3	4962 ± 24	24.5 ± 5.6	9.2 ± 2.7	17.7 ± 4.9	22.5 ± 4.2	0.71 ± 0.08
	P value	0.273	0.392	0.179	0.011	0.783	0.252	0.397
5 mM Arg	0	14.3 ± 3.1	45.2 ± 1.2	32.5 ± 7.5	5.7 ± 1.8	5006 ± 9	19.2 ± 2.4	0.72 ± 0.08
	0.5	15.2 ± 3.2	47.5 ± 1.3	29.2 ± 7.1	27.8 ± 5.1	4626 ± 29	44.7 ± 1.2	1.36 ± 0.14
	2	17.3 ± 3.2	47.8 ± 1.0	23.3 ± 5.4	54.0 ± 4.2	4243 ± 23	73.0 ± 4.0	1.89 ± 0.10
	4	20.0 ± 3.3	50.0 ± 1.5	21.3 ± 4.6	91.0 ± 7.0	3773 ± 87	126 ± 8.1	2.32 ± 0.19
	P value	0.483	0.730	0.969	0.177	0.024	0.001	<0.001

Values, expressed as nmol/ml for amino acids and μmol/ml for ammonia, are means ± SEM, n = 6.

Results for the concentrations of AA in the incubated rumen fluid containing unprotected Gln + Cit showed rapid degradation of Gln, but not Cit, between 0.5 and 4 h of incubation.

This degradation of Gln was accompanied by a sharp increase in the accumulation of Glu in rumen fluid. In contrast, the concentrations of Cit and Gln in the incubated rumen fluid containing protected AA were equivalent to approximately 25% of the encapsulated Cit and Gln. Similar results were obtained when this protected AA product was placed in water without any bacteria (Figure 1A and 1B). Thus, not all AA was encapsulated by the binder in the protected AA preparation. Nonetheless, Gln released from the protected AA product disappeared progressively from the incubated rumen fluid with a concomitant increase in Glu, but the concentration of free Cit in the fluid remained relatively constant during the 4-h period of incubation. This result further supports the notion that extracellular Cit and Glu do not undergo significant catabolism by microbes in the rumen.

A. Rumen-Protected Amino Acids



B. Unprotected Amino Acids

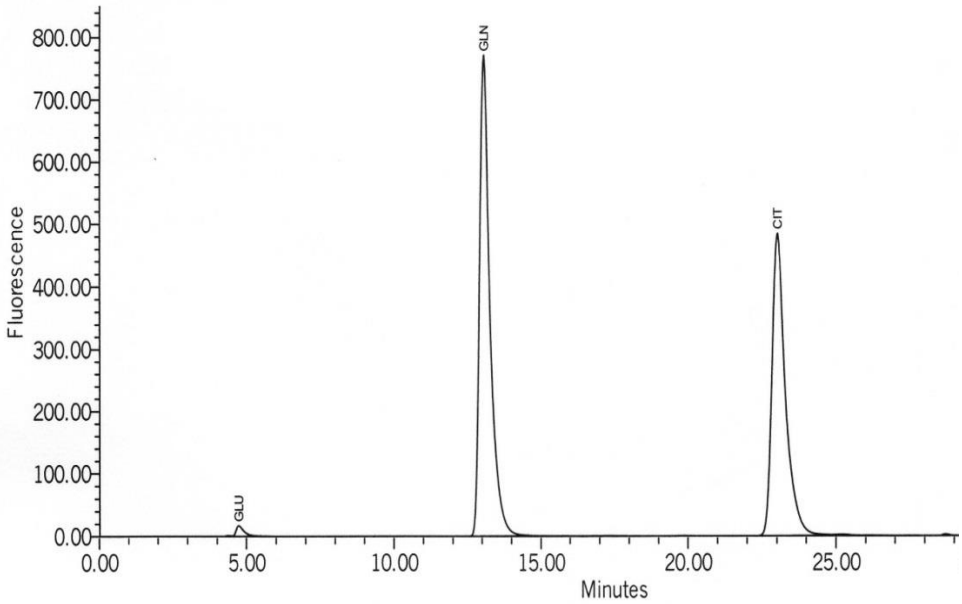


Figure 2.1 Analysis of free glutamine and citrulline in two amino acid products.
10 g of both products (A and B) were mixed with 1 L double-distilled water, and the solution was centrifuged at 600 g for 10 min. The supernatant fluid was used for HPLC analysis of amino acids.

Changes in the concentrations of AA in rumen fluid and plasma of steers following oral administration of RUAA or RPAA.

The concentrations of free amino acids in rumen fluid of steers after consumption of either the RPAA or RUAA supplements are shown in Table 2.2. Glu was the most abundant AA in rumen fluid obtained from both groups of steers, followed by Gln, Gly, Arg, Ala, and Ser in order of decreasing abundance. The concentrations of Asn, Thr, Tau, Trp, Phe, Lys, and total Cys in rumen fluid did not differ ($P > 0.05$) throughout the 6-h sampling period in either group. However, the concentrations of many AA (including Gln, Arg, Cit, Orn and Pro) in rumen fluid showed time-dependent changes ($P < 0.05$), indicating that oral administration of RUAA or RPAA could increase their concentrations in the rumen. In the RUAA group, Gln and Cit were released rapidly from the unprotected AA product to increase their concentrations in rumen fluid, with Gln and Cit values peaking at 0.5 and 1 h, respectively. This is likely because of their different rates of turnover in the rumen or different rates of utilization by rumen microbes. In the rumen of RUAA steers, the extensive catabolism of Gln generated ammonia, Glu, Ser, Arg, Ala, Orn, and Pro. Concentrations of both Gln and Cit in rumen fluid of RUAA steers declined rapidly after 1 h likely because of a rapid flow of these two AA out of the rumen into the other comparts of the forestomach. The magnitudes of changes in the concentrations of Gln and Cit in rumen fluid of RPAA steers were much smaller than those for RUAA steers, indicating that a majority of Gln and Cit in the RPAA product was not released into the rumen fluid. This result is consistent with the in vitro data that most (~75%) of the Gln and Cit in the RPAA product was encapsulated by the binder.

It is clear that a large amount of Glu is produced from unprotected Gln in the rumen of steers. Dietary and rumen-derived Glu can enter the small intestine for subsequent utilization,

and this aspect of Glu metabolism should be quantified in future studies involving cattle with a fitted cannula in the duodenum. In ruminants including sheep (Tagari and Bergman 1978) and cattle (Reynolds 2006), as in nonruminants, such as pigs and rats (Blachier et al. 2009; Burrin and Stoll 2009; Wu 1998), nearly all Glu (95-97%) in the lumen of the small intestine is metabolized during the first pass into the portal vein. Major products of intestinal Glu catabolism include CO_2, Ala, Asp, Orn, Cit, Arg, and Pro (Wu 1998).

The concentrations of free amino acids in the plasma of steers after consumption of either the RPAA or RUAA supplementation are shown in Table 2.2. Gly was the most abundant AA in plasma obtained from both groups of steers, followed by Gln, Val, Pro, Ala, Leu, total Cys, Lys and Cit in order of decreasing abundance. The concentrations of Asp, Glu, Gly, Trp, Met, Pro, total Cys, ammonia, urea or glucose in the plasma did not differ throughout the 6-h sampling period in either group. However, the concentrations of other AA (including Gln, Arg, Cit, and Orn) in plasma showed time-dependent changes, indicating that oral administration of RUAA or RPAA could increase their concentrations. Gln in the rumen of the RUAA group could be used by microbes in the rumen for synthesis of AA and then proteins, which would then be available for digestion in the abomasum and the small intestine to contribute Gln and its precursors (e.g., branched-chain AA). In the RPAA group, the Gln that escaped the rumen could either enter the portal vein or be utilized by the small intestine to synthesize Cit. In both the RUAA and RPAA groups, Cit could escape the rumen to enter the small intestine for absorption into the portal vein and then into the systemic blood circulation (Wu 2018). Cit in the blood is utilized for the synthesis of Arg, which is then converted into Orn. Thus, dietary supplementation with Cit in either the rumen-protected or nonprotected form can effectively increase the concentration of Cit and Arg in plasma. These findings are novel and important, because they indicate that it is not

necessary to protect Cit when it is supplemented to the diets of ruminants. Because Cit is effectively converted into Arg in ruminants (Lassala et al. 2009), expensive and time-consuming procedures for encapsulating Arg as a means of providing enteral Arg to those animals can be eliminated. able 2.2 Concentrations of Amino Acids and Ammonia in the rumen fluid of steers after consumption of amino acid supplement**.**

