2003

# Effects of free methionine and lysine on in vitro fermentation and in vivo performance and ruminal fermentation of late lactation holstein cows

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# EFFECTS OF FREE METHIONINE AND LYSINE ON IN VITRO FERMENTATION AND IN VIVO PERFORMANCE AND RUMINAL FERMENTATION OF LATE LACTATION HOLSTEIN COWS

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agriculture and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science

in

The Interdepartmental Program in Animal and Dairy Sciences

by
Yi-Hua Chung
B.S., Chinese Culture University, Taiwan, 1999
August 2003

## ACKNOWLEGMENTS

Finally, I have reached the point where I can write something without my academic boss proof reading it at least four times. It has taken long time for me to finish my master's degree here in the LSU. I still can remember the nervousness, frustration, and even the fear when I was trying to settle into graduate life here. But, now, I can live with confidence and will be leaving the LSU with my head up. There are too many people who have helped me during my journey that I need to thank. However, there are some people that definitely deserve a place in my thesis because without them, I would never have been able to get my jobs done and present this thesis.

First of all, I would like to express my greatest gratitude to my academic professor, Dr. H. Gale Bateman, II, for his guidance and support throughout my master's program, for his knowledge on planning our research projects, for his patience waiting on me to catch up with his mind, for his trust in my work, and of course, for his passion to push me to my potential and make me believe that I can be a better graduate student than I ever thought I could be. I would also like to express my appreciation to my committee members, Dr. Cathy C. Williams, Dr. Bruce Jenny, and Dr. L. Lee Southern, for their academic guidance, knowledge, and assistance throughout my master's program, especially for Dr. Cathy Williams, for her mental support and for always being my friend.

I would like to thank Mr. Danny Gantt, for his technical assistance and coaching on my on-farm skills. I would like to thank my fellow graduate students, Christie Stanley, Patricia Terrell, Gina Goodier, and Amy Beem, for their help, support, jokes, culture exchange, and life sharing. They opened my mind, fulfilled my boring graduate life, kept me company, and helped me overcome every challenge that I encountered throughout my master's program. I know I can always count on you girls. That meant a lot to me, thanks!

A special appreciation is expressed to Christie and Mike Stanley. Thank you both for bringing Tuxedo into my life. Tuxedo is so sweet and wonderful. He makes me feel responsible and not lonely anymore. I know he is always at home and waiting on me.

I would also like to thank undergraduate students, Andra Ponson (and her Freckles, Dr. Giraffé, Ph.D. x 3), Emily Meyer, Laura Ward, Justin Roberts, and Ofelia Guerra, for their assistance both on-farm and in lab.

I would like to thank my friends, Yen-Ling Cheng, I-Ching Lai, Chih-Chun Huang (Jean), AAA family (Albert, Jane, and Alvin), for their help and care, and Dr. Pou-Lin Wu, for his sharing and helping to take care of Tuxedo every time I need to out of town. Because of you all, I know where to go every time I am homesick.

Finally, I would like to express my gratitude to my parents and my brothers, for their love, support, and encouragement. I could not go so far without them. If I have ever made any achievement in my life, it is all because of them.

Thank you all and thank God above!

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## ABBREVIATION KEY

4% FCM=4% fat corrected milk
AA=Amino acids
ADF=Acid detergent fiber
ADIN=Acid detergent insoluble nitrogen
Arg=Arginine
BCFA=Branched-chain fatty acids
CP=Crude protein
Cys=Cysteine
DIM=Days in milk
DM=Dry matter
DMI=Dry matter intake
EAA=Essential amino acid
ECM=Energy corrected milk
His=Histidine
HMB=DL-2-hydroxy-4- methylthiobutanoic acid
Ile=Isolecuine
IVDMD=In vitro dry matter disappearance
Leu=Leucine
LSU=Louisiana State University
Lys=Lysine
Met=Methionine
MP=Metabolizable protein
N=Nitrogen
NAN=Nonammonia-nitrogen
NEAA=Non-essential amino acid
$NH_3$=ammonia
$NH_4^+$=ammonia-nitrogen ion
NPN=Non-protein nitrogen
OM=Organic matter
OMI=Organic matter intake
Phe=Phenylalanine
RDP=Rumen-degradable protein
RH=Relative humidity
RPLys=Ruminally protected lysine
RPMet=Ruminally protected methionine
RUP=Ruminally undegraded feed protein
S=Sulfur
SCC=somatic cell counts
SCCS=Somatic cell count scores
TDN=Total digestible nutrients
THI=Temperature-humidity index
Thr=Threonine
TMR=Total mixed rations
Trp=Tryptophan
Tyr=Tyrosine
Val=Valine
VLDL=Very low density lipoproteins
VFA=Volatile fatty acid

## ABSTRACT

Two experiments were conducted to investigate the effects of free Met and Lys supplementation on ruminal fermentation in vitro and in vivo. In the in vitro study, supplementation of Met and Lys had effects on concentrations of total VFA and $NH_4^+$, proportions of acetate, propionate, butyrate, isovalerate, valerate, and the ratio of acetate to propionate. Percentages of microbial N synthesis in effluent pellets were not affected by supplementing Lys and Met. The combination of 0.52% Met and 1.03% (90% DM) Lys resulted in the highest concentration of total VFA, second highest concentration of $NH_4^+$, and lowest ratio of acetate to propionate with no decrease in the microbial CP production. In the in vivo study, supplementing 0.29% Met and 2.27% Lys (100% DM) had no impact on mean DMI, OMI, milk yield, milk component production or percentage, SCCS, 4% FCM and ECM production efficiencies, or body weight gain. Although DMI for the treatment group was numerically lower than the control group, milk yield, % milk fat, and % milk protein for the treatment group were numerically higher than the control group and resulted in numerically higher 4% FCM and ECM efficiencies for cows received AA supplementation. There was a statistical interaction of treatment and day observed on DMI, OMI, and 4% FCM and ECM production efficiencies indicating that the treatment group maintained a better production efficiency than the control group throughout the experiment. The concentration of ruminal $NH_4^+$ and the proportion of butyrate were increased, but proportions of acetate and valerate were decreased while the pH, total VFA, proportions of propionate, isobutyrate and isovalerate, and the ratio of acetate to propionate were not affected by supplementing AA.

# CHAPTER 1. INTRODUCTION

Nutrient requirements of the mammary gland for synthesis of milk and milk protein include glucose, acetate, β-hydroxybutyrate, long-chained fatty acids, and amino acids (AA) (Schwab, 1994). Amino acids absorbed by the mammary gland are provided by metabolizable protein (MP), which is the true protein digested post-ruminally (NRC, 2001). Sources of MP are: 1) ruminally synthesized microbial protein, 2) ruminally undegraded feed protein (RUP), and 3) endogenous protein (NRC, 2001). Microbial protein is produced by ruminal microorganisms, which include bacteria, protozoa, and fungi. Ruminal bacteria have a nitrogen (N) requirement for growth, and this requirement can be satisfied by supplying peptides, AA, and/or ammonia ($NH_3$) to the ruminal environment (NRC, 2001).

The basic concept of dietary protein formulation is to optimize the efficiency of dietary N utilization by satisfying the requirements of the ruminal microbes and the host animal. This is accomplished by providing an adequate amount of rumen-degradable protein (RDP) for use by the ruminal microorganisms to improve ruminal fermentation and obtain maximum rumen microbial protein production (NRC, 2001) and also providing an adequate amount of rumen-undegradable protein (RUP) with balanced AA that complement ruminally synthesized microbial protein for the host animal use (Schwab, 1995). There is a growing interest in optimizing the delivery of AA to the duodenum to accurately meet the AA requirements for maintenance and production purposes of ruminant animal. Strategies (Bateman et al., 1999; Vanhatalo et al., 1999) for manipulating the post-ruminal supply of AA include: 1) increasing production of microbial protein, 2) increasing dietary crude protein content with appropriate protein supplements, 3) feeding proteins resistant to ruminal degradation but also providing sufficient rumen-degradable crude protein for rumen microbes, and 4) using rumen-protected AA.

Feeding excess amounts of N as dietary protein has been considered inefficient and has caused environmental N accumulation. It has been proposed that in the US, 90% of the ammonia emission comes from agriculture, and 90% of this emission is produced mainly by livestock husbandry (Meisinger and Jokela, 2000). Based on both economical and environmental standpoints, the fundamental goal for dairy protein formulation is not only to optimize the efficiency of dietary N utilization to maximize growth and milk production per unit N consumed (Schwab, 1995), but also to address experimental concerns in dairy cattle diet formulation to reduce N excretion in manure (Vanhatalo et al., 1999).

Methionine (Met) and lysine (Lys) have been reported as the first two limiting AA for maximum milk yield and milk protein production (Schwab et al., 1992). Supplying these AA may improve microbial protein synthesis and therefore improve milk production without adding excess N to the environment. Therefore, two experiments were conducted to study the effects of free Met and Lys supplementation on both in vitro fermentation and performance and ruminal fermentation of lactating Holstein cows.

# CHAPTER 2. REVIEW OF LITERATURE

## 2.1 Background

Feed cost is the major factor that influences the productivity of the livestock industry. In livestock diet composition, dietary protein accounts for the most expensive proportion among all other nutrients. Digested dietary protein supplies essential AA (EAA), non-essential AA (NEAA), N, and peptides for animal physiological uses. Essential AA, or indispensable AA, are AA that cannot be synthesized by animal tissues in the amounts sufficient for optimum performance especially during the early stage of growth and for high levels of production (NRC, 2001). Non-essential AA, or dispensable AA, are AA that do not necessarily have to be provided in the diet but can be synthesized in adequate amounts by animal tissues.

Dairy cattle have metabolic AA requirements but not metabolic protein requirements (NRC, 2001; Schwab, 1996). Intestinally absorbed AA serve as building blocks for the synthesis of body tissues (Schwab, 1996) and are essential for maintenance, growth, reproduction, and lactation by dairy cattle (NRC, 2001). Metabolizable protein serves as the major source of absorbable AA to the small intestine of ruminants.

Under most commercial settings, Met and Lys have been reported as the two first-limiting AA for maximum milk yield and milk protein production (Schwab et al, 1992). However, dietary protein of a conventional diet varies and differs from microbial and milk protein in AA composition (Schwab, 1995). Common feeds, such as alfalfa (11.1% Lys and 3.8% Met; % of total EAA), corn silage (7.5% Lys and 4.8% Met), yellow corn (7% Lys and 5% Met), and soybean meal (13.7% Lys and 3.1% Met) are often deficient in Lys and Met contents as compared to milk (16% Lys and 5.5% Met) and ruminal bacteria (15.8% Lys and 5.2% Met) in their Lys and Met profiles (Schwab, 1995). Because free AA are unstable and degrade rapidly in the rumen, the effects of supplementing ruminally protected Met (RPMet) and Lys (RPLys)

products on ruminal fermentation and milk performance for high producing dairy cows have been extensively studied.

Early studies on the development of protecting AA have focused on AA derivatives and analogs, especially for Met (Schwab, 1995). The Ca salt of DL-2-hydroxy-4-methylthiobutanoic acid (HMB), also known as methionine hydroxy analog, has been studied as a supplement for increasing milk and milk fat production. From a metabolic standpoint, HMB acts as Met in both chickens and rats. Although HMB has a high ruminal degradation rate, feeding HMB often influences ruminal fermentation, diet digestibility and lipid metabolism (Schwab, 1995). Ca salts of HMB are no longer manufactured, but liquid HMB is available and has been used extensively in both swine and poultry diets as a substitute for Met (NRC, 2001).

The technique of encapsulation of AA with lipids (fat and oil), often in combination with inorganic materials and carbohydrates as stabilizers, softening agents, and fillers, has been pursued, but this technique has been limited to protecting only Met from ruminal degradation (Schwab, 1995). Rumen-protected AA of N-Cap-M and N-Cap-L (Prince Agri Products, Inc.) were Met or Lys encapsulated with hydrogenated vegetable oils, but these two products are no longer on the market. Megalac Plus™ (Church & Dwight Co., Inc.) is a bypass fat product containing 6% methionine hydroxy analog, which is protected by the fatty acids from rumen degradation. Mepron® 85 (Degussa Corp.) is a rumen slow-release product which contains 85% DL-Met and small amounts of ash, starch, fat, and cellulose. This product consists of a nucleus of Met with several thin coats of stearic acid and ethylcellulose. Results of nylon bag studies have shown that the protection rates of Mepron® 85 in the rumen are approximately, 90% at 2 h, 80% at 6 h, and 70% at 15 h (Schwab, 1995). There is an interest in protecting AA by encapsulating with ruminally-inert, pH-sensitive polymers that are resistant to ruminal degradation and deliver intact AA to the small intestine (Schwab, 1995). The result of this

technique is a rumen-stable rather than a slow-release product. Smartamine™ M and Smartamine™ ML (Rhône-Poulenc Animal Nutrition; Antony Cedex, France), rumen-stable forms of Met and Met plus Lys products, have been marketed but are costly (Schwab, 1995), and currently Smartmine™ML is not marketed. Smartamine™ M contains 70% DL-Met, and Smartamine™ ML contains 15% DL-Met plus 50% L-Lys-HCl. Nylon bag studies indicated the rumen protection rates of these two products exceed 90% at 24 h, and intestinal availability values were approximately 90% (Schwab, 1995).

There is not yet an efficient commercially available RPLys product because of the physical-chemical properties of Lys as compared to Met (NRC, 2001). Research data regarding effects of supplementing free Lys as a substitute for RPLys, alone or in combination with either free Met or RPMet on ruminal fermentation and milk performance are limited. While the development of ruminally protected AA still has a long way to go, several relatively inexpensive commercial feed grade sources of crystalline free forms of AA include: L-Lysine-HCl, L-Threonine, L-Tryptophan, and DL-Methionine. These products are marketed and have been used extensively in both the swine and poultry industries. Since there are no direct research results that indicate the ideal Met and Lys profiles for maximum milk production by dairy cattle, it is reasonable to accept the ideal Met and Lys requirements (Met=0.26% of the diet and Lys=1.03% of the diet; 90% DM) for maximum milk production for lactating sows (NRC, 1998; Oldham, 1994). It is probable that the absolute amounts of each AA required for milk production by dairy cattle are greater than that for pigs because of greater milk output and larger size of dairy cattle (Oldham, 1994). However, when these requirements are expressed as percentage of the diet, they may be lower than that of swine due to greater dry matter intake (DMI). It is our interest to determine if the ideal amounts of Met and Lys from dietary AA requirement of

lactating sows (NRC, 1998) will be the ideal requirements of Met and Lys for improving ruminal fermentation and maximizing milk and milk component production in lactating dairy cattle.

This review of literature will focus on protein nutrition of both the ruminant animal and ruminal microorganisms. Research results of rumen-protected AA and free AA supplementation on ruminal fermentation via both in vitro and in vivo techniques and production responses to improved Met and Lys nutrition will be described. Metabolism and utilization of the end products of ruminal fermentation will also be discussed.

## 2.2 Protein Nutrition in the Rumen

Ruminants need to absorb AA at the small intestine to meet their AA requirements for both maintenance and production. The requirement for absorbed AA can be met from three sources: 1) ruminally synthesized microbial protein, 2) RUP that escapes ruminal degradation and flows to the small intestine, and 3) endogenous proteins. Microbial protein usually accounts for the largest portion of the total AA that enters the small intestine (Stern et al., 1994). Clark et al. (1992) reported that about 59% of the nonammonia-N (NAN) that passed to the duodenum of dairy cattle was supplied by microbial protein. Since a larger amount of the absorbed AA is provided by microbial protein than RUP, optimizing ruminal fermentation is extremely important in diet formulation. The ultimate goal for optimal ruminal fermentation is to provide a diet with adequate, but not excessive, amounts of RDP to meet the requirements of the ruminal microorganisms while also providing adequate RUP, in conjunction with the microbial protein, to meet the host animal's AA requirements.

### 2.2.1 Microbial Nitrogen Requirements

The ruminal population of microorganisms consists of bacteria, protozoa, and fungi. The bacteria contribute the major role toward ruminal fermentation. Unlike the host animal, ruminal bacteria do not have an AA requirement but do require N as substrate for growth (NRC, 2001).

Peptides, AA, and $NH_3$ serve as N sources to meet microbial N requirements, and these three N sources are supplied from hydrolysis of recycled N and degradation of RDP that includes both dietary and endogenous proteins.

#### 2.2.1.1 Ammonia, Peptide, and Amino Acid

Although ruminal bacteria do have N requirements, the exact amount of N for maximum microbial protein synthesis is unknown. Recommended concentration of required $NH_3$ for ruminal bacteria are varied and are estimated to be between 0.35 and 29 mg/dl (Owens and Zinn, 1993). Satter and Slyter (1974) and Schaefer et al. (1980) reported that a concentration of ruminal $NH_3$ of 2 to 5 mg/dl was adequate for microbial growth. Petersen (1987) reported 1 to 2 mg/dl of $NH_3$ was required for optimal fiber digestion. However, Demeyer and Van Nevel (1986) and Hespell and Bryant (1979) reported only 0.2 mg/dl of $NH_3$ was needed for ruminal microorganisms to grow.

Salter et al. (1979) indicated that between 18 to 100% of microbial N was derived from $NH_3$, and Nolan (1975) and Leng and Nolan (1984) indicated that 50% or more of the microbial N was derived from $NH_3$, and the rest was from peptides and AA. These researchers used $NH_3$ as the major or sole N source for the ruminal bacteria. Other researchers (Cotta and Russell, 1982; Griswold et al., 1996; Russell and Sniffen, 1984) have demonstrated improved microbial growth or efficiency when peptides or AA replaced $NH_3$ or urea as the major or sole source of N. Maeng and Baldwin (1976) reported increased microbial yields and growth rates when the combination of 75% urea and 25% AA-N replaced 100% urea as dietary N. Argyle and Baldwin (1989) reported that ruminal bacterial growth was increased by AA and peptides, but the increase in growth rates by AA was due to many different AA being available to the bacteria in a given mixture rather than any specific growth limiting AA. An in vitro study by Russell and Strobel (1993) and an in vivo study by Rooke and Armstrong (1989) both reported that microbial protein

synthesis was increased when peptides and AA were substituted for $NH_3$. Cruz Soto et al. (1994) reported that the stimulation of microbial protein synthesis in vitro by using AA and peptides varied with species of bacteria and energy source used. Reviews by Clark et al. (1992) and Stern et al. (1994) indicated that microbial protein flow to the duodenum may be decreased when RDP was replaced by RUP, possibly because the supply of peptides and AA for ruminal microbes were limited.

Based on the results described above, although ruminal microorganisms do not have requirements for preformed AA, growth of microorganisms and synthesis of microbial protein are stimulated by the presence of pre-formed AA and peptides. More research is needed to determine how AA and peptides stimulate the synthesis of microbial protein (Firkins, 1996).

#### 2.2.1.2 Non-Protein Nitrogen and Rumen-Degradable Protein

Ruminants can utilize non-protein nitrogen (NPN) as well as natural dietary protein. Based on the rate and extent of ruminal degradation, NPN in feeds and supplements, such as urea and ammonium salts, are considered to be completely degraded in the rumen (NRC, 2001). Substitution of NPN for natural protein always decreases the cost of dietary protein, but production responses seldom increase with the addition of NPN (Owens and Zinn, 1993). There are some important rules that need to be kept in mind when using NPN (Owens and Zinn, 1993). First, NPN only supplies $NH_3$ for ruminal bacteria but does not supply energy and minerals as do plant and animal protein sources. Second, high producing dairy cattle utilize natural protein more efficiently than NPN because the protein requirements for dairy cattle during early lactation are greater than the protein requirements for the ruminal microorganisms. Third, NPN is degraded faster and more completely than natural protein sources in the rumen. If most of the dietary RDP is supplied by NPN, too much readily soluble N is presented to the ruminal microorganisms, and excess N may be washed out of the rumen as $NH_3$ before it can be incorporated into microbial

protein. But, if most of the dietary RDP is supplied by natural proteins, excess protein may escape or bypass ruminal degradation and still be available to the host animal at the lower gut. This is especially true during early stages of lactation. Fourth, NPN is recommended to be used only during mid or late lactation since protein requirements of the host animal are lower during this time.

### 2.3 Development of Limiting Amino Acid Requirements

Dairy cattle have AA requirements for maintenance, growth, reproduction, and production rather than a protein requirement per se (NRC, 2001). The 10 EAA for dairy cattle are arginine (Arg), histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), threonine (Thr), tryptophan (Trp), and valine (Val). Although the classification of AA as essential or nonessential was based on research with non-ruminant animals, Black et al. (1957) indicated that the classification of AA in dairy cattle and sheep was similar to that of non-ruminants.

The NRC (2001) stated that the requirements for NEAA for both growth and production are met before the requirements for the most limiting AA. The NRC (2001) also discussed a theory that the efficiency of using MP for synthesis of protein will be determined by how well the profile of EAA in MP matches the profile required by the host animal and the amount of total EAA in MP. Growing interest in determination of AA requirements and development of a protein system, which supplies balanced absorbable AA, is based on the knowledge that (Schwab, 1996): 1) production of milk and milk protein are influenced by the pattern of absorbed AA, 2) efficiency of use of RUP for milk protein production is influenced by AA composition and intestinal digestibility of RUP, and 3) the AA profile in digestible RUP is often not adequate for the optimum use of MP for milk protein synthesis.

Enormous amounts of research have been conducted either by direct or indirect approaches to determine the sequence of limiting AA for maximum milk yield and milk protein production. Direct approaches of abomasal or duodenal infusion studies indicate that Met and Lys are the first two limiting AA when lactating cows are fed conventional diets (Schwab et al., 1992). Schwab et al. (1992) using Holstein cows concluded that Lys seemed to be the first-limiting and Met to be the second-limiting AA during peak lactation; Lys was the first- limiting during early lactation; both Lys and Met were co-limiting during midlactation; and there seemed to be no AA deficiencies for Holstein cows fed conventional diets during late lactation. Methionine and Lys were reported as the two limiting AA for synthesis of tissue protein in growing ruminants (Merchen and Titgemeyer, 1992). Methionine and Lys were reported to be the first and second limiting AA respectively in microbial protein for N retention in growing sheep (Nimrick et al., 1970; Storm and Ørskov, 1984) and cattle (Richardson and Hartfield, 1978).

Most feed proteins have lower Lys and Met contents, especially Lys, compared to milk and ruminally synthesized microbial protein (Schwab, 1995; 1996). The contribution of Lys flow to the duodenum was usually lower after feeds were exposed to ruminal degradation (Schwab, 1995; 1996). Lysine is more sensitive to processing and has a lower intestinal digestibility than other EAA in RUP (Schwab, 1995; 1996). Therefore, when cattle are fed conventional diets, Lys supplementation may improve milk and milk protein production.

The sequence of Met and Lys limitation is influenced by dietary composition and is determined by their relative concentrations in RUP (NRC, 2001; Schwab, 1995). It is very important to know the sequence of EAA in a diet with known composition in order to select a protein supplements that will improve the AA profile in MP (NRC, 2001). Lys was the first-limiting for young post-weaned calves (Abe et al., 1997), growing cattle (Abe et al., 1997; Burris et al., 1976), and lactating cows (King et al., 1991; Polan et al., 1991; Schwab et al., 1992)

when corn and corn-byproducts provided most of the RUP. Conversely, Met was the first-limiting for post-weaned calves (Donahue et al., 1985; Schwab et al., 1982), growing cattle (Hopkins et al., 1999; Klemesrud and Klopfenstein, 1994; Lusby, 1994; Robert et al., 1999a), and lactating cows (Armentano et al., 1997; Rulquin and Delaby, 1997; Robert et al., 1994; Schingoethe et al., 1988) when small amounts of corn were fed, when high forage diets were fed, or when all or most of the RUP was provided by legume (soybean products), animal-derived proteins, or a combination of both (Merchen and Titgemeyer, 1992; Rulquin and Vérité, 1993; Schwab et al., 1992). Lysine and Met were identified as co-limiting when lactating cows were fed diets with minimal protein supplementation (Schwab et al., 1976).

There is a need for supplementing Met and Lys to high producing dairy cows because most of the conventional diets fed in the U.S. are deficient in these AA. Moreover, high producing dairy cows have the greatest requirement for RUP, which provides a large portion of absorbed AA to support the high level of production (Schwab, 1995).

### 2.4 Responses to Rumen-Protected Methionine and Lysine Supplementation

Responses of both growing and lactating dairy cattle to post-ruminal supply of Met and Lys have been reported. A summary can be found in NRC (2001). In studies with growing cattle, variable increases in body weight gains and feed efficiency (Hopkins et al., 1999; Robert et al., 1999a; Veira et al., 1991), and variable decreases in urinary N excretion (Abe et al., 1997,1998; Campbell et al., 1996, 1997a; Donahue et al., 1985; Schwab et al., 1982) have been reported. In studies with lactating cows (Chapoutot et al., 1992; Freeden et al., 1999; Garthwaite et al., 1998; Nichols et al., 1998; Nocek et al., 1999; Piepenbrink et al., 1999; Rode et al., 1999; Rulquin and Delaby, 1997; Rulquin and Vérité, 1993; Rulquin et al., 1993; Schwab, 1995; 1996; Sloan et al, 1998; Sniffen et al., 1999a and b; Wu et al., 1999), reported production responses included: 1) milk protein is more sensitive than milk yield to Met and Lys supplementation, especially for

cows in post-peak lactation, 2) increases in milk protein percentage are independent of milk yield, 3) casein is the milk protein fraction was most influenced, 4) increases in milk protein production to increased supplies of either Met or Lys in MP are the most predictable when the resulting predicted supply of the other AA in MP is near or at estimated requirements, 5) milk yield responses to Met and Lys supplementation are more common in cows in early than in mid or late lactation, and 6) production responses to Met and Lys supplementation are greater when CP is about 14 to 18 % of DM than when CP is either lower or higher.

Milk fat percentage has also been reported to increase with Met or Lys supplementation, but the increases are not predictable. Socha et al. (1994a) reported increases in percentage of milk fat when Met was infused post-ruminally. Increased percentage of milk fat was also reported when Met (Samuelson et al., 2001; Yang et al., 1986) or Met and Lys (Bremmer et al., 1997; Canale et al., 1990; Rogers et al., 1987; Xu et al., 1998) were supplied in rumen-protected forms. However, Socha et al. (1994a, b, and c) reported increased percentages of milk fat and milk protein in post-peak lactating cows, but reported an increased percentage of milk protein and an unchanged percentage of milk fat in both peak- and mid-lactation cows infused with Met post-ruminally. The reasons why milk fat was sometimes increased by feeding increased amounts of Met and Lys in MP are still not clear (NRC, 2001). Possible reasons include (NRC, 2001): 1) Met supplementation increases post-ruminal supply of energy as acetate and propionate and post-ruminal supply of microbial protein and lipid for post-ruminal utilization by stimulating rumen fermentation (Chandler et al., 1976); 2) Synthesis of milk fat has a close relationship with blood concentration of acetate (Croom et al., 1981; Ray et al., 1983); 3) Met may have an impact on de novo synthesis of short- and medium-chain fatty acids in the mammary gland (Christensen et al., 1994; Pisulewski et al., 1996); and 4) Met is a methyl donor for the synthesis of choline (Sharma and Erdman, 1988). Choline is essential for the synthesis of phospholipids required for

the synthesis of chylomicrons and very low density lipoproteins (VLDL) and is also possibly a limiting nutrient for milk fat synthesis (Erdman, 1994; Sharma and Erdman, 1988; 1989).

## 2.5 Responses to Free Methionine and Lysine Supplementation

### 2.5.1 In Vitro Free Methionine and Lysine Supplementation

While the main interest of supplementing AA to ruminants has focused on ruminally protected forms of Met or Lys, some researchers have reported that supplementing free forms of Met and Lys altered ruminal fermentation and may have the potential to increase post-ruminal supply of Met, Lys, and other EAA that may limit production.

Chalupa (1976) studied degradation of AA by ruminal microbes in both in vitro and in vivo systems. Under in vitro conditions, Lys was degraded at a moderate rate (0.2 to 0.3 m*M*/hr) and Met was degraded at a relatively lower rate (0.1 to 0.14 m*M*/hr). Also, the degradation rate of Met was approximately twice as great when fermented alone than in conjunction with other EAA in both the in vitro and in vivo systems. Chalupa (1976) concluded that free AA could not survive ruminal degradation with the possible exception of Met, especially if large amounts were administered.

Bach and Stern (1999) observed decreased true microbial digestion of Met, cysteine (Cys), tyrosine (Tyr), and Ile when the level of free Met supplementation was high in the diet. Bach and Stern (1999) concluded that dietary Met supplementation could increase the flow of Met, Cys, Tyr, and Ile to the duodenum.

Robert et al. (1999b) reported that DL-Met (Rhodimet$^{TM}$ NP99) was equally as effective at stimulation of ruminal digestibility of organic matter (OM) as two HMB sources when normal quality corn silage was used as substrate. Koenig et al. (1999) reported that neither HMB nor DL-Met had a stimulatory effect on in vitro ruminal fermentation when S was adequate in the substrate.

### 2.5.2 In Vivo Free Methionine and Lysine Supplementation

Although Chalupa (1976) indicated about a 1.5x greater degradation rate of EAA in vivo than estimates derived in vitro, Cottle and Velle (1989) reported substantial amounts of intact AA flowed out of the rumen when the free AA mixture of Lys, Thr, and Met was administered to three hay-fed sheep in moderate intraruminal doses. Campbell et al. (1997b) reported an increase in ruminal outflow of Lys, Met, and Thr when moderate doses of Lys, Met, Thr, and Trp were added to steers fed dry-rolled corn. Cottle and Velle (1989) also reported that the relative rate of apparent ruminal degradation in the first 4 h after intraruminal infusion was highest for Lys and lowest for Met, but was highest for Lys and lowest for Thr after 24 h. Cottle and Velle (1989) concluded that rates of apparent ruminal degradation of free AA as well as outflow rates of the free AA were dose-dependent. Velle et al. (1997) reported the mean escape rate of AA increased from 9% at the lowest dosage to 21% at the highest dosage during the first 8 h after eighteen AA were intraruminally administrated to two non-pregnant, non-lactating cows. Velle et al. (1997) reported that Met caused a net increase in the concentration of eleven other AA in ruminal fluid. Velle et al. (1997) also reported a relatively low ruminal degradation rate and a correspondingly high ruminal escape rate for Met, Lys, and Thr when eighteen AA were administered intraruminally in a rumen-unprotected form. Velle et al. (1997) concluded that high amounts of some AA caused considerable increases in concentrations in ruminal fluid of other AA, which may contribute to improve of yield of milk and milk components. Velle et al. (1998) studied the apparent ruminal degradation and escape of AA by administrating nine different mixtures of EAA and eight different mixtures of NEAA to two cows. The researchers reported the mean initial ruminal degradation rate (1 h after administration) of EAA was 26% when the AA were administered in mixtures and 45% when the AA were administered individually, and for NEAA, 34% when administered in mixtures and 54% when administered individually. In the same study,

mean ruminal escape rate during the first 8 h after administration of EAA was 22% when administered in mixtures and 16% when administered individually, and for NEAA, 13% when administered in mixtures and 11% when administered individually. Velle et al. (1998) concluded that ruminal escape of limiting AA could be increased when administered together with larger amounts of low cost, non-limiting AA. Volden et al. (1998) proposed that considerable amounts of free Lys, Met, and Thr escaped ruminal degradation when administered intraruminally in an unprotected form to six cows in both peak (DIM=106±3) and late lactation (DIM=288±3). Volden et al. (1998) reported that the mean ruminal escape values of Lys, Met, and Thr across dosages and feeding levels were 20.5%, 22.1%, and 16.7%, respectively. Sulu et al. (1989) reported that the mean ruminal escape values were 25% for Lys, 31% for Met, 18% for Tyr, and 37% for Ile when a single, graded dosage of AA was administrated to nonlactating cows fed hay and concentrate. In a study by Cottle and Velle (1989), the mean ruminal escape rates for Lys, Met, and Thr were 10%, 30%, and 17%, respectively. Velle et al. (1997) suggested that moderate amounts of free AA might be practical as feed additives and can be a realistic alternative to the use of rumen-undegraded feed protein and expensive rumen-protected AA depending on the cost (Velle et al., 1998; Volden et al., 1998). Results above also suggest that supplementing a substantial amount of free AA alone or in combination with other non-limiting AA may decrease ruminal degradation of AA and also inhibit other AA from being digested by ruminal microbes.