Table 2.2 Concentrations of amino acids, ammonia and urea the rumen fluid of steer after consumption of amino acid supplement

AA	RUAA						RPAA						*P*-values		
	0 h	0.5 h	1 h	2 h	4 h	6 h	0 h	0.5 h	1 h	2 h	4 h	6 h	AA	Time	AA x Time
Asp	16 ± 0.9	26 ± 5.0	35 ± 7.3	20 ± 1.3	18 ± 1.0	15 ± 0.9	16 ± 1.2	16 ± 1.4	16 ± 1.6	15 ± 1.4	16 ± 1.7	16 ± 2.1	0.062	<0.001	0.023
Glu	44 ± 0.8	338 ± 48	523 ± 45	243 ± 22	29 ± 2.2	32 ± 4.4	47 ± 1.0	46 ± 2.3	48 ± 3.1	46 ± 0.9	39 ± 2.1	46 ± 1.7	<0.001	<0.001	<0.001
Asn	6.2 ± 0.4	6.8 ± 0.3	6.4 ± 0.4	6.6 ± 0.5	7.2 ± 0.4	6.9 ± 0.2	6.4 ± 0.2	6.3 ± 0.3	6.7 ± 0.3	6.6 ± 0.1	6.4 ± 0.1	6.3 ± 0.4	0.258	0.681	0.406
Ser	17 ± 2.3	25 ± 2.0	32 ± 4.2	27 ± 3.8	23 ± 7.1	18 ± 4.1	18 ± 0.9	19 ± 1.2	18 ± 1.4	15 ± 1.6	15 ± 2.1	14 ± 1.8	0.072	<0.001	0.015
Gln	31 ± 1.3	3570 ± 887	2108 ± 432	228 ± 80	102 ± 29	35 ± 4.9	34 ± 2.8	120 ± 26	61 ± 27	32 ± 2.5	28 ± 2.6	30 ± 3.7	0.001	<0.001	<0.001
His	8.4 ± 0.9	14 ± 2.3	15 ± 1.9	11 ± 1.3	10 ± 2.3	8.8 ± 1.7	7.7 ± 0.7	8.4 ± 0.7	8.2 ± 0.9	8.6 ± 1.1	8.0 ± 0.7	7.9 ± 1.0	0.002	<0.001	<0.001
Gly	25 ± 3.5	27 ± 3.3	28 ± 4.5	26 ± 4.8	26 ± 3.5	23 ± 3.9	26 ± 3.4	29 ± 2.0	30 ± 2.0	28 ± 1.2	28 ± 2.1	26 ± 1.3	0.420	0.009	0.529
Thr	14 ± 2.1	15 ± 2.5	15 ± 2.5	15 ± 2.9	14 ± 2.3	14 ± 2.5	17 ± 2.4	16 ± 2.8	15 ± 1.8	15 ± 2.0	15 ± 2.2	15 ± 1.4	0.641	0.096	0.166
Cit	5.4 ± 0.4	4481±1095	5436±1187	2800 ± 439	761 ± 389	20 ± 6.9	6.2 ± 0.7	575 ± 62	693 ± 85	513 ± 94	106 ± 63	6.6 ± 1.1	<0.001	<0.001	<0.001
Arg	20 ± 2.2	32 ± 2.4	37 ± 3.8	26 ± 4.5	20 ± 5.7	18 ± 5.4	21 ± 3.0	21 ± 3.0	22 ± 2.0	22 ± 3.3	21 ± 3.5	20 ± 2.6	0.244	<0.001	0.002
β-Ala	12 ± 1.1	20 ± 3.0	26 ± 7.7	21 ± 3.0	19 ± 2.7	14 ± 1.1	11 ± 1.2	11 ± 1.2	14 ± 3.1	15 ± 3.5	13 ± 2.8	12 ± 2.4	0.092	<0.001	0.002
Tau	1.3 ± 0.2	1.4 ± 0.1	1.3 ± 0.1	1.3 ± 0.1	1.3 ± 0.1	1.4 ± 0.1	1.4 ± 0.1	1.3 ± 0.1	1.2 ± 0.1	1.3 ± 0.1	1.1 ± 0.1	1.2 ± 0.1	0.369	0.324	0.716
Ala	17 ± 1.9	48 ± 9.3	56 ± 10	33 ± 10	26 ± 7.1	22 ± 5.7	16 ± 2.4	16 ± 1.8	15 ± 1.5	15 ± 1.3	15 ± 1.6	14 ± 1.8	0.028	<0.001	<0.001
Tyr	12 ± 1.4	14 ± 1.7	12 ± 1.7	13 ± 1.5	12 ± 1.4	12 ± 1.0	11 ± 1.5	12 ± 1.7	12 ± 2.4	12 ± 2.2	11 ± 2.1	10 ± 2.5	0.614	<0.001	<0.001
Trp	8.7 ± 1.0	8.0 ± 0.9	8.0 ± 0.6	8.7 ± 1.1	8.4 ± 1.1	8.2 ± 1.5	8.7 ± 0.8	8.6 ± 0.7	8.1 ± 0.7	8.3 ± 0.7	8.2 ± 0.4	7.7 ± 0.4	0.950	0.087	0.681
Met	7.3 ± 0.8	7.4 ± 0.9	7.2 ± 1.1	6.9 ± 0.8	6.9 ± 0.8	6.6 ± 0.7	7.1 ± 0.4	7.1 ± 0.6	7.0 ± 0.9	6.9 ± 0.7	6.6 ± 0.5	6.5 ± 0.6	0.882	0.009	0.718
Val	11 ± 1.4	11 ± 1.0	11 ± 1.5	9.5 ± 1.5	9.8 ± 1.2	10 ± 0.8	9.8 ± 0.6	11 ± 1.0	9.5 ± 0.7	8.8 ± 0.6	8.1 ± 0.4	9.0 ± 0.7	0.661	0.007	0.030
Phe	7.2 ± 0.8	7.6 ± 0.8	7.5 ± 0.9	7.4 ± 0.5	8.0 ± 0.8	8.0 ± 0.8	7.8 ± 0.5	8.7 ± 0.4	8.4 ± 1.1	8.0 ± 0.6	7.7 ± 0.4	7.4 ± 0.4	0.645	0.341	0.032
Ile	9.5 ± 1.6	10 ± 1.4	9.6 ± 1.4	9.3 ± 1.7	9.1 ± 1.7	8.2 ± 1.2	8.6 ± 0.5	9.1 ± 0.5	9.9 ± 0.4	8.9 ± 0.5	8.3 ± 0.4	8.5 ± 0.7	0.969	0.021	0.196
Leu	14 ± 1.3	15 ± 1.9	15 ± 1.7	13 ± 1.8	13 ± 1.8	12 ± 1.5	13 ± 1.7	13 ± 2.1	13 ± 1.5	12 ± 1.6	13 ± 2.2	12 ± 1.9	0.603	<0.001	0.199
Orn	11 ± 1.7	27 ± 6.3	49 ± 4.1	20 ± 3.4	13 ± 1.5	14 ± 2.0	12 ± 3.2	13 ± 3.4	12 ± 2.6	11 ± 2.7	12 ± 2.9	11 ± 3.0	0.045	<0.001	<0.001
Lys	10 ± 1.9	10 ± 2.1	10 ± 2.0	9.7 ± 1.8	9.4 ± 1.6	9.5 ± 1.7	9.9 ± 1.7	9.6 ± 1.1	10 ± 1.4	9.4 ± 1.1	9.3 ± 1.6	9.5 ± 2.0	0.943	0.480	0.857
Pro	14 ± 1.0	21 ± 1.5	35 ± 1.9	19 ± 1.3	17 ± 1.1	15 ± 1.3	13 ± 1.2	14 ± 1.0	14 ± 1.3	15 ± 1.7	13 ± 1.4	12 ± 1.4	0.039	0.027	0.038
Cys	6.2 ± 0.5	6.5 ± 0.6	6.4 ± 0.6	6.8 ± 0.8	6.3 ± 0.5	6.1 ± 0.6	6.5 ± 0.6	6.8 ± 0.8	6.7 ± 0.6	6.3 ± 0.5	6.2 ± 0.7	6.0 ± 0.8	0.922	0.854	0.761
NH_3	0.84 ± 0.06	1.21 ± 0.05	1.45 ± 0.09	1.06 ± 0.06	0.83 ± 0.07	0.85 ± 0.06	0.86±0.06	0.90±0.06	0.92±0.06	0.87±0.06	0.85±0.06	0.83±0.07	0.041	<0.018	0.035

Values, expressed as nmol/ml for amino acids and µmol/ml for ammonia, are means ± SEM, n = 8.