Several studies of supplementing Met, Lys, or other AA on ruminal fermentation and production responses have been conducted. Lundquist et al. (1985) reported increased numbers of ciliated protozoa and increased concentrations of butyrate, isobutyrate, and isovalerate in the rumen when three Holstein cows in late lactation were fed DL-Met compared to HMB or $Na_2SO_4$. Studies with steers by Campbell et al. (1997b) reported fatter carcasses and better marbling scores when steers were supplemented with a free AA mixture of DL-Met, L-Lys,

L-Thr, and L-Trp suggesting some improvement in energy availability although no significant differences in ruminal fermentation and performance were observed. Serial studies with Angora goats by Carneiro et al. (1998a, b, and c) have shown that supplementing DL-Met in water or feed (top-dressed) increased plasma Met concentrations of adult goats, but ruminal $NH_3$ decreased and VFA ratios, protozoa numbers, and pH were not altered by adding Met. Plasma Met concentrations were increased by supplementing Met in drinking water, but mohair production was not increased suggesting that the post-ruminal Met supply was already adequate for maximum growth of mohair (Carneiro et al., 1998c). In the study with growing Angora goats by Carneiro et al. (1998b), feed intake tended to increase when Met was provided either in drinking water or in the feed.

The effects of Met supplementation and timing of supplementation on ruminal fermentation in beef cattle have been studied. Clark and Peterson (1988) reported an increased rate of fermentation of DM of low quality grass hay and improved weight gains in crossbred beef cattle supplemented with urea and 15 g of DL-Met compared with soybean meal. Wiley et al. (1991) reported increased in situ dry matter (DM) and neutral detergent fiber (NDF) disappearance rates for beef cattle fed low-quality, cool-season forages with supplementation of DL-Met at 1200 h and 1500 h compared with supplementation at 0800 h, suggesting an improvement in fermentation when availability of carbohydrates and N sources to ruminal microbes were synchronized. McCracken et al. (1993) reported greater in situ rates of DM and NDF disappearance in Met-supplemented steers than in un-supplemented steers. However, steers supplemented in A.M. exhibited faster rates of disappearance than steers supplemented in P.M. Disagreement of Wiley et al. (1991) and McCracken et al. (1993) may be due to differences in carbohydrate availability. Carbohydrates were more readily available with actively growing forage in the study of McCracken et al. (1993) than with the low-quality hay and straw diet in the

study of Wiley et al. (1991). Also in the study of McCracken et al. (1993), although Met supplementation did not increase rates of DM and NDF disappearance, ruminal purine concentration was greater in Met-supplemented heifers than in un-supplemented heifers fed low-quality forage, which implies an increased microbial mass.

Uchida et al. (2001) reported that DL-Met performed as well as HMB in improving milk yield and contents of milk fat and crude protein when the post-ruminal supply of Met was similar to HMB. Whiting et al. (1971) reported no significant variation in milk yield and milk contents when 12 Holstein cows in mid-lactation were supplemented with DL-Met or HMB. Results of Whiting et al. (1971) suggested that supplementing either the free form or ruminally protected AA at mid-lactation was not beneficial, but implicated that DL-Met may have the same effect on production response as HMB.

Although free AA are considered highly degradable in the rumen, co-supplementing ruminally protected AA with free AA tends to improve production responses by supplying readily available sources of AA for ruminal bacteria to improve microbial protein production while also providing AA for the host animal. Samuelson et al. (2001) reported reduced milk yield and milk protein production by supplementing with RPMet (Mepron®85) alone in mid-lactation and reduced milk protein percentage by supplementing with free DL-Met alone in Holstein cows. In contrast, in the same experiment, co-supplementing with RPMet and DL-Met to cows in mid-lactation did not influence milk fat percentage but increased yield and percentage of milk protein (+0.1%) in both Holstein and Brown Swiss cows. This combination also increased lactose percentage (+0.18%) in Holstein cows fed an alfalfa hay and corn grain-based diet. Harrison et al. (2000) reported that the highest production of milk, 3.5% FCM, and milk components were observed from ninety-eight Holstein cows fed animal-based bypass protein (Prolak™) co-supplemented with RPMet (Alimet®) and free L-Lys-HCl from 3 wk prepartum

through 17 wk postpartum. Koudele et al. (1999) reported increased milk protein percentage but no significant differences on milk yield and milk fat percentage when sixty lactating Holstein cows were co-supplemented with RPMet (Mepron®85) and L-Lys compared to no AA supplementation or Lys supplementation alone. In the same study, Koudele et al. (1999) proposed that it was impossible to determine if the production response described above was due solely to Met or Met in conjunction with Lys.

### 2.6 End Products of Ruminal Fermentation

Ruminal microorganisms convert dietary carbohydrate and protein to volatile fatty acids (VFA), microbial protein, and gases. The VFA and microbial protein can be absorbed and utilized by the host animal while the gases are lost to the environment. The end products of ruminal fermentation include useful compounds, such as VFA, microbial protein, and water-soluble vitamins, useless compounds, such as $CH_4$ and $CO_2$, and even compounds harmful to the host animal, like $NH_3$ and nitrate (Owens and Goetsch, 1993). A large proportion of the energy and protein requirements of the host ruminant can be satisfied via the end products of ruminal fermentation. Microbial protein supplies high quality protein to the host animal. About 50 to 85% of the requirement of the metabolizable energy for ruminants is provided by VFA when forage diets are fed (Owens and Goetsch, 1993).

#### 2.6.1 Ruminally Synthesized Microbial Protein

Microbial protein is an end product (or by-product) of the ruminal fermentation of cellulose. Microbial protein that reaches the duodenum contributes the greatest amount of protein for the ruminant animal (Firkins, 1996). Schwab (1996) indicated that ruminally synthesized microbial protein supplies 50% or more of absorbed AA when rations are balanced properly. Microbial protein is considered to be a consistent (Schwab, 1996) and high quality protein source with a balanced AA profile (Clark et al., 1992) and is also relatively less expensive to produce

compared to other protein sources. Ruminal microorganisms do not have AA requirements but do have requirements for branched-chain fatty acids (BCFA) and $NH_3$ for microbial protein synthesis.

Microbial protein has the same intestinal digestibility as RUP at 80 to 90% (Schwab, 1996). The biological value of microbial protein ranges from 66 to 87%, and microbial protein is approximately 80% AA (Owens and Zinn, 1993). Microbial protein synthesis is limited by ATP production and depends largely on the ruminal availability of N and carbohydrates (NRC, 2001) that can provide carbon skeletons and energy (Stern et al., 1994). Therefore, synchronization of ruminally degradable carbohydrates with RDP is extremely important for optimizing microbial protein synthesis (Firkins, 1996; NRC, 2001; Stern et al., 1994). Factors that influence ruminal fermentation and microbial protein synthesis include amount and type of supplemental protein, carbohydrate sources and availability in the rumen, and ruminal pH (Bateman et al., 1999).

### 2.6.2 Ruminally Synthesized Volatile Fatty Acids

Ruminally synthesized VFA include the short-chain fatty acids, acetate, propionate, and butyrate and the BCFA, isobutyrate and isovalerate. Major VFA, acetate, propionate, and butyrate supply energy for the host animal after being absorbed and metabolized, and also influence the biosynthesis of milk, milk fat, and milk protein.

Tissues of the adult ruminant obtain energy for maintenance and metabolism from the absorbed VFA by oxidative metabolism. The efficiency of energy utilization for each individual VFA is different. Propionate has the highest efficiency of energy utilization and is followed by butyrate and acetate. The rumen is the major site for absorption of VFA. After absorption across ruminal epithelial cells, VFA are transported into ruminal capillaries, the portal vein, and then into the liver.

Acetate is the major product of carbohydrate digestion during ruminal fermentation and is the only VFA that can be found in large amounts in the peripheral tissues of the host animal. The major site for acetate metabolism is in peripheral tissues. Leng and West (1969) reported that 1 to 4% of acetate was converted to ketone bodies in ruminal epithelial cells. Pethick et al. (1981), Smith and Crouse, (1984), and Johnson et al. (1990) indicated that 70% of acetate could escape ruminal and hepatic metabolism and go into peripheral tissues to be metabolized.

Acetate supplies both energy and acetyl units for the synthesis of milk and milk components. The majority of the energy for mammary gland synthesis of milk is obtained from oxidation of acetate (Schwab, 1994), and a smaller amount of energy is obtained from oxidation of β-hydroxybutyrate, AA, and glucose. Unlike non-ruminants, the mammary gland of ruminants can utilize acetate directly to synthesize milk fat. Therefore, a sufficient supply of acetate is very important for milk yield and milk fat synthesis. Palmquist and Jenkins (1980) reported that ruminal infusion of acetate or supplementing acetate bicarbonate in the diet increased milk yield and milk fat percentage, but this increase was limited by the proportion of acetate in the rumen.

Propionate is the major precursor of glucose synthesis in ruminants and ruminal fermentation is the major source of propionate to these animals. The liver is the major site for propionate metabolism. Bergman and Wolff (1971) reported that more than 50% of propionate is metabolized in the liver and utilized for the synthesis of glucose. In ruminal fermentation, a high proportion of propionate may result in a low proportion of acetate. A high proportion of propionate in the rumen is beneficial for dairy cattle in late lactation because lipid synthesis by adipose tissues can be stimulated by the presence of a high proportion of propionate (McCartor et al., 1979). Propionate is also important for the synthesis of lactose in milk.

Similar to propionate, ruminal fermentation is the major source of butyrate in ruminants. Weigand et al. (1972) reported that large amounts of butyrate, 33 to 78%, are converted to ketone

bodies (acetone, acetoacetate, and β-hydroxybutyrate) in ruminal epithelial cells and the liver. Giesecke et al. (1985) reported that β-hydroxybutyrate is transported through the blood and supplies energy and fatty acids for organs and peripheral tissues. In the mammary gland, β-hydroxybutyrate can be converted to long-chain fatty acids to become a part of milk fat.

## 2.7 Fermentation Techniques

### 2.7.1 In Vitro System

An in vitro system can be defined as a system in which ruminal microbes are incubated with feedstuffs or test components in a batch, a continuous or discontinuous culture. Compared to in vivo systems, in vitro systems are generally lower in cost, faster, and more repeatable (Owens and Goetsch, 1993). There are two styles of in vitro systems that have been studied and used extensively. They include the batch incubation, and the continuous culture system.

The batch incubation, as described by Tilley and Terry (1963), is a procedure commonly used in forage evaluation. The method involves two stages: 24 or 48 h digestion of test feedstuffs with buffered ruminal fluid followed by 48 h digestion with pepsin-HCl to solubilize proteins (Owens and Goetsch, 1993; Van Soest, 1994). The Tilley-Terry system only needs small amounts of test feedstuffs, and this system can be ran rapidly compared to in vivo evaluations. In vitro dry matter disappearance (IVDMD) can be measured by this system and is highly correlated with in vivo digestibility (Owens and Goetsch, 1993). A modified procedure has been developed to shorten and simplify the original procedure. It includes substitution of buffered ruminal fluid with a high-phosphate buffer, which acidifies directly and supplies pepsin during the second stage, and decreases pepsin digestion time to one day (Van Soest, 1994). In general, the Tilley-Terry system has been a relatively accurate procedure for appraising digestibility in the laboratory when applied to reasonable-quality feedstuffs without supplements (Van Soest, 1994).

Compared to the Tilley-Terry system, the continuous culture system has been studied more extensively not only for nutrient digestibility but also for ruminal microorganism metabolism. The operating conditions of the continuous culture system are more similar to the in vivo environment than closed vessel incubation. The continuous culture system described by Slyter et al. (1964) had been reported to maintain similar bacterial numbers as in the rumen (Slyter and Putnam, 1967), but protozoa numbers were decreased markedly compared to ruminal protozoa numbers. Other studies (Abe and Kumeno, 1973) also reported decreased protozoa numbers using a similar continuous culture system. The dual flow continuous culture system described by Hoover et al. (1976) was designed to stimulate the differential dilution rates of liquids and solids that occur in the rumen and has been reported to improve the maintenance of protozoa numbers compared to the single overflow system. Hannah et al. (1986) used a modified dual flow continuous culture system to evaluate the feasibility of in vitro fermentation data compared to in vivo data. Hannah et al. (1986) reported similar true OM digestibility and crude protein (CP) and AA degradability in both the in vitro and in vivo systems. This indicated that the dual flow continuous culture system could provide reasonable estimates of ruminal fermentation. However, results of in vitro fermentation should be evaluated in vivo. Concepts which are ineffective in vitro should be ineffective in vivo, but concepts which are effective in vitro may not be effective in vivo because of microbial or animal adaptations and changes in the ruminal environment (Owens and Goetsch, 1993).

### 2.7.2 In Situ (In Sacco) Technique

The in situ (in sacco) procedure, also called the cloth bag method, is the technique of suspending a polyester bag filled with test components in the rumen through a fistula for a specific time period (Owens and Goetsch, 1993; Van Soest, 1994). Ruminal microorganisms, ruminal fluid, and end products of digestion can be flushed in and out via the small pores of the

cloth bag. The portion of the test component that disappeared from the bag is considered to have been digested. The in situ system provides the advantages of: 1) rapid measuring of the digestibility of forages and proteins, 2) digesting test components in a real ruminal environment, and 3) avoiding rapid changing in microbial types, which may occur in a batch culture systems (Owens and Goetsch, 1993; Van Soest, 1994). The in situ system also has disadvantages that include: 1) accumulation of lignified materials or microbes in the bag, 2) low or even negative digestibility estimates, and 3) efflux of small particles without being digested (Owens and Goetsch, 1993). An improved method by Udén et al. (1974) and Van Hellen and Ellis (1977) used specific pore sizes and controlled the ratio of sample weight to surface area of the bag. Errors can be minimized by using bags with larger ratio of surface area to sample size, and the optimal pore size is about 30 μm (Van Soest, 1994).

## 2.8 Objectives

Two experiments were conducted to study effects of free Met and Lys supplementation on ruminal fermentation and milk production response using both in vitro and in vivo techniques. The objectives of in vitro experiment were 1) to study the effects of free Met and Lys on in vitro fermentation and 2) to determine an optimal level of free Met and Lys in combination for improving ruminal fermentation. The objective of the in vivo experiment was to determine if the optimal concentrations of free Met and Lys from the in vitro experiment would impact in vivo ruminal fermentation and the milk production response of Holstein cows.

# CHAPTER 3. EFFECTS OF FREE METHIONINE AND LYSINE ON IN VITRO FERMENTATION

## 3.1 Objectives

This experiment was conducted to study the effects of free Met and Lys supplementation on ruminal fermentation in vitro. The objectives were 1) to study the effects of free Met and Lys on in vitro fermentation and 2) to determine an optimal combination of free Met and Lys for improving ruminal fermentation.

## 3.2 Materials and Methods

### 3.2.1 Continuous Culture System and Operation

Two single-effluent continuous culture fermentors (maximum capacity, 3 L; Biovessel with microbial package; Applikon Inc.; Foster City, CA) were used to investigate the effects of AA supplementation on in vitro fermentation. Twenty-four hours prior to inoculation, 25 g of treatment diets were added to 2 L of buffer (Goering and Van Soest, 1970), and allowed to hydrate. Trypticase was removed from the original recipe of Goering and Van Soest (1970) to maximize any impact of supplemental AA in this study. The buffer and the assigned treatment were allowed to stand at 39 $^{o}$C under aerobic conditions and were constantly stirred at 90 RPM during the hydration period. Immediately prior to inoculation, 100 ml of reducing solution (Goering and Van Soest, 1970) was added to the buffer solution of each fermentor, and then each fermentor was inoculated with 500 ml of the strained ruminal fluid mixture. Approximately 45 ml of the remainder of the ruminal fluid mixture was sampled and stored frozen (-20 $^{o}$C) until further analysis.

During each experimental period, each fermentor was fed 20 g of its assigned treatment diet twice daily at 0800 h and 1600 h. The fluid was constantly stirred at 200 RPM throughout the fermentation period. Fermentation was maintained at 39 $^{o}$C under anaerobic conditions by

bubbling $CO_2$ through the fermentor fluid for 5 minutes to force out any $O_2$ every time the system was opened to the atmosphere. Fermentor fluid pH was maintained at 6.5 or higher by infusing 0.5 M phosphate (pH=8) and 0.5 M bicarbonate (pH=10) buffers at 1:1 ratio. Phosphate and bicarbonate buffers were infused automatically using peristaltic pumps. Excess amounts of fermentor fluid were removed and recorded daily during the stabilization period in order to maintain a working volume of 2 L.

### 3.2.2 Inoculant Donor and Animal Care

Inoculant was obtained from three ruminally-cannulated, non-lactating Holstein cows maintained on bermudagrass pasture at the Louisiana State University (LSU) Dairy Science Research and Teaching Farm in Baton Rouge, Louisiana, between January and September of 2002. Ruminal fluid was collected via the rumen cannula by hand from each donor cow. Ruminal contents were strained through four layers of cheesecloth in order to separate fluid from large feed particles, and the fluid from all three cows was combined. Strained ruminal fluid mixture (total volume equal 1500 ml) was immediately transported to the laboratory and mixed well before inoculation. All donor cows were handled and housed under protocols approved by the Institutional Animal Care and Use Committee of the LSU Agricultural Center.

### 3.2.3 Dietary Treatments and Arrangement

One basal diet, which consisted of 50% (as-fed basis) commercial chopped alfalfa hay (90% DM) (Bert & Wetta Larned, Inc.; Larned, KS) and 50% (as-fed basis) concentrate pellet (90% DM), was used as the base for formulating experimental treatments. Composition of the concentrate is listed in Table 3-1. Concentrate and hay were ground to pass a 2 mm screen. The AA were mixed with the basal diet. Methionine was provided as DL-Met, which was 89% pure AA, and Lysine was provided as L-Lys-Hydrochloride, which was 78.8% pure AA. Three levels of Met, 0%, 0.26%, and 0.52% of the diet (as-fed basis; 90% DM) and three levels of Lys, 0%,

Table 3-1. Composition of concentrate used in formulation of the basal diet.

| Ingredient | % of DM |
|---|---|
| Ground corn | 61.46 |
| Soybean meal | 34.40 |
| Molasses | 3.00 |
| Rock phosphate | 0.40 |
| Salt | 0.38 |
| Trace mineral pack[1] | 0.04 |
| Vitamin pack[2] | 0.03 |
| Limestone | 0.29 |

[1] Contained 8.44% Ca, 6.65% P, 4.33% Mg, 2.64% S, 0.34% I, 12.59% Mn, 0.85% Zn, 1.62% Fe, 0.064% Co, and 2.44% Cu.

[2] Contained 11,137 kIU vitamin A/kg, 2,784 kIU vitamin D/kg, 30,928 IU vitamin E/kg, and 680 ppm Se.

1.03%, and 2.06% of the diet (as-fed basis; 90% DM) were added to the basal diet in a replicated 3 × 3 factorial arrangement to formulate nine dietary treatments. Arrangement of treatments is shown in Table 3-2. In this study, three concentrations of Met and three concentrations of Lys were chosen to evaluate the effects of free Met and Lys supplementation on in vitro ruminal fermentation. The intermediate concentrations of Met (0.26%) and Lys (1.03%) were chosen based on the ideal dietary requirements of Met and Lys for lactating sows consuming a corn-soybean based diet with 90% DM (NRC, 1998). Because of the loss of AA during ruminal fermentation, 0 and 2x the "ideal" concentrations were also evaluated to allow for defining the optimal concentrations for ruminants. Crystalline DL-Met and L-Lys-HCl were added to the basal diet and mixed well by hand. The AA treatments were randomly assigned to each fermentor, and each fermentor was used twice with each of the nine treatments. Therefore, there were four observations per treatment.

### 3.2.4 Experimental Period and Sample Collection

Experimental period length for each fermentation run in the first replicate was 5 d, which included 4 d of stabilization followed by 1 d of sampling. Experimental period length for each fermentation run in the second replicate was 6 d, which included 4 d of stabilization followed by 1 d of sampling and 1 day of microbial isolation.

#### 3.2.4.1 Volatile Fatty Acid, Ammonia-Nitrogen, and Fermentor Fluid Collection

On the day of sampling for each replicate, two samples of fermentor fluid (approximately 45 ml each) were collected immediately prior to the morning feeding at 0800 h (corresponding to 0 hr post feeding). Fermentor fluid then was collected again at 0830, 0900, 0930, 1000, 1030, 1100, 1130, 1200, 0100, 0200, 0300, 0400 h, which corresponded to 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 7, and 8 hrs post feeding, respectively. Fermentor pH was recorded at each sampling time. One of the samples of fermentor fluid was sub-sampled (4 ml) and prepared for VFA analysis.

Table 3-2. Dietary arrangement of concentrations of Met and Lys supplemented in experimental diets. (90% DM).

| **Methionine** / **Lysine** | **None** (0 %) | **Low** (0.26 %) | **High** (0.52 %) |
|---|---|---|---|
| **None** (0 %) | **Diet 1** (Lys 0 %; Met 0 %) | **Diet 4** (Lys 0 %; Met 0.26 %) | **Diet 7** (Lys 0 %; Met 0.52 %) |
| **Low** (1.03 %) | **Diet 2** (Lys 1.03 %; Met 0 %) | **Diet 5** (Lys 1.03 %; Met 0.26 %) | **Diet 8** (Lys 1.03 %; Met 0.52 %) |
| **High** (2.06 %) | **Diet 3** (Lys 2.06 %; Met 0 %) | **Diet 6** (Lys 2.06 %; Met 0.26 %) | **Diet 9** (Lys 2.06 %; Met 0.52 %) |

The remainder of the fermentor fluid sample was then acidified by adding 1 ml of 20% (vol/vol) $H_3PO_4$ and stored frozen (-20 $^oC$) for $NH_4^+$ analysis. The second sample of fermentor fluid was stored frozen (-20 $^oC$) immediately without any reagent addition for later analyses of purine and total N content.

### 3.2.4.2 Fermentor Bacteria Harvesting

A modified isolation procedure adapted from Zinn and Owens (1980) and Steinhour et al. (1982) was used to isolate bacteria from the fermentor on the second day of sampling. The entire fermentor contents were blended in a Waring commercial blender at low speed for 5 min. to separate the bacteria from the feed particles. Sufficient quantities of 0.9% saline solution were added as needed to reduce the viscosity of the blended fluid. The blended fluid was strained through four layers of cheesecloth and solids were discarded. Strained fluid was centrifuged at 500x g for 5 min to remove the remaining feed particles and protozoa. The supernatant was decanted, and the pellet was discarded. The supernatant was re-centrifuged at 20,000x g for 20 min. The supernatant from this second centrifugation step was decanted and discarded. The pellet was re-suspended in 0.9% saline solution and re-centrifuged at 20,000x g for 20 min. Again, the supernatant was decanted and discarded. This step was repeated once more prior to harvesting the bacterial pellet, which was stored frozen (-20 $^oC$) until later analysis.

### 3.2.5 Analytical Procedures

#### 3.2.5.1 Chemical Analysis of Ingredients and Experimental Diets

Both the concentrate pellet and alfalfa hay along with the nine experimental diets were analyzed for total N, acid detergent insoluble nitrogen (ADIN), ash, DM (AOAC, 1980), and acid detergent fiber (ADF) (Van Soest et al., 1991). Total N was tested in order to calculate the CP content. The CP content was calculated as: N × 6.25. Acid detergent insoluble nitrogen was

tested in order to separate the available CP from the bound CP. Bound CP was calculated as: ADIN × 6.25. The content of available CP was calculated as: CP – bound CP.

#### 3.2.5.2 Total Volatile Fatty Acids

A 4 ml sample of fermentor fluid was mixed with 1 ml of 25% (wt/wt) meta-phosphoric acid containing 10 g/L 2-ethylbutyric acid, which was used as an internal standard for VFA quantification. The mixture of fermentor fluid and meta-phosphoric acid was then centrifuged at 30,000×g for 25 min. Concentrations of individual VFA were measured by GLC using a Shimadzu GC17-A equipped with a 15-m EC-1000 column that had an internal diameter of 0.53 mm and a film thickness of 1.2 μm (Alltech Associates, Inc.; Deerfield, IL). The reagent preparation procedure and temperature gradient program for VFA analysis was adapted from Grigsby et al. (1992) and Bateman et al. (2002), respectively (Appendix A.).

#### 3.2.5.3 Ammonia-Nitrogen

Before $NH_4^+$ analysis, acidified fermentor fluid was thawed at room temperature and clarified by centrifuging at 30,000× g for 20 min. The clarified supernatants were then decanted and analyzed for $NH_4^+$ using a modified phenol-hypochlorite reaction adapted from Broderick and Kang (1980) and Bateman et al. (1996, 2002) (Appendix B.).

#### 3.2.5.4 Purine and Nitrogen

Non-acidified fermentor effluents from each time point were thawed at room temperature and composited. Composited effluents were then centrifuged at 20,000× g for 25 min. to remove any large solids. Effluent pellets and isolated bacteria were dried at 55 $^oC$ overnight, and dry weight was recorded. Oven dried effluent pellets and bacteria were ground by hand. Effluent pellets and bacteria were then dried at 105 $^oC$ and analyzed for their contents of ash, purine, and N. Ash was analyzed according to an AOAC procedure (1980). Purines were measured according to the procedure of Zinn and Owens (1986) (Appendix C.). Concentrations of purines in bacteria

and fermentor effluent were measured in order to separate N into microbial and dietary fractions (Zinn and Owens, 1986). Contents of N in effluent and bacterial pellets were analyzed using a N auto-analyzer (Quickchem 8000; Lachat Instruments; Loveland, Colo.) equipped with Quickchem® Method (TKN 13-107-06-2-D).

### 3.2.6 Experimental Design and Statistical Analysis

Data for $NH_4^+$, total and individual VFA, and bacterial N synthesis were analyzed as a replicated 3 × 3 factorial arrangement of treatment by ANOVA. The statistical model included terms of Lys, Met, time of sampling, and all of the interactions of Lys, Met, and time of sampling. Chemical analyses of experimental diets were also analyzed using ANOVA. All data are presented as least squares means. All calculations were completed using SAS (SAS Institute Inc., 1990). Significance was declared at $P < 0.05$.

## 3.3 Results and Discussion

### 3.3.1 Chemical Analyses of Ingredients and Experimental Diets

Chemical analyses of alfalfa hay and concentrate are presented in Table 3-3. Both the alfalfa hay and concentrate used in this study had 90% of DM. Chemical analyses of the nine dietary treatments are presented in Table 3-4. All experimental diets had similar ($P > 0.05$) nutrient contents.

### 3.3.2 Total and Individual VFA Production

In a closed fermentation system, total VFA concentration can be related to carbohydrate fermentation (Owens and Goetsch, 1993). Least squares means for concentrations of total VFA and proportions of individual VFA are presented in Table 3-5. No effect of time of sampling ($P = 0.88$) on concentrations of total VFA or proportions of individual VFA was observed. Mean concentrations of total VFA were significantly affected by the interaction of Lys and Met supplementation ($P < 0.01$) (Figure 3-1) or the addition of Lys ($P < 0.05$) or Met ($P < 0.01$)

Table 3-3. Chemical analysis of alfalfa hay and concentrate mixture fed to fermentors.

| | Alfalfa hay | Concentrate mixture |
|---|---|---|
| DM, % | 89.51 | 89.92 |
| | % of DM | |
| N | 2.80 | 2.72 |
| CP[1] | 17.50 | 17.00 |
| ADIN[2] | 0.35 | 0.19 |
| Bound CP[3] | 2.19 | 1.19 |
| Available CP[4] | 15.31 | 15.81 |
| ADF | 34.41 | 3.38 |
| Ash | 12.24 | 5.77 |

[1] CP = N × 6.25.

[2] ADIN = acid detergent insoluble nitrogen.

[3] Bound CP = ADIN × 6.25.

[4] Available CP = CP – Bound CP.

Table 3-4. Least squares means for chemical analysis of the experimental diets fed to fermentors.

| | | Experimental diets | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Diet1 | Diet2 | Diet3 | Diet4 | Diet5 | Diet6 | Diet7 | Diet8 | Diet9 | | | | |
| | Lys | 0 | 1.03 | 2.06 | 0 | 1.03 | 2.06 | 0 | 1.03 | 2.06 | | *P*-value | | |
| Item | Met | 0 | 0 | 0 | 0.26 | 0.26 | 0.26 | 0.52 | 0.52 | 0.52 | SEM | Lys | Met | Lys*Met |
| DM, % | | 89.89 | 90.83 | 90.33 | 90.49 | 90.17 | 90.51 | 90.88 | 91.08 | 90.24 | 0.58 | 0.7640 | 0.6860 | 0.7212 |
| | | % of DM | | | | | | | | | | | | |
| N | | 3.10 | 3.27 | 3.52 | 3.19 | 3.30 | 3.38 | 3.22 | 3.35 | 3.38 | 0.04 | 0.0002 | 0.7978 | 0.0843 |
| CP[1] | | 19.40 | 20.45 | 22.00 | 19.95 | 20.60 | 21.15 | 20.10 | 20.95 | 21.10 | 0.27 | 0.0002 | 0.7978 | 0.0843 |
| ADIN[2] | | 0.34 | 0.33 | 0.35 | 0.42 | 0.33 | 0.29 | 0.33 | 0.38 | 0.38 | 0.04 | 0.8093 | 0.8284 | 0.2544 |
| Bound CP[3] | | 2.12 | 2.08 | 2.22 | 2.63 | 2.05 | 1.84 | 2.05 | 2.36 | 2.37 | 0.24 | 0.8093 | 0.8284 | 0.2544 |
| Available CP[4] | | 17.28 | 18.37 | 19.78 | 17.32 | 18.55 | 19.31 | 18.05 | 18.59 | 18.73 | 0.37 | 0.0011 | 0.9614 | 0.2325 |
| ADF | | 19.82 | 17.71 | 17.53 | 16.85 | 17.25 | 17.07 | 18.19 | 17.09 | 16.35 | 0.69 | 0.1113 | 0.0921 | 0.3674 |
| Ash | | 8.69 | 8.86 | 8.62 | 8.78 | 8.65 | 8.49 | 8.83 | 8.73 | 8.49 | 0.13 | 0.1024 | 0.7314 | 0.7191 |

[1] CP = N × 6.25.

[2] ADIN = acid detergent insoluble nitrogen.

[3] Bound CP = ADIN × 6.25.

[4] Available CP = CP – Bound CP.

Table 3-5. Least squares means for ruminal performances of the continuous culture system fed diets with or without supplemental Met and Lys.

| | Experimental diets | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Diet1 | Diet2 | Diet3 | Diet4 | Diet5 | Diet6 | Diet7 | Diet8 | Diet9 | | | | |
| Lys | 0 | 1.03 | 2.06 | 0 | 1.03 | 2.06 | 0 | 1.03 | 2.06 | | *P*-value | | |
| Item Met | 0 | 0 | 0 | 0.26 | 0.26 | 0.26 | 0.52 | 0.52 | 0.52 | SEM | Lys | Met | Lys*Met |
| Total VFA, m*M* | 269.01$^{c}$ | 274.06$^{c}$ | 339.02$^{b}$ | 316.06$^{b}$ | 321.77$^{b}$ | 333.71$^{b}$ | 333.53$^{b}$ | 371.97$^{a}$ | 320.20$^{b}$ | 10.69 | 0.0162 | < 0.0001 | < 0.0001 |
| Acetate, % | 60.67$^{ab}$ | 59.68$^{c}$ | 60.97$^{a}$ | 60.96$^{a}$ | 59.56$^{c}$ | 59.81$^{bc}$ | 59.96$^{bc}$ | 59.97$^{bc}$ | 60.37$^{abc}$ | 0.33 | 0.0076 | 0.3624 | 0.0437 |
| Propionate, % | 16.92$^{c}$ | 17.43$^{bc}$ | 15.94$^{d}$ | 17.95$^{ab}$ | 17.33$^{c}$ | 17.01$^{c}$ | 17.95$^{ab}$ | 18.14$^{a}$ | 16.96$^{c}$ | 0.20 | < 0.0001 | < 0.0001 | 0.0218 |
| Isobutyrate, % | 1.62 | 1.54 | 1.43 | 1.48 | 1.52 | 1.64 | 1.52 | 1.41 | 1.38 | 0.07 | 0.5678 | 0.1025 | 0.0905 |
| Butyrate, % | 14.58$^{ef}$ | 15.37$^{cd}$ | 16.39$^{a}$ | 14.22$^{f}$ | 15.68$^{bcd}$ | 15.80$^{bc}$ | 15.04$^{de}$ | 15.24$^{cd}$ | 16.06$^{ab}$ | 0.23 | < 0.0001 | 0.4235 | 0.0481 |
| Isovalerate, % | 3.24$^{a}$ | 3.19$^{a}$ | 2.73$^{c}$ | 2.75$^{c}$ | 3.03$^{ab}$ | 3.09$^{a}$ | 2.83$^{bc}$ | 2.66$^{c}$ | 2.62$^{c}$ | 0.08 | 0.0517 | < 0.0001 | < 0.0001 |
| Valerate, % | 2.98$^{a}$ | 2.79$^{bc}$ | 2.54$^{e}$ | 2.64$^{de}$ | 2.88$^{ab}$ | 2.65$^{cde}$ | 2.70$^{cd}$ | 2.58$^{de}$ | 2.61$^{de}$ | 0.05 | < 0.0001 | 0.0023 | < 0.0001 |
| Ratio[1] | 3.60$^{b}$ | 3.43$^{cde}$ | 3.86$^{a}$ | 3.41$^{de}$ | 3.53$^{bcd}$ | 3.53$^{bcd}$ | 3.36$^{e}$ | 3.32$^{e}$ | 3.57$^{bc}$ | 0.05 | < 0.0001 | < 0.0001 | 0.0027 |
| $NH_4^+$, mg/dl | 4.74$^{e}$ | 5.13$^{d}$ | 5.65$^{bc}$ | 4.60$^{e}$ | 5.55$^{c}$ | 6.06$^{a}$ | 5.24$^{d}$ | 5.95$^{ab}$ | 5.41$^{cd}$ | 0.11 | < 0.0001 | 0.0003 | < 0.0001 |

[1] Ratio of the acetate to propionate in fermentor effluent.