Table 2.3 Concentrations of amino acids, ammonia and urea the plasma of steer after consumption of amino acid supplement

AA	RUAA						RPAA						*P*-values		
	0 h	0.5 h	1 h	2 h	4 h	6 h	0 h	0.5 h	1 h	2 h	4 h	6 h	AA	Time	AA x Time
Asp	5.4 ± 0.5	4.3 ± 0.5	3.9 ± 0.5	4.0 ± 0.5	4.4 ± 0.4	3.9 ± 0.5	4.0 ± 0.4	3.7 ± 0.4	3.7 ± 0.3	4.0 ± 0.5	4.7 ± 1.7	4.4 ± 1.6	0.696	0.092	0.235
Glu	52 ± 4.2	47 ± 1.8	43 ± 2.5	45 ± 3.3	43 ± 4.0	40 ± 2.2	47 ± 3.5	50 ± 2.5	43 ± 3.9	42 ± 2.8	46 ± 15	43 ± 14	0.937	0.278	0.841
Asn	31 ± 2.1	29 ± 1.6	28 ± 1.4	25 ± 1.5	23 ± 0.8	22 ± 1.5	27 ± 2.5	28 ± 1.8	28 ± 2.3	26 ± 1.8	26 ± 9.3	23 ± 8.3	0.933	<0.001	0.162
Ser	67 ± 2.3	70 ± 3.6	68 ± 4.2	59 ± 3.3	57 ± 2.9	59 ± 3.4	60 ± 2.9	62 ± 3.1	63 ± 4.6	62 ± 3.6	62 ± 2.2	58 ± 2.0	0.105	0.001	0.105
Gln	286 ± 17	294 ± 18	311 ± 22	311 ± 19	296 ± 17	289 ± 17	268 ± 18	262 ± 14	277 ± 17	274 ± 15	302 ± 20	288 ± 17	0.432	0.003	0.090
His	67 ± 5.2	66 ± 5.3	64 ± 6.1	63 ± 4.9	57 ± 4.9	56 ± 4.8	56 ± 5.8	55 ± 5.5	57 ± 4.7	56 ± 4.9	57 ± 19	53 ±18	0.378	0.036	0.112
Gly	347 ± 25	345 ± 30	344 ± 32	339 ± 22	356 ± 25	342 ± 36	325 ± 28	347 ± 33	354 ± 42	345 ± 36	378 ± 26	370 ± 34	0.866	0.366	0.602
Thr	62 ± 3.0	64 ± 4.8	64 ± 5.9	61 ± 5.6	56 ± 4.3	53 ± 5.7	60 ± 6.4	64 ± 6.3	68 ± 5.4	61 ± 6.1	62 ± 2.1	56 ± 1.9	0.781	0.015	0.797
Cit	87 ± 1.8	94 ± 3.7	101 ± 5.2	102 ± 4.3	106 ± 3.5	97 ± 2.7	97 ± 5.6	103 ± 6.4	112 ± 6.6	113 ± 5.3	117 ± 4.8	107 ± 7.0	0.112	<0.001	0.978
Arg	121 ± 5.1	131 ± 6.3	133 ± 9.5	134 ± 6.4	138 ± 5.9	126 ± 4.9	122 ± 6.2	131 ± 6.3	141 ± 7.2	146 ± 8.9	156 ± 5.4	134 ± 5.3	0.363	<0.001	0.064
β-Ala	12 ± 2.1	12 ± 2.4	14 ± 2.5	15 ± 2.3	17 ± 2.4	16 ± 2.8	12 ± 1.2	12 ± 1.1	13 ± 1.8	14 ± 1.7	15 ± 5.1	15 ± 5.1	0.791	<0.001	0.939
Tau	39 ± 5.9	29 ± 3.8	26 ± 3.9	29 ± 3.9	28 ± 3.2	28 ± 3.5	27 ± 2.0	28 ± 2.6	25 ± 3.5	26 ± 2.9	33 ± 11	29 ± 9.5	0.633	0.108	0.081
Ala	181 ± 10	184 ± 13	190 ± 12	241 ± 25	169 ± 8.3	168 ± 6.6	164 ± 6.9	158 ± 8.8	169 ± 12	180 ± 16	164 ± 10	169 ± 9.2	0.168	<0.001	0.025
Tyr	70 ± 3.0	67 ± 2.6	66 ± 3.0	64 ± 2.9	55 ± 2.0	53 ± 1.5	65 ± 3.4	66 ± 2.8	65 ± 3.0	62 ± 2.2	58 ± 19	54 ± 18	0.847	<0.001	0.635
Trp	49 ± 3.2	47 ± 3.1	49 ± 3.0	50 ± 3.6	47 ± 4.0	48 ± 4.5	48 ± 3.8	50 ± 6.3	54 ± 5.1	52 ± 5.5	52 ± 17	48 ± 16	0.701	0.555	0.582
Met	27 ± 0.8	27 ± 0.8	27 ± 1.4	26 ± 1.0	26 ± 1.1	25 ± 1.2	25 ± 2.0	26 ± 1.7	27 ± 1.4	26 ± 1.3	26 ± 0.9	26 ± 0.9	0.872	0.701	0.701
Val	224 ± 11	221 ± 9.1	215 ± 9.8	207 ± 9.6	180 ± 6.7	171 ± 6.5	196 ± 12	206 ± 8.7	210 ± 12	201 ± 8.2	185 ± 6.2	178 ± 5.9	0.520	<0.001	0.238
Phe	51 ± 2.6	52 ± 2.5	52 ± 2.6	51 ± 2.5	44 ± 1.4	43 ± 2.2	49 ± 1.4	52 ± 1.9	53 ± 3.6	52 ± 2.2	49 ± 16	45 ± 15	0.643	<0.001	0.426
Ile	100 ± 4.9	95 ± 3.3	92 ± 4.4	83 ± 3.1	71 ± 2.4	67 ± 2.3	87 ± 5.3	90 ± 3.5	87 ± 5.6	84 ± 4.2	81 ± 2.7	74 ± 2.5	0.860	<0.001	0.026
Leu	148 ± 6.9	141 ± 6.4	138 ± 6.5	128 ± 5.7	110 ± 5.6	104 ± 5.3	130 ± 8.2	136 ± 6.2	132 ± 7.5	129 ± 5.2	122 ± 11	111 ± 10	0.855	<0.001	0.037
Orn	106 ± 8.0	95 ± 5.6	99 ± 7.0	88 ± 5.7	89 ± 6.3	83 ± 6.0	103 ± 10	101 ± 8.0	99 ± 7.8	95 ± 8.0	93 ± 8.2	92 ± 8.8	0.717	0.012	0.424
Lys	104 ± 5.8	96 ± 5.5	98 ± 8.8	83 ± 4.4	77 ± 5.1	70 ± 5.6	87 ± 10	88 ± 7.8	90 ± 3.3	81 ± 5.7	73 ± 7.0	72 ± 6.6	0.885	<0.001	0.586
Pro	184 ± 11	186 ± 12	189 ± 9.2	181 ± 5.6	178 ± 5.6	175 ± 5.7	186 ± 9.5	190 ± 7.4	189 ± 9.1	182 ± 6.9	180 ± 6.7	177 ± 8.5	0.713	0.248	0.519
Cys	132 ± 4.1	135 ± 11	140 ± 8.0	135 ± 6.6	130 ± 7.2	133 ± 6.5	134 ± 8.3	140 ± 9.6	143 ± 6.7	136 ± 7.5	129 ± 7.1	131 ± 10	0.604	0.197	0.442
NH_3	86 ± 4.7	92 ± 6.6	102 ± 5.9	98 ± 4.9	93 ± 4.5	87 ± 5.1	84 ± 4.8	87 ± 5.0	95 ± 3.5	92 ± 6.6	87 ± 4.8	85 ± 5.5	0.674	0.154	0.706
Urea	6.08±0.35	6.22±0.29	6.38±0.32	6.70±0.20	6.34±0.12	6.52±0.15	6.16±0.23	6.34±0.19	6.70±0.19	6.68±0.18	6.24±0.31	6.06±0.24	0.559	0.183	0.624
Gluc	3.42±0.12	3.58±0.13	3.72±0.15	3.62±0.18	3.54±0.12	3.46±0.14	3.52±0.13	3.60±0.10	3.84±0.14	3.70±0.16	3.65±0.10	3.56±0.09	0.682	0.136	0.572

Values, expressed as nmol/ml for amino acids and ammonia and as µmol/ml for glucose and urea, are means ± SEM, n = 8.

Changes in the concentrations of AA in plasma of adult sheep receiving oral administration of Cit, Gln or urea.

Concentrations of AA in the plasma of sheep after oral administration of Cit, Gln or urea are summarized in Tables 2.4, 2.5 and 2.6, respectively. It should be noted that AA used in this experiment were not encapsulated to decrease degradability within the rumen. Because only a small amount of Cit, Gln, or urea (8 g) were fed to sheep, their oral intake did not affect concentrations of ammonia, urea, glucose or any AA in plasma other than the AA that was in the dietary supplement. As baseline values, Gly was consistently the most abundant AA in plasma among all treatments, followed by Gln, Arg, Ala, Pro, Cit, Val, total Cys, and Leu in in order of decreasing abundance. Concentrations of Cit in plasma increased progressively by 117% at 4 h, compared with the baseline value ($P < 0.001$). The increases in the concentrations of Arg in the plasma of sheep were significant ($P < 0.05$) at 2 h (+14%) and 4 h (+23%) after oral administration of Cit. Oral administration of Cit did not affect ($P > 0.05$) the concentrations of other AAs, in plasma. Oral administration of Gln only slightly increased ($P < 0.05$) the concentration of Gln in plasma of sheep at 4 h by 6%, compared with the baseline value. This may not be interpreted to indicate that Gln escapes the rumen to enter the blood. It is possible that orally administered Gln was utilized by bacteria in the rumen to synthesize AA (including branched-chain AA), which subsequently served as substrates for protein synthesis. The microbial protein would then be terminally hydrolyzed by proteinases and peptidases in the small intestine to release Gln and other AAs. Of note, oral administration of 8 g urea was not effective in enhancing the concentrations of anyAA in the plasma of sheep within an 8-h period. As noted previously, this small amount of urea may not be sufficient to increase the synthesis of microbial proteins in the rumen.

In summary, the results of our in vitro and in vivo experiments indicated that, in contrast to Gln and Arg, extracellular Glu and Cit do not undergo significant catabolism by bacteria in the rumen of steers and sheep. These findings revise the traditional view that all AA in diets are unable to avoid metabolism in the rumen. This new concept has far-reaching implications in the nutrition of ruminants and their dietary supplementation with selected amino acids. Thus, Cit, without encapsulation, can be effectively supplemented to the diets of ruminants to increase its concentrations and that of Arg in plasma. Dietary Cit (a stable and neutral nonproteinogenic AA) is an excellent source of Arg for utilization and metabolism by ruminants to enhance their growth and productivity.

Table 2.4 Concentrations of amino acids, ammonia, urea, and glucose in plasma of sheep after consumption of a citrulline supplement

	Time after Citrulline administration					*P*-Values
AA	0 h	0.5 h	1 h	2 h	4 h	
ASP	11 ± 1.1	11 ± 0.7	10 ± 0.8	10 ± 1.1	11 ± 1.2	0.973
GLU	61 ± 1.2	62 ± 1.9	62 ± 3.5	61 ± 2.4	62 ± 3.0	0.844
ASN	33 ± 3.4	35 ± 3.3	31 ± 2.3	33 ± 3.0	35 ± 3.7	0.809
SER	75 ± 3.7	79 ± 2.4	74 ± 1.6	77 ± 3.2	79 ± 6.6	0.534
GLN	372 ± 33	385 ± 29	392 ± 32	389 ± 32	386 ± 30	0.784
HIS	62 ± 7.0	63 ± 7.7	61 ± 8.1	61 ± 7.7	63 ± 8.9	0.932
GLY	511 ± 73	531 ± 79	532 ± 77	515 ± 79	527 ± 74	0.894
THR	60 ± 5.7	61 ± 6.1	60 ± 3.6	62 ± 5.0	63 ± 4.5	0.715
CIT	140 ± 11	147 ± 12	162 ± 12	213 ± 16	304 ± 29	<0.001
ARG	190 ± 25	195 ± 27	202 ± 28	217 ± 31	233 ± 32	0.893
β ALA	18 ± 3.2	18 ± 3.3	20 ± 2.8	19 ± 3.7	19 ± 3.1	0.902
TAU	77 ± 4.9	73 ± 5.9	71 ± 5.9	67 ± 4.7	66 ± 6.2	0.702
ALA	182 ± 21	184 ± 26	176 ± 26	173 ± 24	167 ± 22	0.352
TYR	61 ± 7.8	64 ± 9.0	61 ± 8.1	61 ± 8.2	61 ± 6.9	0.711
TRP	39 ± 1.9	39 ± 2.4	38 ± 2.2	39 ± 2.9	37 ± 1.9	0.569
MET	24 ± 1.6	26 ± 1.5	25 ± 1.7	24 ± 1.8	25 ± 2.1	0.818
VAL	128 ± 9.4	137 ± 13	135 ± 10	139 ± 13	139 ± 7.6	0.477
PHE	36 ± 2.4	37 ± 2.7	37 ± 1.9	37 ± 1.9	36 ± 3.7	0.616
ILE	62 ± 3.6	70 ± 3.6	66 ± 5.0	68 ± 2.3	69 ± 2.0	0.343
LEU	107 ± 7.5	115 ± 8.7	113 ± 7.5	112 ± 7.9	108 ± 5.7	0.496
ORN	78 ± 6.6	80 ± 6.9	84 ± 7.0	97 ± 8.3	107 ± 8.6	0.114
LYS	94 ± 13	97 ± 15	94 ± 13	94 ± 13	93 ± 13	0.819
Pro	156 ± 11	154 ± 13	159 ± 14	157 ± 14	152 ± 16	0.996
Cys	114 ± 9.2	118 ± 12	121 ± 10	109 ± 13	116 ± 14	0.963
Ammonia	91 ± 4.3	93 ± 5.0	88 ± 5.6	95 ± 6.2	90 ± 5.7	0.908
Urea	6.82 ± 0.51	6.94 ± 0.48	7.03 ± 0.64	6.98 ± 0.57	6.63 ± 0.72	0.976
Glucose	3.38 ± 0.28	3.41 ± 0.32	3.45 ± 0.34	3.29 ± 0.30	3.26 ± 0.35	0.981

Values, expressed as nmol/mol for amino acids and µmol/ml for urea and glucose are means ± SEM, n = 6.