$^{a, b, c, d, e, f}$: means in the same row with different superscripts are significantly different ($P < 0.05$).

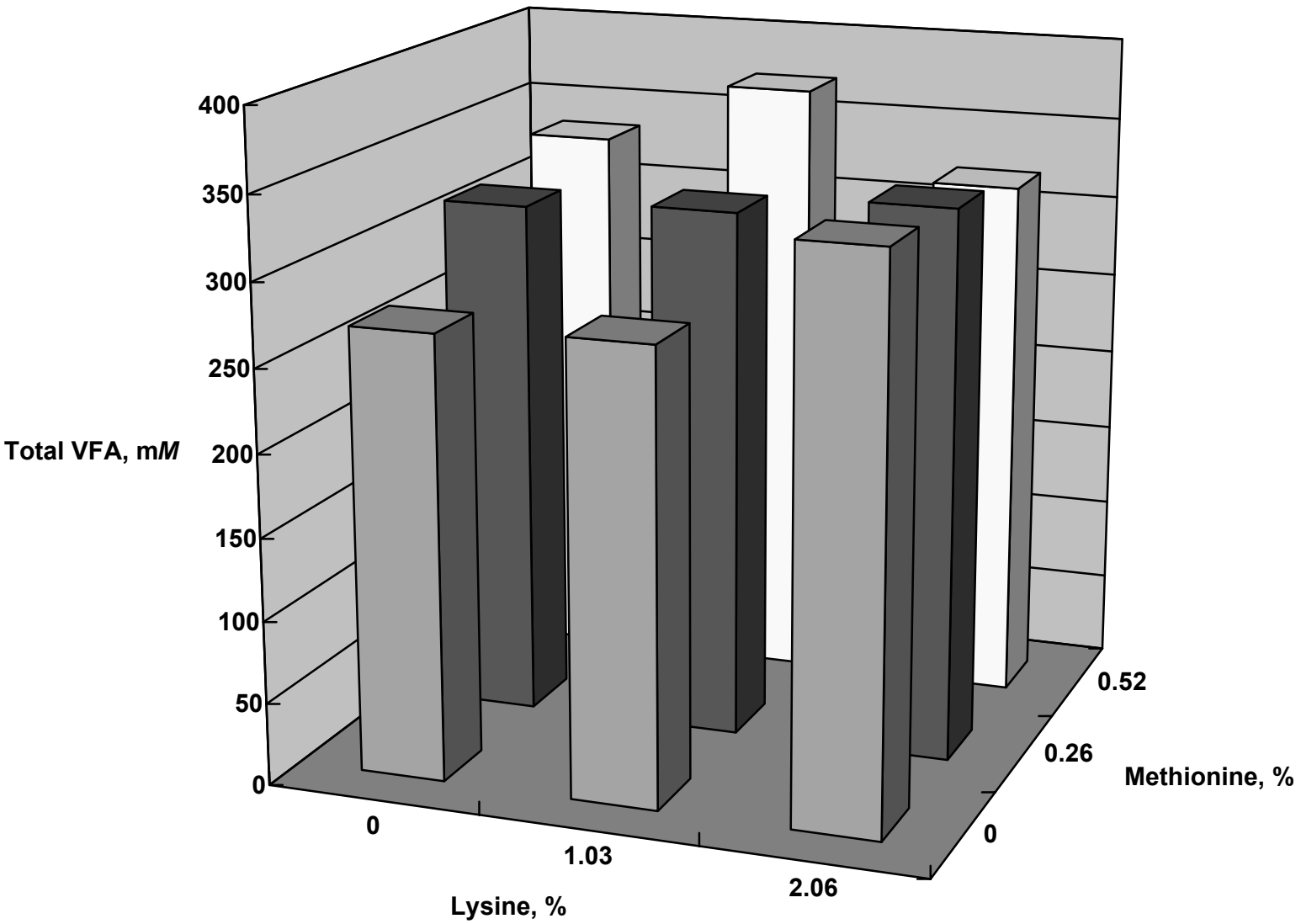


Figure 3-1. Least squares means for concentration of total VFA of diets with or without supplemental lysine and methionine (SEM=10.6885).

(Figure 3-2). Supplementing either Lys or Met increased the production of total VFA. The highest mean concentration of total VFA was observed in diet 8 with 1.03% Lys and 0.52% Met supplementation and the numerically lowest mean concentration of total VFA was observed in the control diet with no AA supplementation. However, the hypothesized ideal combination of Lys and Met (1.03% and 0.26%, respectively; diet 5) did not result in the highest concentration of total VFA as expected.

Least squares means for concentrations of total VFA from replicate 1 and 2 are presented in Table 3-6 and Table 3-7, respectively. There was only the effect of Lys supplementation ($P < 0.01$) observed on the concentrations of total VFA in replicate 1. However, in contrast to replicate 1, effects of the interaction of Lys and Met ($P < 0.01$) or Met ($P< 0.01$) supplementation were observed on the concentrations of total VFA in replicate 2. The diet with the numerically highest total VFA production shifted from diet 6 (2.06% Lys and 0.26% Met) in replicate 1 to diet 8 (1.03% Lys and 0.52% Met) in replicate 2. Because donor cows were maintained on bermudagrass pasture from February to September 2002, collections of inoculant were conducted during different seasons, and the degree of maturity for the bermudagrass pasture that the donor cows consumed may have contributed to the change in VFA. Therefore, VFA from inoculants for replicate 1 and 2 were analyzed (Table 3-8) in order to determine if there was an effect of replicate. There was no effect of replicate ($P > 0.05$) on the concentration of total VFA, and only minor shifts in the proportions of individual VFA were observed between inoculants indicating that the shift of the numerically highest concentration of total VFA was probably not associated with the inoculant used in the replicates. One possible reason for the shift may be changes within the microbial species, but the real reason to cause this shift is still unknown.

The proportion of acetate was affected by both the interaction of Lys and Met (Figure 3-3) and Lys supplementation ($P< 0.01$) but was not affected by Met supplementation (Figure 3-4).

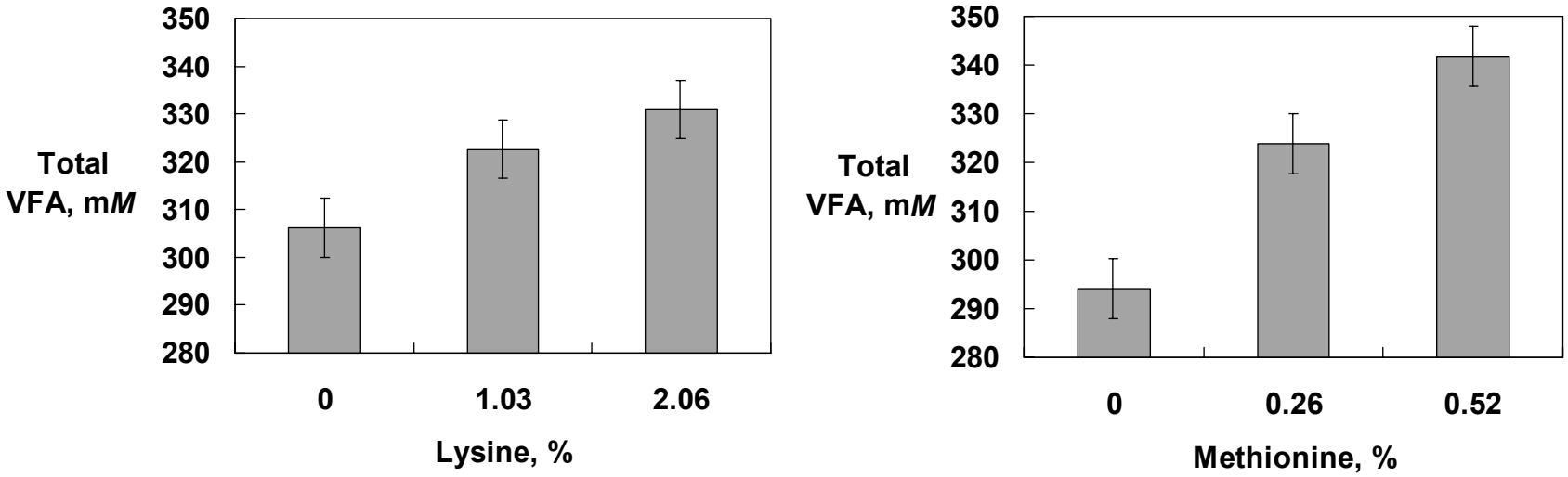


Figure 3-2. Comparison of least squares means for total VFA concentration of diets with or without supplemental lysine or methionine.

Table 3-6. Least squares means for ruminal performances of the continuous culture system fed diets with or without supplemental Met and Lys from replicate 1.

| | Experimental diets | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Diet1 | Diet2 | Diet3 | Diet4 | Diet5 | Diet6 | Diet7 | Diet8 | Diet9 | | | | |
| Lys | 0 | 1.03 | 2.06 | 0 | 1.03 | 2.06 | 0 | 1.03 | 2.06 | | *P*-value | | |
| Item Met | 0 | 0 | 0 | 0.26 | 0.26 | 0.26 | 0.52 | 0.52 | 0.52 | SEM | Lys | Met | Lys*Met |
| Total VFA, m*M* | 292.67 | 310.34 | 362.13 | 295.03 | 342.34 | 388.50 | 303.10 | 352.93 | 343.07 | 14.96 | < 0.0001 | 0.2557 | 0.1693 |
| Acetate, % | 60.75 | 59.81 | 60.43 | 60.14 | 60.89 | 58.99 | 59.37 | 58.96 | 59.58 | 0.55 | 0.6445 | 0.0704 | 0.1277 |
| Propionate, % | 15.97$^{e}$ | 16.96$^{bc}$ | 16.19$^{de}$ | 17.63$^{a}$ | 15.21$^{f}$ | 17.39$^{ab}$ | 17.41$^{ab}$ | 17.72$^{a}$ | 16.67$^{cd}$ | 0.22 | 0.1192 | < 0.0001 | < 0.0001 |
| Isobutyrate, % | 1.47 | 1.38 | 1.21 | 1.36 | 1.73 | 1.61 | 1.35 | 1.23 | 1.32 | 0.12 | 0.7372 | 0.0145 | 0.1028 |
| Butyrate, % | 15.27 | 15.92 | 16.88 | 15.12 | 16.20 | 16.24 | 16.06 | 16.85 | 17.06 | 0.36 | 0.0002 | 0.0195 | 0.7471 |
| Isovalerate, % | 3.39 | 3.24 | 2.76 | 2.98 | 3.20 | 3.09 | 3.00 | 2.70 | 2.69 | 0.15 | 0.0653 | 0.0106 | 0.0801 |
| Valerate, % | 3.14$^{a}$ | 2.68$^{bcd}$ | 2.52$^{d}$ | 2.78$^{b}$ | 2.76$^{bc}$ | 2.69$^{bcd}$ | 2.80$^{b}$ | 2.53$^{cd}$ | 2.68$^{bcd}$ | 0.08 | < 0.0001 | 0.2645 | 0.0040 |
| Ratio[1] | 3.81$^{b}$ | 3.54$^{de}$ | 3.76$^{bc}$ | 3.42$^{de}$ | 4.04$^{a}$ | 3.42$^{de}$ | 3.42$^{de}$ | 3.34$^{e}$ | 3.58$^{cd}$ | 0.07 | 0.3195 | 0.0001 | < 0.0001 |
| $NH_4^+$, mg/dl | 4.65$^{c}$ | 5.93$^{ab}$ | 5.95$^{ab}$ | 4.55$^{c}$ | 5.71$^{b}$ | 6.28$^{a}$ | 5.66$^{b}$ | 5.81$^{b}$ | 5.64$^{b}$ | 0.16 | < 0.0001 | 0.2469 | < 0.0001 |

[1] Ratio of the acetate to propionate in fermentor effluent.

$^{a, b, c, d, e, f}$: means in the same row with different superscripts are significantly different ($P < 0.05$).

Table 3-7. Least squares means for ruminal performances of the continuous culture system fed diets with or without supplemental Met and Lys from replicate 2.

| | Experimental diets | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Diet1 | Diet2 | Diet3 | Diet4 | Diet5 | Diet6 | Diet7 | Diet8 | Diet9 | | | | |
| Lys | 0 | 1.03 | 2.06 | 0 | 1.03 | 2.06 | 0 | 1.03 | 2.06 | | *P*-value | | |
| Item Met | 0 | 0 | 0 | 0.26 | 0.26 | 0.26 | 0.52 | 0.52 | 0.52 | SEM | Lys | Met | Lys*Met |
| Total VFA, m*M* | 245.35$^{ef}$ | 237.78$^{f}$ | 315.92$^{cd}$ | 337.10$^{bc}$ | 301.20$^{cd}$ | 278.93$^{de}$ | 363.96$^{ab}$ | 391.01$^{a}$ | 297.33$^{cd}$ | 14.68 | 0.3058 | < 0.0001 | < 0.0001 |
| Acetate, % | 60.58$^{bc}$ | 59.55$^{c}$ | 61.50$^{ab}$ | 61.77$^{a}$ | 58.22$^{d}$ | 60.63$^{bc}$ | 60.55$^{bc}$ | 60.98$^{ab}$ | 61.17$^{ab}$ | 0.40 | < 0.0001 | 0.1107 | < 0.0001 |
| Propionate, % | 17.87$^{bc}$ | 17.91$^{bc}$ | 15.68$^{e}$ | 18.27$^{b}$ | 19.46$^{a}$ | 16.63$^{d}$ | 18.48$^{b}$ | 18.56$^{b}$ | 17.26$^{c}$ | 0.27 | < 0.0001 | < 0.0001 | 0.0235 |
| Isobutyrate, % | 1.77$^{a}$ | 1.69$^{a}$ | 1.64$^{a}$ | 1.60$^{ab}$ | 1.31$^{c}$ | 1.68$^{a}$ | 1.69$^{a}$ | 1.59$^{ab}$ | 1.43$^{bc}$ | 0.07 | 0.0441 | 0.0160 | 0.0076 |
| Butyrate, % | 13.89$^{cd}$ | 14.82$^{b}$ | 15.91$^{a}$ | 13.33$^{d}$ | 15.15$^{b}$ | 15.36$^{ab}$ | 14.02$^{c}$ | 13.63$^{cd}$ | 15.05$^{b}$ | 0.22 | < 0.0001 | 0.0024 | < 0.0001 |
| Isovalerate, % | 3.08$^{ab}$ | 3.14$^{a}$ | 2.71$^{cd}$ | 2.52$^{d}$ | 2.85$^{bc}$ | 3.09$^{a}$ | 2.66$^{cd}$ | 2.62$^{cd}$ | 2.54$^{d}$ | 0.08 | 0.1918 | < 0.0001 | < 0.0001 |
| Valerate, % | 2.81$^{b}$ | 2.89$^{ab}$ | 2.56$^{c}$ | 2.51$^{c}$ | 3.00$^{a}$ | 2.62$^{c}$ | 2.60$^{c}$ | 2.62$^{c}$ | 2.55$^{c}$ | 0.06 | < 0.0001 | 0.0047 | 0.0005 |
| Ratio[2] | 3.40$^{cd}$ | 3.33$^{d}$ | 3.97$^{a}$ | 3.40$^{cd}$ | 3.02$^{e}$ | 3.65$^{b}$ | 3.30$^{d}$ | 3.29$^{d}$ | 3.55$^{bc}$ | 0.07 | < 0.0001 | 0.0006 | 0.0050 |
| $NH_4^+$, mg/dl | 4.83$^{c}$ | 4.32$^{e}$ | 5.35$^{b}$ | 4.64$^{de}$ | 5.40$^{b}$ | 5.84$^{a}$ | 4.82$^{cd}$ | 6.09$^{a}$ | 5.19$^{bc}$ | 0.15 | < 0.0001 | < 0.0001 | < 0.0001 |

[1] Ratio of the acetate to propionate in fermentor effluent.