Table 2.5 Concentrations of amino acids, ammonia, urea, and glucose in plasma of sheep after consumption of glutamine supplement

	Time after Glutamine administration					*P*-Values
AA	0 h	0.5 h	1 h	2 h	4 h	
Asp	13 ± 1.7	13 ± 1.4	13 ± 1.8	13 ± 1.2	9.8 ± 0.3	0.141
Glu	60 ± 5.8	62 ± 4.3	60 ± 5.4	59 ± 4.5	58 ± 4.7	0.366
Asn	24 ± 2.9	24 ± 2.1	26 ± 2.5	25 ± 2.2	28 ± 2.3	0.285
Ser	72 ± 3.1	77 ± 3.8	77 ± 5.1	78 ± 3.6	76 ± 4.2	0.148
Gln	362 ± 6.2	364 ± 8.7	370 ± 9.0	373 ± 8.7	383 ± 11	0.012
His	61 ± 3.9	62 ± 4.1	62 ± 3.6	62 ± 3.7	60 ± 2.7	0.849
Gly	515 ± 82	527 ± 86	528 ± 93	540 ± 98	533 ± 94	0.646
Thr	62 ± 3.8	62 ± 3.3	63 ± 4.3	64 ± 3.7	62 ± 2.7	0.765
Cit	143 ± 14	148 ± 17	142 ± 12	144 ± 14	153 ± 12	0.352
Arg	186 ± 13	193 ± 15	192 ± 14	193 ± 11	196 ± 11	0.383
β Ala	18 ± 1.8	20 ± 1.3	19 ± 2.3	19 ± 1.5	19 ± 1.3	0.619
Tau	69 ± 5.6	74 ± 6.1	73 ± 4.7	71 ± 3.2	71 ± 4.4	0.988
Ala	186 ± 19	188 ± 18	186 ± 18	178 ± 16	174 ± 15	0.448
Tyr	64 ± 6.5	66 ± 6.9	67 ± 8.4	63 ± 7.2	63 ± 7.3	0.603
Trp	37 ± 2.2	38 ± 2.7	39 ± 2.3	39 ± 2.4	38 ± 2.0	0.814
Met	25 ± 1.8	26 ± 1.5	25 ± 2.1	24 ± 2.1	24 ± 1.3	0.705
Val	125 ± 8.5	133 ± 9.0	136 ± 9.5	133 ± 13	135 ± 14	0.950
Phe	37 ± 2.2	38 ± 1.9	39 ± 1.3	39 ± 3.0	38 ± 2.7	0.677
Ile	67 ± 4.0	70 ± 2.5	70 ± 2.4	70 ± 1.6	69 ± 2.6	0.857
Leu	106 ± 5.8	113 ± 5.2	114 ± 4.7	109 ± 3.9	106 ± 5.7	0.216
Orn	78 ± 2.6	80 ± 2.2	84 ± 3.6	90 ± 2.7	87 ± 2.6	0.437
Lys	93 ± 10	92 ± 9.7	91 ± 9.6	92 ± 9.5	92 ± 11	0.925
Pro	152 ± 14	149 ± 11	155 ± 13	157 ± 14	150 ± 13	0.987
Cys	108 ± 8.5	113 ± 9.7	114 ± 8.8	117 ± 9.3	102 ± 8.0	0.778
Ammonia	88 ± 5.0	90 ± 5.6	91 ± 5.9	93 ± 6.5	86 ± 6.4	0.930
Urea	6.67 ± 0.58	6.75 ± 0.62	6.81 ± 0.60	6.52 ± 0.73	6.44 ± 0.69	0.964
Glucose	3.41 ± 0.26	3.37 ± 0.23	3.48 ± 0.27	3.53 ± 0.28	3.32 ± 0.25	0.985

Values, expressed as nmol/mol for amino acids and μmol/ml for urea and glucose are means ± SEM, n = 6.

Table 2.6 Concentrations of amino acids, ammonia, urea, and glucose in plasma of sheep after consumption of urea supplement

	Time after Urea Administration					*P*-Values
AA	0 h	0.5 h	1 h	2 h	4 h	
Asp	11 ± 0.8	11 ± 0.7	9.9 ± 1.0	10.6 ± 0.7	10 ± 0.6	0.609
Glu	59 ± 4.0	55 ± 3.9	51 ± 3.3	55 ± 2.9	52 ± 3.0	0.405
Asn	29 ± 2.7	27 ± 2.1	26 ± 2.5	26 ± 2.3	30 ± 2.2	0.689
Ser	76 ± 2.7	75 ± 2.9	74 ± 4.1	75 ± 3.7	76 ± 4.8	0.826
Gln	377 ± 41	382 ± 41	385 ± 42	388 ± 43	387 ± 44	0.621
His	61 ± 3.2	60 ± 2.4	60 ± 2.5	60 ± 2.7	59 ± 3.7	0.861
Gly	517 ± 77	512 ± 68	526 ± 68	531 ± 76	542 ± 76	0.845
Thr	60 ± 3.0	61 ± 2.4	62 ± 2.5	61 ± 3.7	60 ± 3.8	0.596
Cit	143 ± 11	140 ± 8.7	142 ± 9.1	142 ± 9.2	149 ± 9.9	0.701
Arg	188 ± 23	189 ± 23	189 ± 21	184 ± 25	193 ± 23	0.824
β Ala	18 ± 0.6	19 ± 1.3	18 ± 1.5	19 ± 1.5	18 ± 0.7	0.981
Tau	73 ± 7.1	73 ± 8.2	72 ± 8.2	73 ± 6.1	70 ± 7.4	0.463
Ala	180 ± 26	181 ± 26	179 ± 27	168 ± 27	162 ± 25	0.301
Tyr	60 ± 4.0	61 ± 4.4	60 ± 4.7	59 ± 4.2	59 ± 4.6	0.327
Trp	38 ± 3.2	39 ± 3.2	38 ± 3.5	37 ± 2.5	38 ± 3.0	0.924
Met	23 ± 1.6	23 ± 1.3	23 ± 1.5	22 ± 1.8	23 ± 1.7	0.716
Val	129 ± 12	132 ± 10	129 ± 9.2	123 ± 4.8	121 ± 5.0	0.945
Phe	38 ± 1.3	39 ± 1.8	39 ± 2.2	38 ± 2.5	39 ± 2.6	0.226
Ile	68 ± 4.2	69 ± 3.6	67 ± 2.9	66 ± 3.0	64 ± 3.1	0.618
Leu	107 ± 3.5	112 ± 5.5	108 ± 4.6	104 ± 3.3	102 ± 3.4	0.224
Orn	79 ± 6.8	80 ± 8.0	79 ± 4.6	78 ± 3.0	80 ± 2.7	0.852
Lys	93 ± 6.7	95 ± 7.0	96 ± 7.3	94 ± 6.0	91 ± 5.5	0.538
Pro	144 ± 10	147 ± 11	148 ± 9.7	151 ± 12	142 ± 9.2	0.973
Cys	110 ± 8.6	112 ± 9.0	114 ± 10	119 ± 11	106 ± 9.8	0.910
Ammonia	90 ± 5.1	91 ± 5.8	96 ± 6.3	98 ± 6.5	93 ± 6.0	0.744
Urea	6.63 ± 0.49	6.71 ± 0.55	6.88 ± 0.57	6.92 ± 0.64	6.57 ± 0.52	0.978
Glucose	3.46 ± 0.25	3.40 ± 0.27	3.51 ± 0.26	3.65 ± 0.29	3.40 ± 0.28	0.964

Values, expressed as nmol/mol for amino acids and µmol/ml for urea and glucose are means ± SEM, n = 6.

Table 2.7 Percentage changes in the concentrations of Cit and Arg in plasma of adult sheep after oral administration of Cit

AA	Time after Citrulline administration					P-value
	0 h	0.5 h	1 h	2 h	4 h	
Cit	100 ± 0^{d}	105 ± 2.3^{cd}	116 ± 3.9^{c}	152 ± 5.4^{b}	217 ± 6.6^{a}	< 0.001
Arg	100 ± 0^{d}	103 ± 2.1^{cd}	106 ± 2.5^{c}	114 ± 3.8^{b}	123 ± 4.4^{a}	< 0.001

Values are mean ± SEM, n = 5.

a-d: Within a row, means not sharing the same superscript letters differ ($P < 0.05$).