[a, b, c, d, e, f]: means in the same row with different superscripts are significantly different ($P < 0.05$).

Table 3-8. Least squares means for concentrations of $NH_4^+$ and VFA of inoculants used in replicate 1 and 2.

| | Inoculants | | | |
|---|---|---|---|---|
| Item | Replicate 1 | Replicate 2 | SEM | *P*-value |
| $NH_4^+$, mg/dl | 1.18 | 0.87 | 0.13 | 0.1156 |
| Total VFA, m*M* | 89.25 | 84.91 | 5.73 | 0.5995 |
| Acetate, % | 65.52 | 68.86 | 1.09 | 0.0458 |
| Propionate, % | 17.52 | 16.11 | 0.49 | 0.0571 |
| Isobutyrate, % | 1.15 | 1.43 | 0.10 | 0.0725 |
| Butyrate, % | 11.89 | 10.50 | 0.86 | 0.2721 |
| Isovalerate, % | 2.46 | 2.02 | 0.15 | 0.0603 |
| Valerate, % | 1.46 | 1.07 | 0.14 | 0.0677 |
| Ratio[1] | 3.79 | 4.28 | 0.13 | 0.0194 |

[1] Ratio of the acetate to propionate.

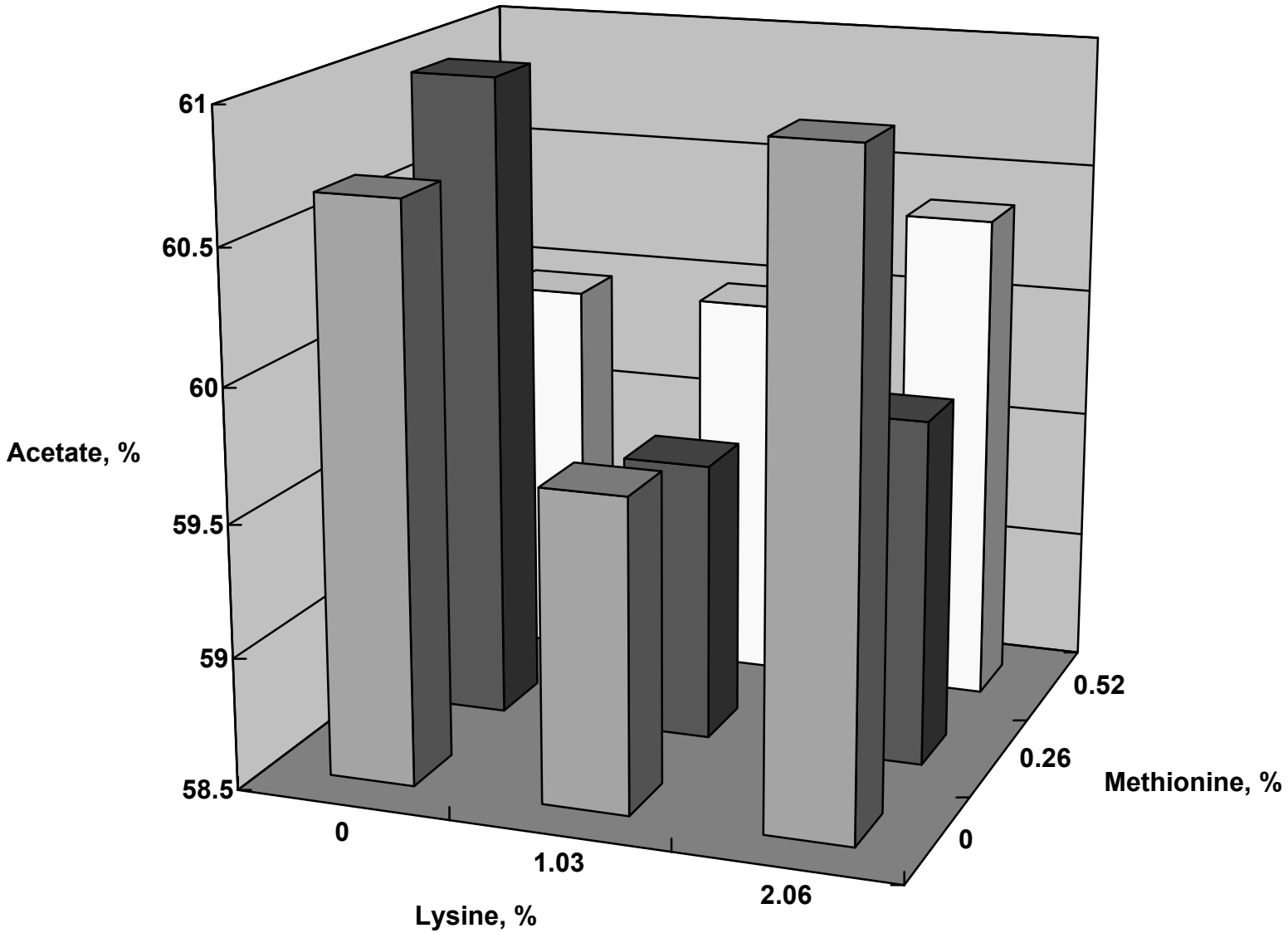


Figure 3-3. Least squares means for proportion of acetate of diets with or without supplemental lysine and methionine (SEM=0.3279).

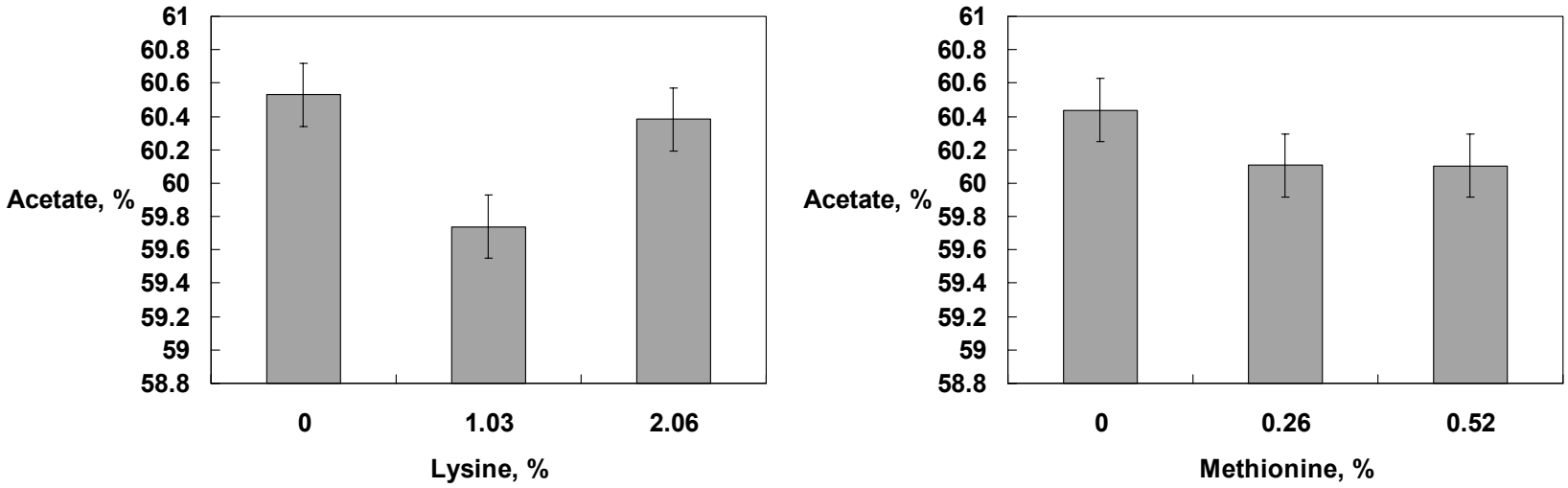


Figure 3-4. Comparison of least squares means for proportion of acetate of diets with or without supplemental lysine or methionine.

The lowest proportion of acetate was observed when 1.03% Lys was added to the diet ($P < 0.05$). The proportion of propionate was influenced by the interaction of Lys and Met ($P < 0.05$) (Figure 3-5) and Lys ($P < 0.01$) and Met ($P < 0.01$) supplementation (Figure 3-6). The proportion of propionate was numerically highest when 1.03% Lys was added to the diet but kept increasing as the amount of Met supplementation increased ($P < 0.01$).

The ratio of acetate to propionate was altered by the interaction of Lys and Met ($P < 0.01$) (Figure 3-7) and Lys ($P < 0.01$) and Met ($P < 0.01$) supplementation (Figure 3-8). The ratio of acetate to propionate was numerically lowest when1.03% Lys added to the diet. The ratio of acetate to propionate decreased as the amount of Met supplementation increased ($P < 0.01$). This is in agreement with other studies that reported a lower ratio of acetate to propionate when rapidly degradable Met (Bach and Stern, 1999) or HMB (Vázquez-Añón et al., 2001) was added to continuous culture systems. Diet 8 resulted in the numerically lowest ratio of acetate to propionate and the highest proportion of propionate suggesting that more dietary energy was captured in the VFA and available to the cow (Bateman et al, 2002).

There were no effects of the interaction of Lys and Met ($P = 0.09$) nor Lys ($P = 0.57$) nor Met ($P = 0.10$) supplementation observed in the proportion of isobutyrate. The proportion of butyrate was affected by both the interaction of Lys and Met ($P < 0.05$) (Figure 3-9) and Lys supplementation ($P < 0.01$) (Figure 3-10) but was not influenced by Met ($P = 0.42$) (Figure 3-10). The proportion of butyrate increased as the amount of Lys addition increased. Proportions of both butyrate and isobutyrate were not affected by DL-Met supplementation in this study. This is in contrast to Noftsger et al. (2002) who reported lower proportions of butyrate and isobutyrate when Met was provided as DL-Met. The proportion of isovalerate was not affected by Lys ($P = 0.05$) but was influenced by both the interaction of Lys and Met ($P < 0.01$) (Figure 3-11) and Met ($P < 0.01$) supplementation (Figure 3-12). The proportion of isovalerate decreased as the amount

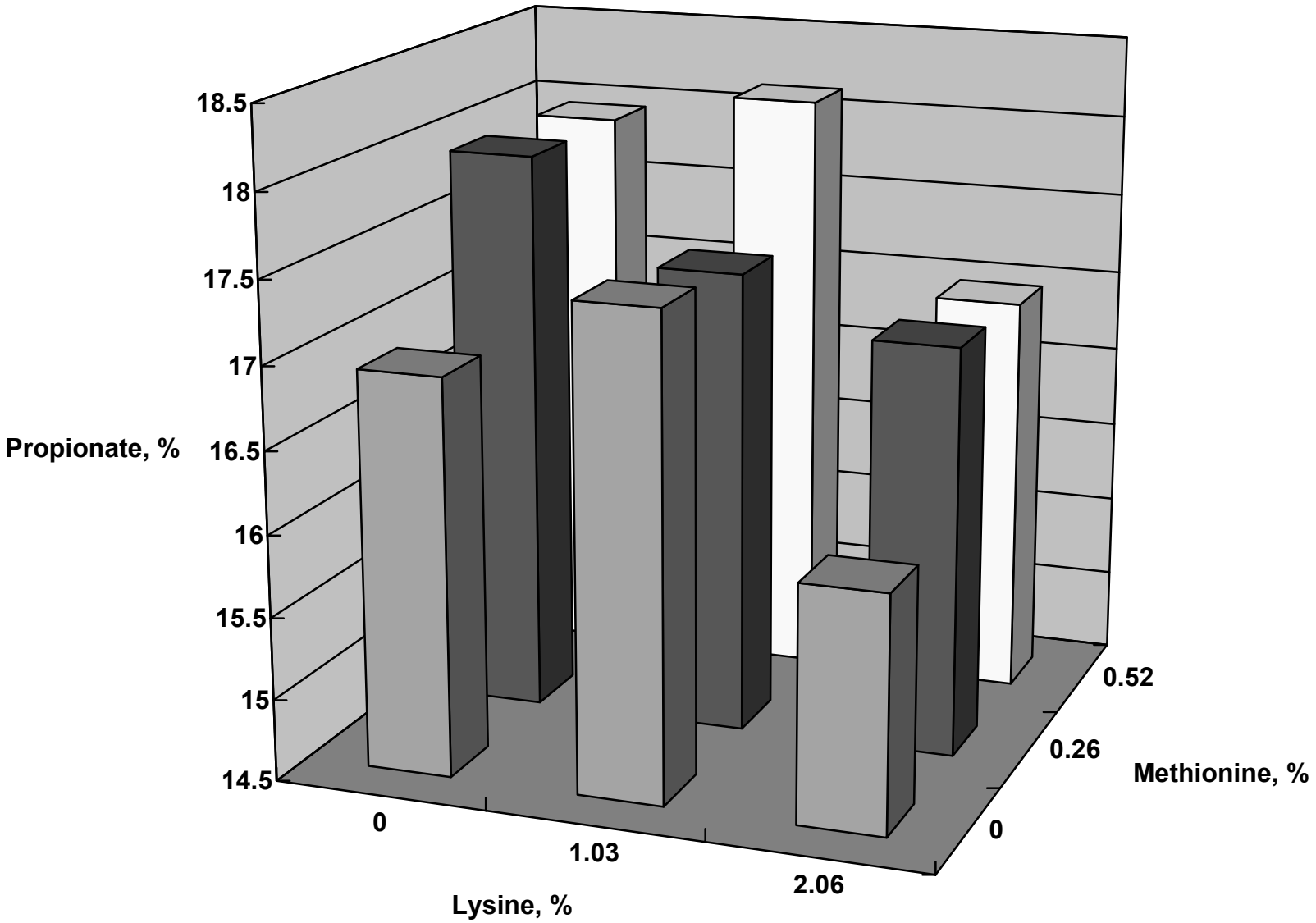


Figure 3-5. Least squares means for proportion of propionate of diets with or without supplemental lysine and methionine (SEM=0.2007).

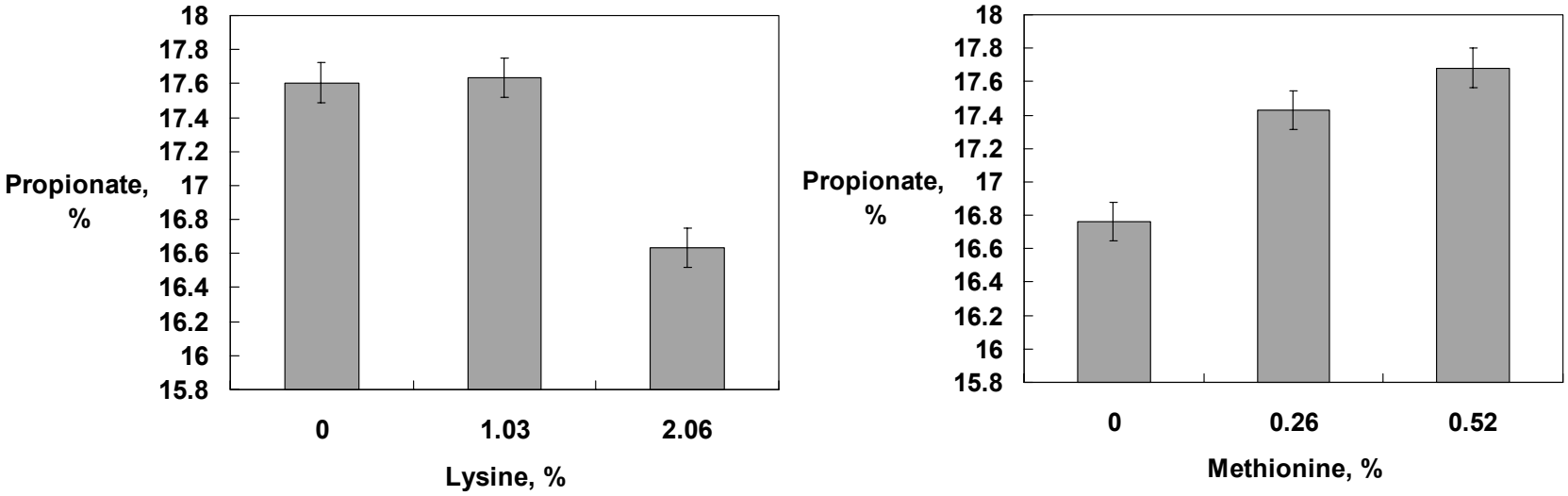


Figure 3-6. Comparison of least squares means for proportion of propionate of diets with or without supplemental lysine or methionine.