CHAPTER III

DIETARY SUPPLEMENTATION OF CITRULLINE AND GLUTAMINE ENHANCES EMBRYONIC SURVIVAL IN GESTATING BEEF COWS

Abstract: Low fertility limits reproductive efficiency in cattle. This study was conducted with multiparous Brangus cows between May 26, 2016 and March 17, 2017 to test the hypothesis that dietary supplementation of rumen-protected AA (Cit+Gln, RPAA) or unprotected AA (RUAA) may improve embryonic survival in lactating beef cows. During the entire experimental period, cows grazed green pasture and had free access to drinking water and mineral blocks. At the onset of lactation, 107 cows were assigned randomly to one of three treatment groups: dried distillers grain (DDG) only (n = 36), DDG top-dressed with RUAA product (n = 35); and DDG top-dressed with RPAA product (n = 36). After two months of lactation, all cows underwent estrous synchronization and were then bred via artificial insemination (AI; the day of breeding = Day 0 of gestation). Each cow received AI only once. On the day of AI, the average body condition score (BCS) was recorded to be 4.56 ± 0.08. One day after breeding until Day 60 of gestation, cows were fed daily 0.64 kg DDG, 0.56 kg DDG + 0.28 kg RUAA (2% of estimated daily intake of 14 kg dry matter from pasture), or 0.56 kg DDG + 0.28 kg RPAA (2% of estimated daily intake of 14 kg dry matter from pasture). On each day of the supplementation period, cows were moved to pens to receive supplement once and then returned to their original pasture. On Days 40 and 60 of gestation, ultrasound determination of pregnancy was made. On Day 60 of gestation, blood samples (10 ml) were obtained from the jugular vein. Cows in all treatment groups grazed normally and appeared healthy. The pregnancy rate (25%) in the control group was relatively low on Day 40. One calf was born dead in the control group, and all calves were born alive in the RUAA and RPAA groups. Dietary supplementation of RUAA or RPAA

enhanced the birth rate of live-born calves from 22% in cows fed DGG alone to 34% ($P = 0.079$) and 36% ($P = 0.045$), respectively. The beneficial effects of the AA supplement were associated with increases in the concentrations of insulin in serum and of Cit, Arg, Orn and Pro in plasma, but decreases in the concentrations of ammonia in plasma ($P < 0.05$). Thus, dietary Cit in either a rumen-protected or unprotected form escaped the rumen, entered the portal circulation, and served as the immediate precursor for synthesis of Arg in extrahepatic tissues of beef cows. Based on the cost of Cit+Gln ($10/kg) and the daily use of 0.14 kg/day for 60 days, the net income from an operation of 1,000 beef cows would be $20,250 and $89,750, respectively, at the price of $750 and $1250 per calf. This simple nutritional method is expected to increase the reproductive efficiency of beef cows and their profitability. These findings may also have important implications for improving both lactation and fertility in dairy cows.

Introduction

The greatest limitation to reproductive efficiency across mammalian livestock species is embryonic mortality, which amounts to 25% to 60% (Bazer et al. 2015). In particular, the rate of pregnancy loss has been estimated to be 30-60% in beef cattle (e.g., 43% for lactating beef cows), with most losses occurring during the first month of gestation (Santos et al. 2004; Thatcher et al. 2001). Of interest, pregnancy loss can be as high as 80% in some heifers due to genetic and environmental factors (Moraes et al. 2018; Diskin et al. 2011). The improvement of functional traits using conventional approaches of phenotypic testing and quantitative genetics is difficult, because most reproductive traits are complex (polygenic) with low heritabilities (Diskin et al. 2011).

Research with swine, sheep and rats has shown that arginine is a nutritionally essential amino acid for the growth, development and survival of the conceptus (embryo/fetus and

placenta) (Bazer et al. 2015; Lassala et al. 2011; Satterfield et al. 2013; Wu et al. 2013, 2017). Of note, dietary supplementation with 0.83% arginine to gilts between days 30 and 114 of gestation (Mateo et al. 2007) or with 1.07% Arg to rats between days 1 and 21 of gestation (Zheng et al. 2008) increased the number of live-born offspring by 2.0 and 3.4 per litter, respectively. Arginine can be metabolized to both nitric oxide (NO) and polyamines which act directly on the conceptus to activate the mechanistic target of rapamycin (MTOR) cell signaling pathway to stimulate proliferation, migration, and protein synthesis by trophectoderm cells that are essential for elongation of the blastocyst and pregnancy recognition signaling (Kim et al., 2011a; Kong et al. 2012, 2014; Wang et al. 2014a,b). Importantly, arginine stimulates the expression of interferon tau in the ruminant conceptus (Kim et al. 2011b). Besides serving as the pregnancy recognition signal in ruminants, interferon tau acts in concert with progesterone to regulate the expression of a multitude of genes critical to growth and development of the conceptus, including transporters of nutrients into the uterine lumen (Bazer et al. 2015).

Under practical production conditions, a disadvantage of supplementing arginine to the diet for beef cows is that this basic amino acid may disturb the acid-base balance in the rumen and the body (Wu et al. 2007b). An alternative to arginine is the use of citrulline, which is a neutral amino acid that is converted into arginine at a nearly 100% efficiency in the presence of intracellular aspartic acid (Wu and Morris 1998). Because aspartic acid is a highly acidic amino acid, its supplementation to the diet may disturb acid-base balance in the body (Wu 2013). This problem is overcome by using glutamine (a neutral amino acid), whose metabolism generates aspartic acid in animals (Wu 2013). Thus, the combination of citrulline and glutamine would be an excellent source of arginine in beef cattle, while maintaining an appropriate pH in the rumen. The feasibility of this novel concept is supported by our results from a study with lactating dairy

cows, which indicated that supplementing rumen-protected citrulline+glutamine to their diet enhanced arginine synthesis (Chapter 2) and, consequently, milk production (Keith et al. 2018), as reported for lactating sows receiving dietary supplementation with arginine (Mateo et al. 2008).

Based on the foregoing results, the present study was conducted to test the hypothesis that dietary supplementation with rumen-protected AA (Cit+Gln, RPAA) or unprotected AA (RUAA) may improve embryonic survival in cattle. Lactating beef cows were chosen because they generally exhibit the highest embryonic wastage among beef cattle (Santos et al. 2004; Thatcher et al. 2001).

Materials and Methods

One hundred and seven (107) multiparous Brangus cows with an average BW of 463.4 ± 7.4 kg and an average BCS of 4.56 ± 0.08 (mean ±SEM) were used for this study between May 26, 2016 and March 17, 2017. During the entire experimental period, they grazed green pasture and had free access to drinking water and mineral blocks. A total of 107 cows were blocked according to BCS and BW, and were randomly assigned to one of three treatment groups: dried distillers grain (DDG) only (n = 36); DDG top-dressed with RUAA product (n = 35); or DDG top-dressed with RPAA product (n = 36). After two months of lactation, all cows underwent estrous synchronization and were then bred via artificial insemination (AI; the day of breeding = Day 0 of gestation) by the same technician. Each cow received AI only once. One day after breeding, cows were fed 0.64 kg DDG, 0.56 kg DDG + 0.28 kg RUAA (2% of estimated daily intake of 14 kg dry matter from pasture), or 0.56 kg DDG + 0.28 kg RPAA (2% of estimated daily intake of 14 kg dry matter from pasture). The AA supplement was provided in two equally divided doses per day. Both RUAA and RPAA were manufactured by Mr. Natale Vittori, a

biochemical engineer (Biotechnology Services and Consulting, Inc., Coppell, TX). The ratio of DDG to an AA supplement product was 2:1 to facilitate their consumption by cows. Distillers' grain was selected as a supplement because it is readily available and commonly used in beef cattle operations. Throughout the duration of the 60-day period of dietary supplementation, cows were brought from pasture to a pen, sorted from their calves, and fed their respective supplement individually. Once the supplement had been consumed, cows along with their calves, were returned to their original pasture. The first two months of gestation were chosen for supplementation because most pregnancy losses in beef cattle occur during this period.

On Days 40 and 60 of gestation, ultrasound determination of pregnancy was made. On Day 60 of gestation, blood samples (10 ml) were obtained from the jugular vein into tubes without coagulants or with heparin, and those cows that were not pregnant were removed from the study. Plasma or serum was obtained after centrifugation at 600 g for 10 min. Plasma was analyzed for amino acids, ammonia, urea, and glucose (Satterfield et al. 2013). Serum was analyzed for insulin (Mercodia Insulin ELISA, Uppsala, Sweden) and progesterone (Abnova Progesterone ELISA, Walnut, CA).