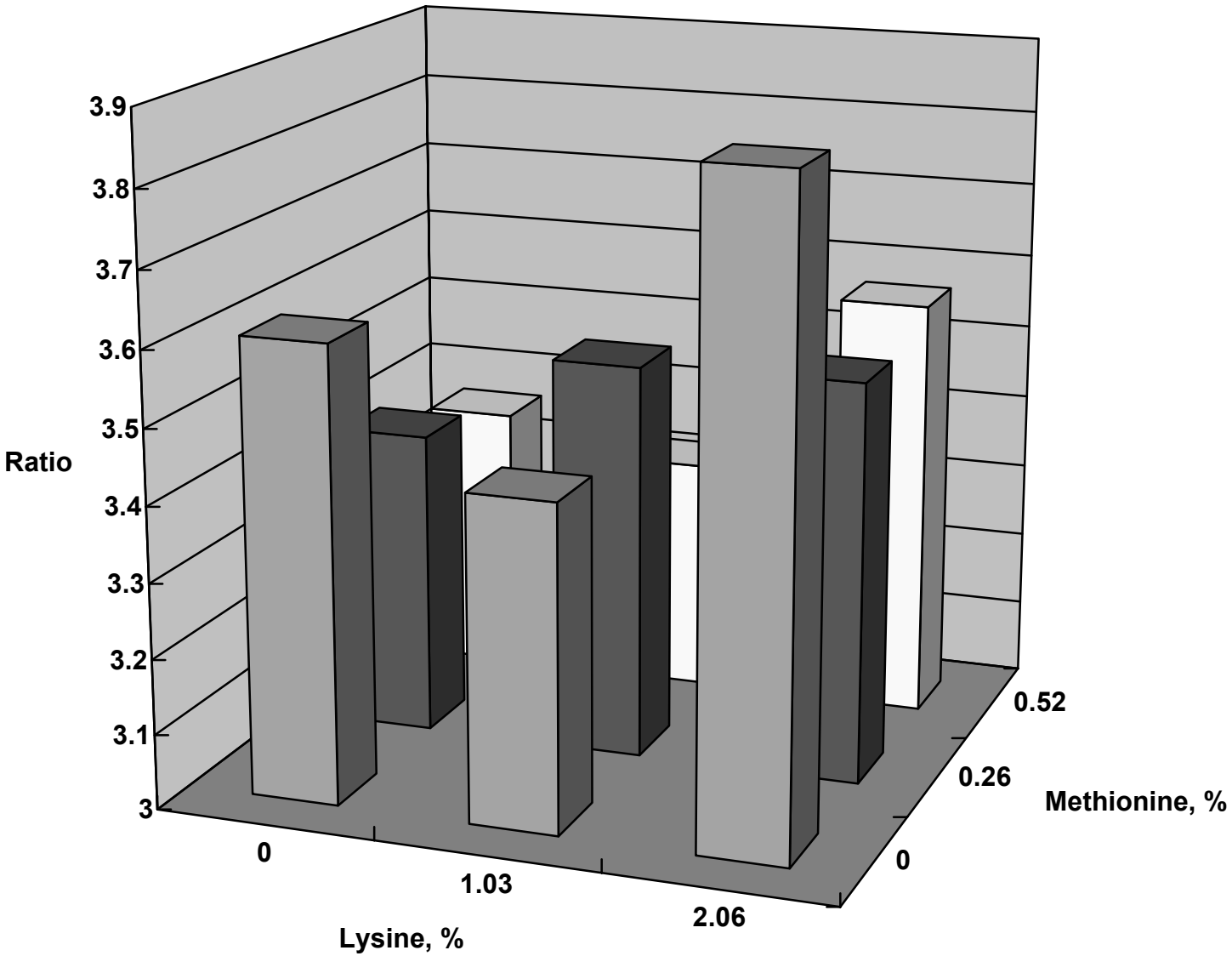


Figure 3-7. Least squares means for ratio of acetate to propionate of diets with or without supplemental lysine and methionine (SEM=0.0535).

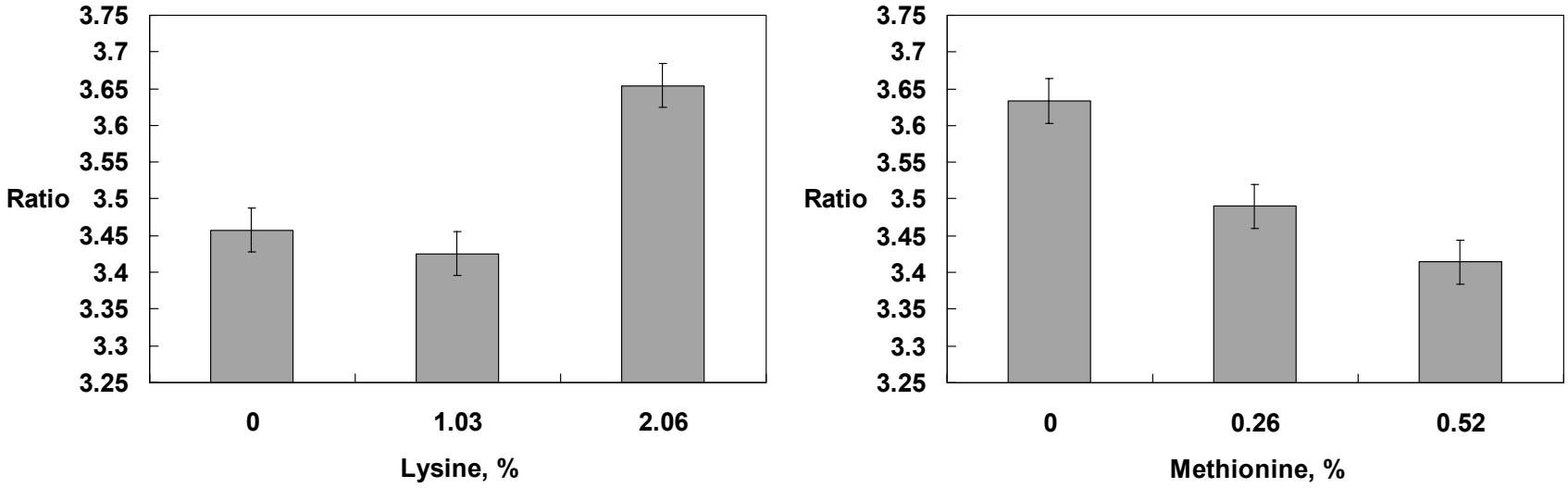


Figure 3-8. Comparison of least squares means for ratio of acetate to propionate of diets with or without supplemental lysine or methionine.

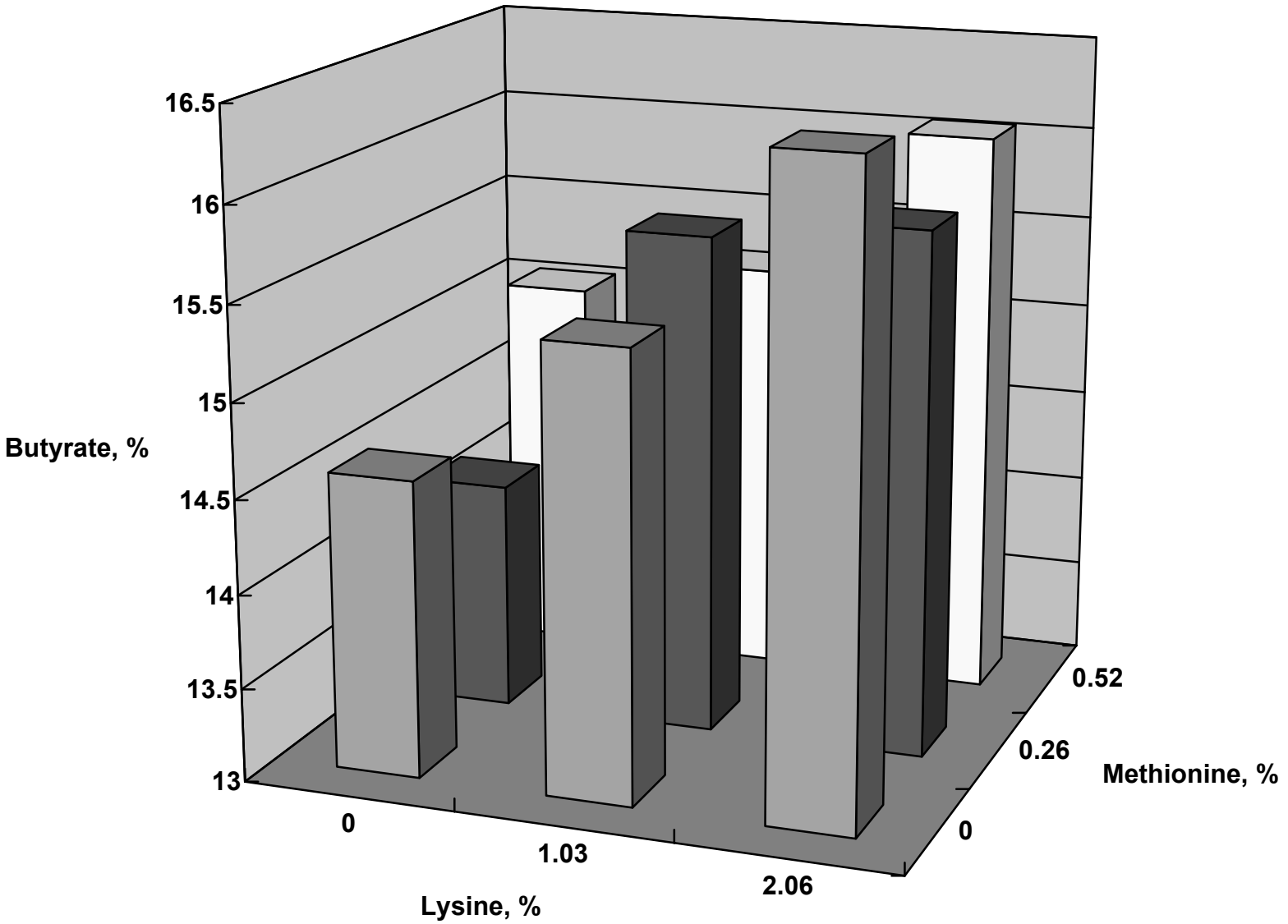


Figure 3-9. Least squares means for proportion of butyrate of diets with or without supplemental lysine and methionine (SEM=0.2325).

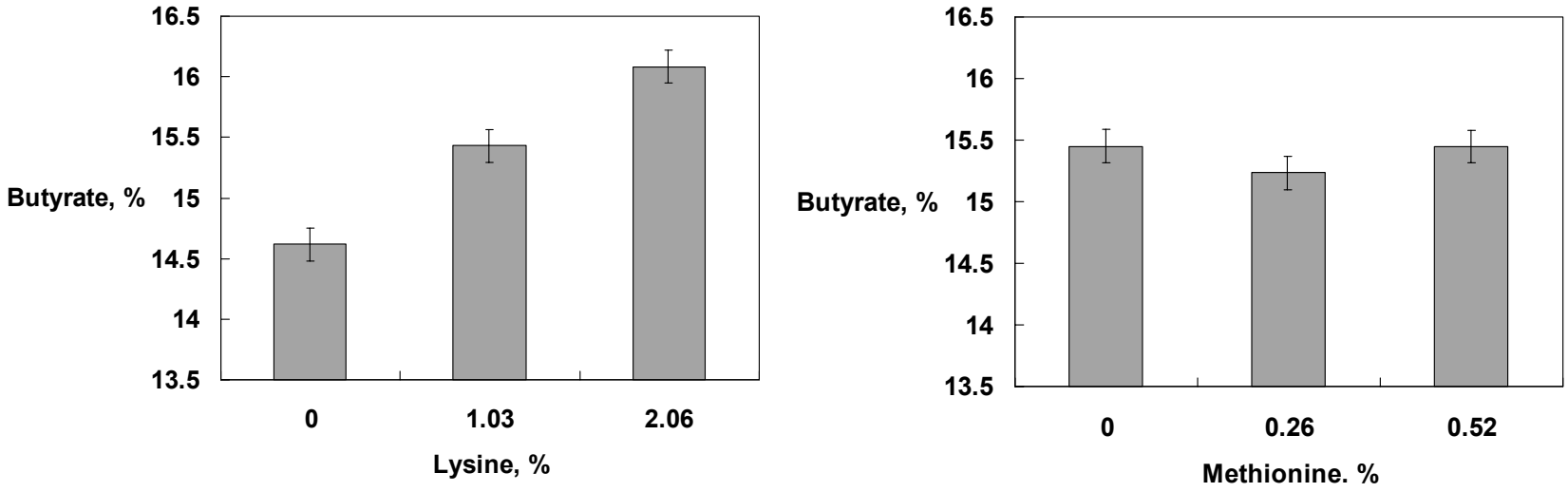


Figure 3-10. Comparison of least squares means of proportion of butyrate of diets with or without supplemental lysine or methionine.

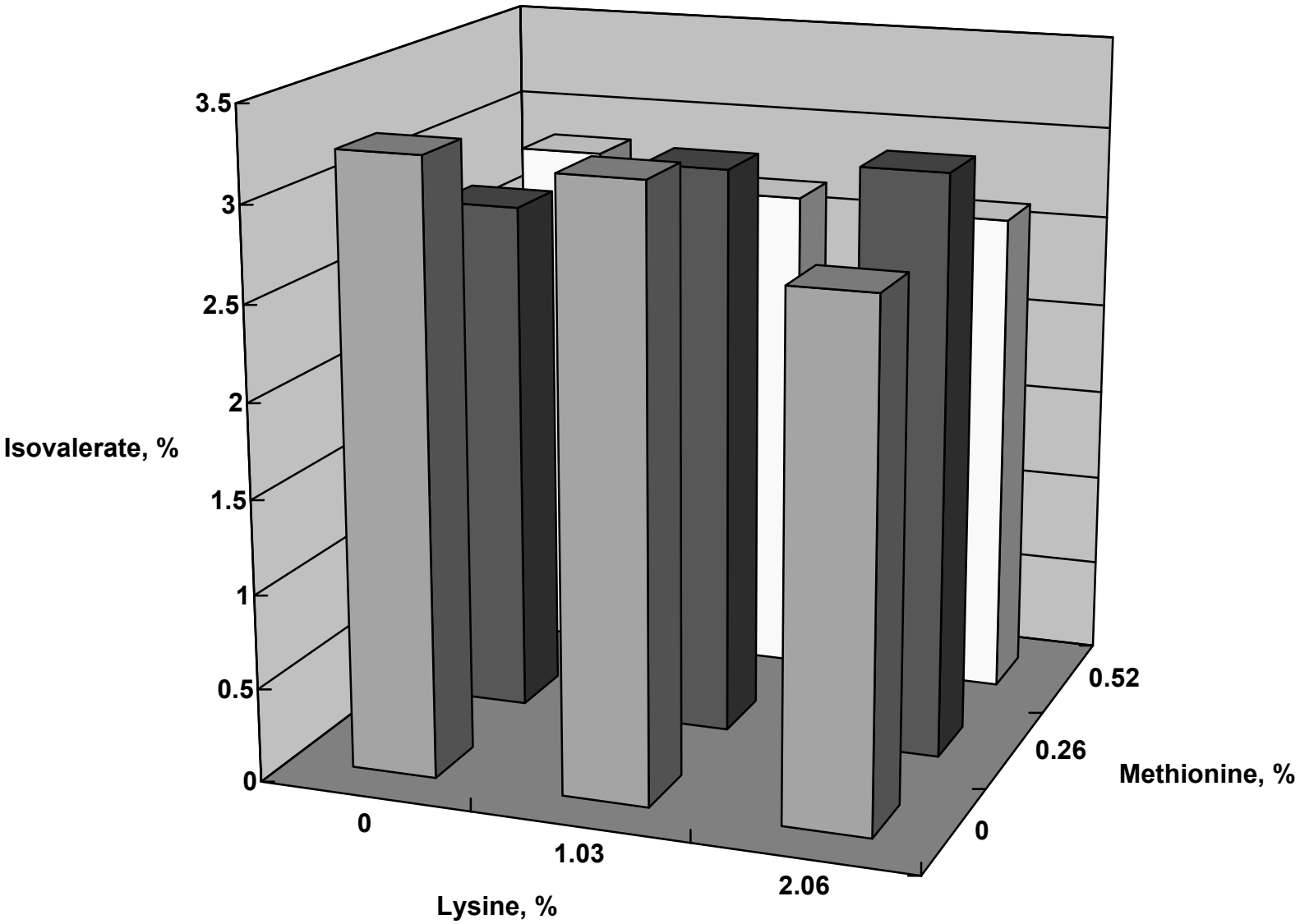


Figure 3-11. Least squares means for proportion of isovalerate of diets with or without supplemental lysine and methionine (SEM=0.0786).