Calves from the previous pregnancy stayed with their mothers until they were weaned at 6 months of age. Those calves did not consume any supplements provided to their mothers. When the cows in the current study gave birth to new calves, gestation length, birth weight of calf, and the number of calves born alive or dead were recorded.

Statistical analysis.

Data on the concentrations of metabolites and hormones in the plasma were analyzed by one-way analysis of variance and the Student-Newman-Keuls multiple comparison test. Data on the pregnancy rate (the number of pregnant cows/the total number of cows receiving AI) or birth

rate (the number of live-born calves/the total number of cows receiving AI) were analyzed by X^2 analysis. Probability values ≤0.05 were taken to indicate statistical significance.

Results and Discussion

Beef cows in all the treatment groups grazed normally and appeared healthy. The average days postpartum for the beef cows used was 68.28 ± 2.2 days. On Days 40 and 60 of gestation, confirmed pregnancies were the same (Table 3.1). The ultrasound analysis showed that all cows carried a single fetus. The pregnancy rate (25%) in the control group was relatively low. This may be related to a number of factors, including climtate, stress induced from daily handling, and the low BCS of cow herd. High temperatures (e.g., high temperature of 30.6^{o}C and humidity of 96% on the day of AI; and high temperature of 35.0^{o}C and a humidity of 43% on July 10) in the Texas' summer. Also there was inclimate weather on the day of AI with heavy thunderstorms and rain, likely causing added stress to the cows. The BCS (a 9-point scale) of the cows used on the project were recorded as 4.56 ± 0.08 on the day of AI (Table 3.5), with a score 1 being extremely thin while a score 9 being extremely fat. This was lower than the ideal BCS of cows at breeding which would be between 5 and 6

There were no pregnancy losses in the cattle between Days 40 and 60 of gestation (Table 3.1). Gestation length did not differ (P > 0.05) among the three groups of cows, and averaged 281.5 ± 1.7 days (n = 34 cows). One calf was born dead in the control group, but all calves were born alive in the RUAA and RPAA groups. Live-born calves grew well and had no mortality within 3 months after birth.

A low rate of pregnancy in lactating beef cattle with heat stress (e.g., 16% in AI-bred beef cows in July in Central Texas) has been reported by other investigators (Sprott et al. 2001). For AI cows, low pregnancy rates may result from many factors, including ovarian dysfunction,

chromosomally abnormal or meiotically immature oocytes, impaired embryonic development, impaired implantation, embryonic/fetal death, and abortion of the fetal/placental tissue (Amundson et al. 2006). Of note, dietary supplementation with RUAA or RPAA enhanced pregnancy rate at Day 40 from 25% to approximately 34 and 36% ($P < 0.05$), respectively; and the birth rate of live-born calves from 22% to 34 and 36% ($P < 0.01$), respectively (Table 3.1). The beneficial effects of the AA supplement were associated with increases in the concentrations of insulin in serum (Table 3.2) and of Cit, Arg, Orn and Pro in plasma (Table 3.3), and, importantly, a decrease in the concentrations of ammonia (Table 3.3) in plasma. No differences in concentrations of progesterone in serum were detected among the three groups of cows (Table 3.2). Thus, dietary Cit in either a rumen-protected or unprotected form escaped the rumen, entered the portal circulation, and served as the immediate precursor for synthesis of Arg in extrahepatic tissues. Arg is known to stimulate the secretion of insulin from pancreatic β-cells (Flynn et al. 2002). As explained in Chapter 2, rumen-protected Gln in the RPAA group can be utilized in the small intestine for synthesis of Cit, and unprotected Gln in the RUAA group can contribute to intestinal Cit production through the synthesis of microbial proteins in the rumen. At the supplemental dose in the present study, neither RUAA nor RPAA affected concentrations of other AA, including Lys and His, indicating that these products did not result in an AA imbalance. Arg can stimulate the conversion of ammonia into urea in the liver (Wu and Morris 1998), thereby beneficially reducing concentrations of ammonia in plasma to prevent hyperammonemia which is highly toxic to embryos and fetuses (Herring et al. 2018).

Embryonic loss represents the single greatest economic loss for beef cows (Looney et al. 2006; Santos et al. 2004; Thatcher et al. 2001). Cows that become pregnant after the first AI, embryo transfer, or natural service are more profitable, because additional costs due to more days

on feed, synchronization of estrus, AI or embryo transfer, or human labor are incurred with each unsuccessful approach to establish pregnancy (Dahlen et al. 2014; Lamb and Mercadante 2016). Also with increased pregnancies resulting from the initial AI, producers can effectively shorten their breeding season which then results in heavier weaning weights and a uniform calf crop to increase their marketability (Troxel et al, 2006).

A successful pregnancy yielding a healthy weaned calf in beef or dairy cows is currently estimated to be worth $750 (Dr. Jason Cleere, Texas A&M AgriLife Extension, personal communication). Based on the cost of Cit+Gln ($10/kg) and the daily use of 0.14 kg/day for 60 days, the total expense for feeding one cow would be $84. For an operation with 1,000 beef cows, the net income would be $62,250 and $131,750, respectively, at the price of $750 and $1250 per calf (Table 3.4). Additional benefits that are not included in the margin of profit calculation include reductions in management and labor costs, improvements in herd health, an increase in cow numbers, and the prospect of high fertility in the next pregnancy. Based on the results of this research, we now know that citrulline without any encapsulation is able to bypass the rumen and, therefore, will be more affordable for use by producers. Thus, the price for feed-grade citrulline without encapsulation will be substantially reduced (e.g., $5/kg). Thus, a nutrition-based management system to increase embryonic survival will have an enormous impact on the global beef industry. These findings also have important implications for enhancing both milk production and fertility in lactating dairy cows, because they also have very low pregnancy rates [e.g., 16% in the U.S. in the summer (Stewart et al. 2011)].

In summary, dietary supplementation with Cit+Gln in either a rumen-protected or unprotected form to lactating beef cattle between Days 1 and 60 of gestation can increase the concentrations of Cit and Arg in the plasma and improve embryonic survival in lactating beef cattle. This simple method is expected to reduce early pregnancy losses and increase reproductive efficiency and profitability in livestock enterprises. Large-scale experiments are using ruminant species are warranted to optimize the supplemental doses and estimate economic returns from the nutritional treatment.

Table 3.1 Calving Data Following Artificial Insemination

Treatment	No. of Cows Receiving AI	Confirmed Pregnancies from AI Service (d 40)[a]	No. of Cows Reaching Term	No. of Live Calves at Birth	Birth Rate of Live Born Calves (%)	Birth Weight of Live Born Calves (kg)	No. of Calves Born Dead
Control	36	9 (25.0%)	9	8	22.2	29.0 ± 1.3	1
RUAA	35	12 (34.3%)†	12	12	34.3*	25.9 ± 1.1	0
RPAA	36	13 (36.1%)†	13	13	36.1*	27.8 ± 0.9	0

All calves born were singles.
Each amino acid (AA) supplement was mixed with 0.56 kg dried distillers grain (DDG) at 2% of estimated total daily intake of dry matter from pasture, while control cows only received 0.56 kg DDG.
[a] The number within the parenthesis refers to a pregnancy rate (the number of pregnant cows/the total number of cows receiving AI). Ultrasound analysis showed that all cows carried singletons.

† $P < 0.05$ vs the Control group.
*$P < 0.01$ vs the Control group.

Table 3.2 Concentrations of progesterone and insulin in serum of gestating beef cows

Hormone	Control (n = 9)	RUAA (n = 12)	RPAA (n = 13)	P-value
Progesterone (ng/ml)	1.99 ± 0.12	2.00 ± 0.11	2.01 ± 0.12	0.996
Insulin (µIU/ml)	132 ± 16[b]	259 ± 30[a]	221 ± 29[a]	< 0.001

Serum samples were obtained from beef cows on Day 6o of gestation. Values, expressed as nmol/ml for amino acids and ammonia and as µmol/ml for glucose and urea, are means ± SEM.
a-b: Within a row, means not sharing the same superscript letters differ ($P < 0.05$).

Table 3.3 Concentrations of amino acids, ammonia, urea, and glucose in plasma of gestating beef cows

Concentration of Amino Acids

AA	Control (n = 9)	RUAA (n = 12)	RPAA (n = 13)	*P*-Values
Asp	9.3 ± 0.5	9.6 ± 0.7	9.4 ± 0.5	0.938
Glu	60 ± 2.6	60 ± 3.2	61 ± 3.1	0.963
Asn	32 ± 1.9	35 ± 2.2	34 ± 2.2	0.535
Ser	57 ± 2.8	59 ± 3.2	60 ± 2.8	0.946
Gln	328 ± 16	336 ± 12	341 ± 13	0.804
His	42 ± 2.2	42 ± 1.8	44 ± 1.4	0.635
Gly	196 ± 7.8	201 ± 7.2	206 ± 8.2	0.684
Thr	58 ± 2.5	60 ± 2.1	61 ± 2.2	0.662
Citrulline	57 ± 2.3[b]	67 ± 2.8[a]	68 ± 3.1[a]	0.030
Arg	80 ± 3.6[b]	95 ± 3.8[a]	97 ± 3.7[a]	0.009
β-Alanine	16 ± 2.0	18 ± 1.4	17 ± 1.7	0.727
Taurine	26 ± 1.4	27 ± 1.6	27 ± 1.6	0.890
Ala	235 ± 8.7	239 ± 10	234 ± 9.6	0.923
Tyr	58 ± 2.3	61 ± 2.2	62 ± 2.6	0.524
Trp	52 ± 2.9	54 ± 2.4	56 ± 2.8	0.602
Met	27 ± 1.0	29 ± 1.5	28 ± 1.2	0.587
Val	196 ± 12	207 ± 11	203 ± 9.2	0.783
Phe	50 ± 2.2	53 ± 2.4	52 ± 1.8	0.641
Ile	103 ± 4.9	106 ± 4.2	108 ± 5.5	0.792
Leu	128 ± 5.4	130 ± 4.9	133 ± 6.6	0.852
Orn	70 ± 3.2[b]	82 ± 3.8[a]	84 ± 4.0[a]	0.033
Lys	97 ± 5.0	99 ± 4.7	101 ± 4.4	0.831
Pro	142 ± 6.4[b]	167 ± 7.1[a]	165 ± 7.3[a]	0.032
Cys	103 ± 4.8	106 ± 5.2	107 ± 5.6	0.874
Ammonia	87 ± 4.1[a]	75 ± 3.5[b]	74 ± 3.8[b]	0.042
Urea	6.05 ± 0.37	6.02 ± 0.44	6.08 ± 0.49	0.985
Glucose	3.47 ± 0.30	3.40 ± 0.33	3.52 ± 0.38	0.968

Plasma samples were obtained from beef cows on Day 6o of gestation. Values, expressed as nmol/ml for amino acids and ammonia and as μmol/ml for glucose and urea, are means ± SEM.

a-b: Within a row, means not sharing the same superscript letters differ ($P < 0.05$).

Table 3.4 Economic return from using Cit+Gln supplement to lactating beef cows

1,000 Beef	Live-born	Income	Supplement	Net income
Cows	calves	$	cost, $	gain, $
$750/calf				
Control	222	166,500	0	166,500
Cit+Gln	361	270,750	42,000	228,750
Difference	139	104,250	42,000	62,250
$1,250/calf				
Control	222	277,500	0	277,500
Cit+Gln	361	451,250	42,000	409,250
Difference	139	173,750	42,000	131,750

Note: 8.4 kg of Cit+Arg is supplemented to one cow for 60 days. The total cost of the supplement is $5/kg.

Table 3.5 Characteristics of beef cows that produced calves

Hormone	Control (n = 9)	RUAA (n = 12)	RPAA (n = 13)
Days Postpartum (Days)	67.9 ± 3.9	70.9 ± 3.6	66.3 ± 3.6
Body Weight (kg)	472. 8 ± 12.3	469.7 ± 12.7	474.7 ± 13.6
BCS at the time of AI	4.5 ± .16	4.5 ± .18	4.5 ± .21

AI = artificial insemination

CHAPTER IV

DISCUSSION AND CONCLUSION

The potential use of L-arginine (Arg) to increase embryonic survival and reproductive efficiency in beef cattle remains to be seen on a large scale. The ability of Arg to supply vital molecules (e.g., NO and polyamines) to both dam and conceptus during the period increasing growth is partly the reason why this amino acid (AA) has been considered a conditionally essential AA during pregnancy (Wu et al. 2013). Past research involving rats, pigs, and sheep have shown promising results for Arg to enhance reproductive performance in those species (Zeng et al., 2008, Wu et al., 2007, 2013). To our knowledge, there are no reports of effects of dietary Arg supplementation on embryonic/fetal survival in beef or dairy cattle.

Results from the experiment with pregnant beef cows were promising for enhancing pregnancy rate after one administration of artificial insemination. Several environmental factors experienced during the study could potentially have affected the results. For example, the daily handling of the cow-calf pairs before administering their respective treatments has the potential to affect pregnancy rates due to subjecting cows to relatively high levels of stress. Also, the hot and humid climate in which the experiment was conducted has the potential to lower pregnancy rates in cattle as demonstrated by Biggers et al. (1987). Cattle located on pasture with lower forage quality have a higher risk of having a deficient diet. This suboptimal diet would have had the potential to negatively affect the cows' ability to establish and maintain a pregnancy. The temperament of the cattle being used coupled with the fact that there was daily handling during the 60-d feeding period. Cattle with an excitable temperament have been shown to have a hyper-active hypothalamic-pituitary-axis when exposed to handling stress from humans (Federenko et al., 2004). The result of this is impairment of mechanisms that are necessary for pregnancy, like

the resumption of the animals' estrous cycles, oocyte ovulation, and establishing the pregnancy (Dobson et al., 2000). It is plausible that if any of the forementioned mechanisms were effected in our study, it could have potentially affected the pregnancy rates seen in the beef cow experiment.

To our knowledge and understanding, results of this study are the first to indicate that extracellular Cit is not subject to significant degradation or metabolism by microbes in the rumen. This unexpected finding was validated in experiments involving incubation of Cit *in vitro* in whole rumen fluid and *in vivo* feeding experiments involving beef cattle, as well as sheep. Our findings also indicated limited catabolism of extracellular Glu by rumen microbes. Thus, the traditional view that all dietary AA undergo degradation in the rumen of ruminants is not correct. This new finding that Cit and Glu escape degradation in the rumen has far-reaching implications in rumen nutrition using dietary supplementation to enhance the provision of Glu and Arg to the gut, and of Arg to the extra-intestinal tissues. At present, there is limited research involving Cit and other so-called "nutritionally nonessential AA" in the diet of ruminants. Results of the present study are novel in revealing that unprotected Cit can be used as a dietary supplement to increase circulating concentrations of Arg in ruminants to enhance production traits such as reproductive performance.

The mechanism(s) responsible for the ability of Cit to by-pass degradation in the rumen remains unclear and requires further study. The fact that no encapsulation or modification treatment is needed for Cit to bypass the rumen allows it to be an economical option for use by producers in the cattle industry. The intravenous administration of Cit (Lassala et al. 2009) or its enteral feeding (this Chapter) to adult sheep can increase the concentration of Cit in plasma, suggesting that sufficient Asp is available for the conversion of Cit into Arg in tissues of

ruminants. Thus, dietary Gln is not needed for the synthesis of Arg from Cit in ruminants, as is the case for other animal species, including pigs, rats and chickens (Wu and Morris 1998). In practice, this can reduce the cost of AA supplement by at least 50%, further enhancing the economic returns of the dietary supplementation with Cit alone. Altering dietary supplements with AA in large-scale experiments with greater numbers of supplemented cows is necessary to establish the effectiveness of this strategy. Results of the present study clearly suggest that the use of Cit as a new dietary supplement to ruminants, including beef cattle, dairy cattle, sheep and goats, will improve their growth and production efficiencies.

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