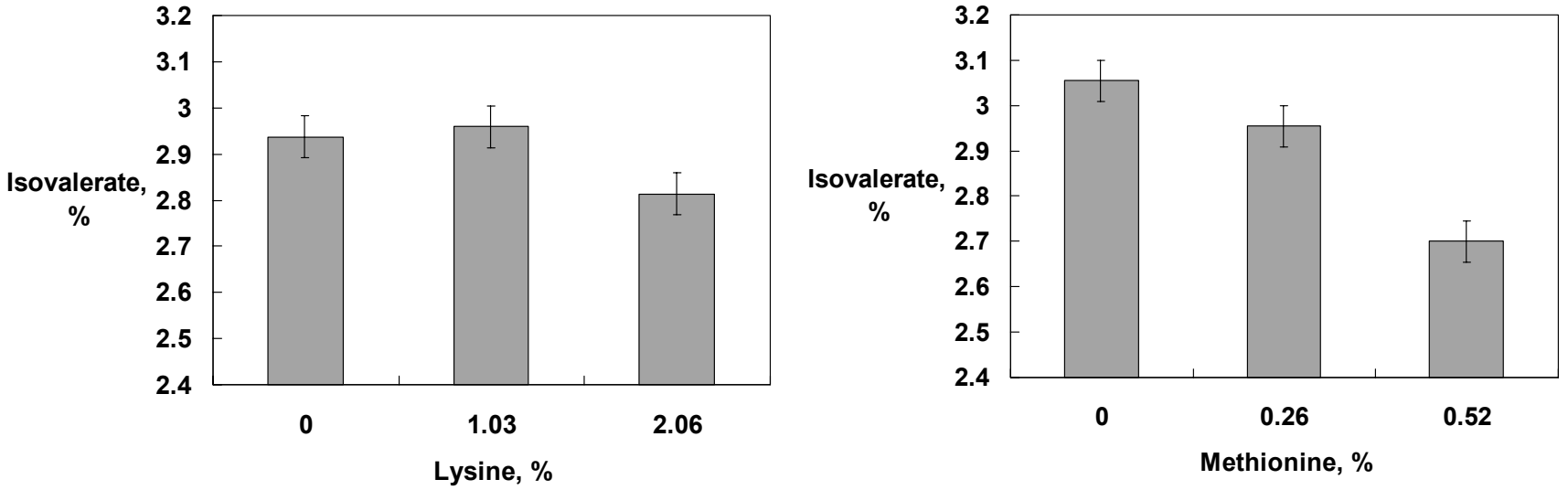


Figure 3-12. Comparison of least squares means for proportion of isovalerate of diets with or without supplemental lysine or methionine.

of Met addition increased. The proportion of valerate was affected by the interaction of Lys and Met ($P < 0.01$) (Figure 3-13) and Lys ($P < 0.01$) and Met ($P < 0.01$) supplementation (Figure 3-14). The proportion of valerate decreased as the amount of Lys or Met or both increased.

### 3.3.3 Ammonia-Nitrogen Production

The interaction of Lys and Met was significant ($P < 0.01$) (Figure 3-15) for the least squares means of $NH_4^+$ concentration from the continuous culture system. Diet 6 resulted in the numerically highest concentration of $NH_4^+$ among treatments studied in this experiment (Table 3-5). There were no effects of time of sampling ($P = 0.90$) observed on the concentrations of $NH_4^+$. Mean concentration of $NH_4^+$ increased as both the amount of Lys ($P < 0.01$) and Met ($P < 0.01$) supplementation increased (Figure 3-16). This is in agreement with Windschitl and Stern (1988) who reported a numerically greater ruminal $NH_4^+$ when DL-Met was supplemented to the continuous culture system.

Diet 6 from replicate 1 and diet 8 from replicate 2 which resulted in the numerically highest concentration of total VFA and also resulted in the numerically highest concentration of $NH_4^+$. However, overall, diet 8, which had the highest mean concentration of total VFA, did not also have the highest mean concentration of $NH_4^+$. This is because diet 6 in replicate 2 still had the second highest concentration of $NH_4^+$ even though the concentration of total VFA for diet 6 in replicate 2 dropped drastically compared to replicate 1. The reason why the concentration of total VFA of diet 6 in replicate 2 dropped while the concentration of $NH_4^+$ was still high is unclear. More research is needed to determine if there is a positive correlation between concentrations of total VFA and $NH_4^+$ in the continuous culture system.

Based on the results discussed above, supplementing either Lys or Met or both increased productions of total VFA and $NH_4^+$ in the continuous culture system. However, the higher concentration of Lys (2.06%) supplementation increased the ratio of acetate to propionate

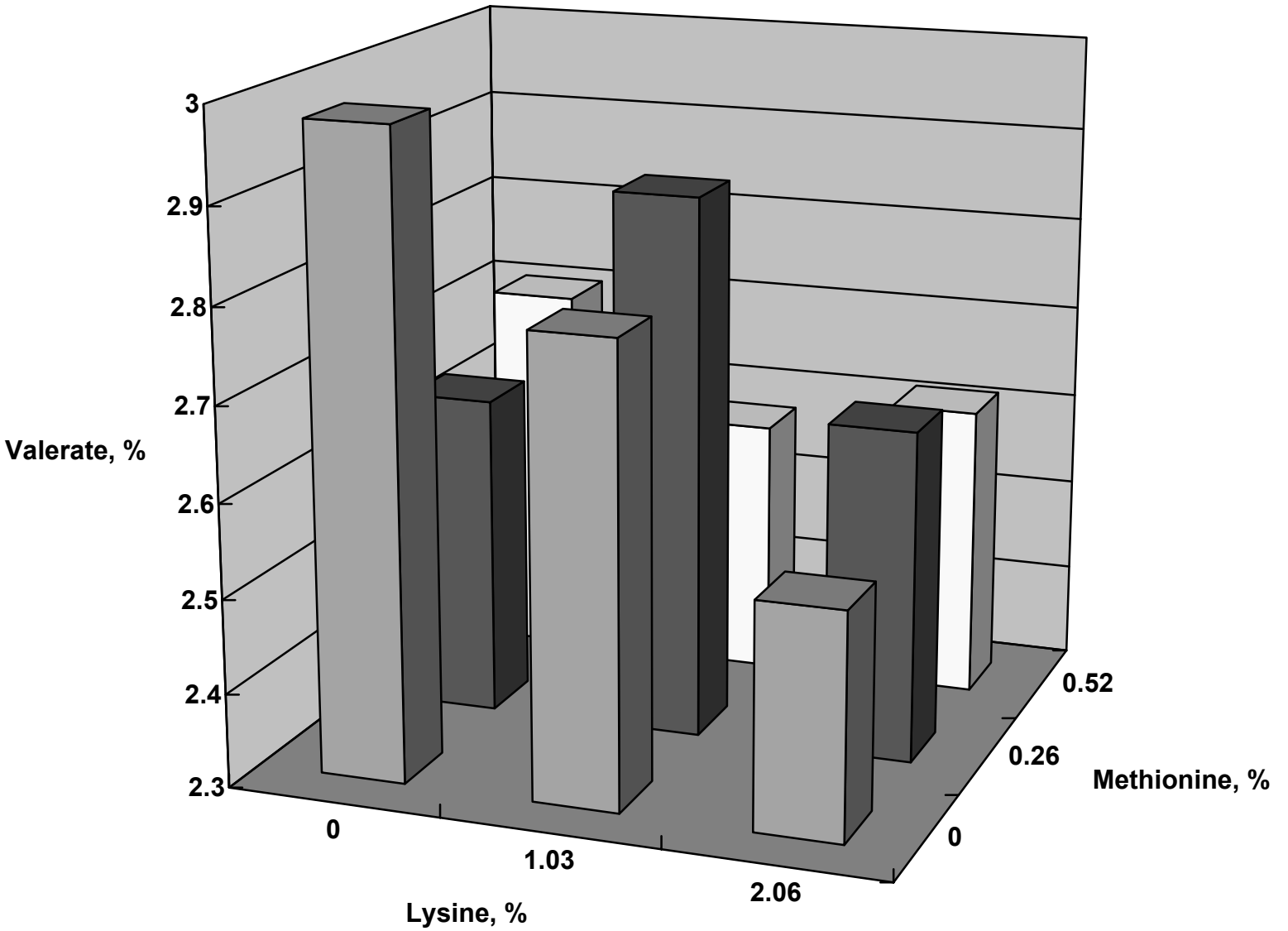


Figure 3-13. Least squares means for proportion of valerate of diets with or without supplemental lysine and methionine (SEM=0.0490).

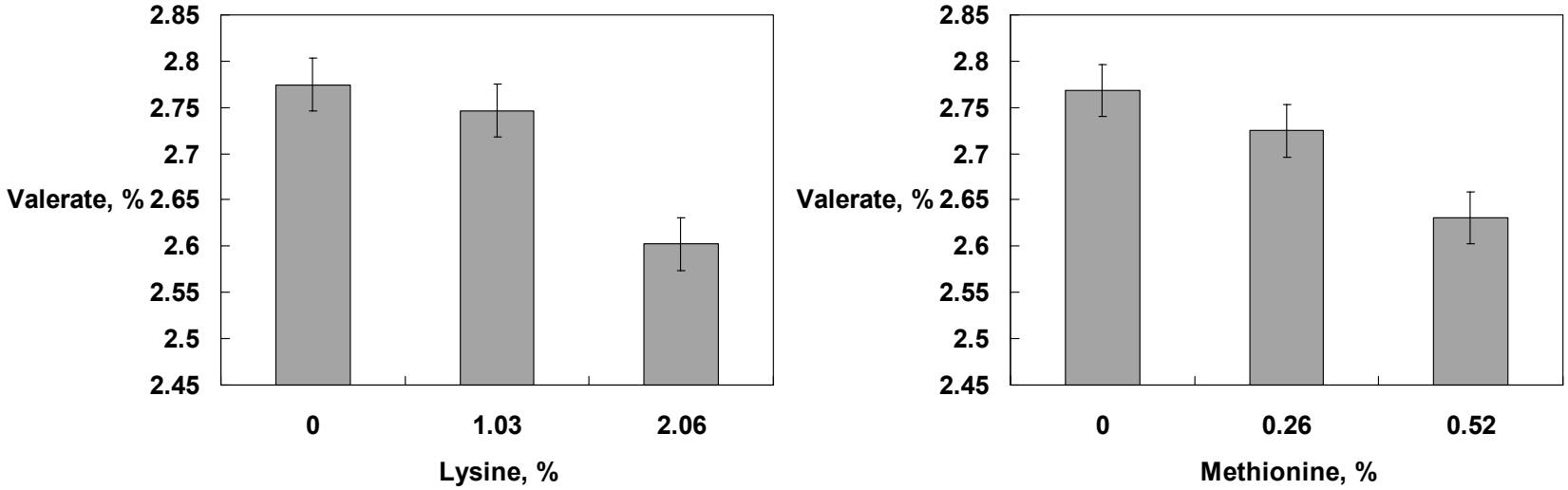


Figure 3-14. Comparison of least squares means for proportion of valerate of diets with or without supplemental lysine or methionine.

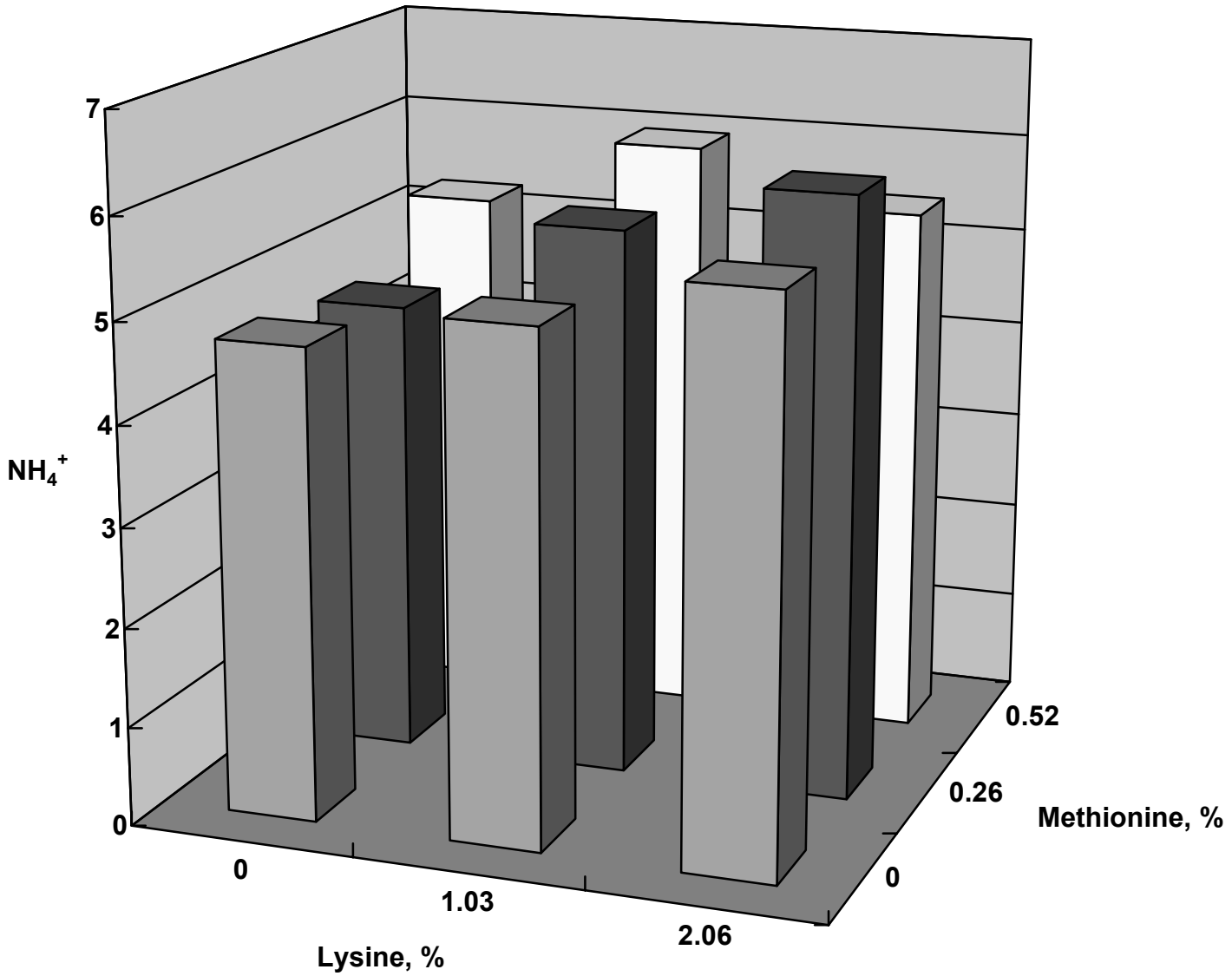


Figure 3-15. Least squares means for concentration of $NH_4^+$ of diets with or without supplemental lysine and methionine (SEM=0.1102).

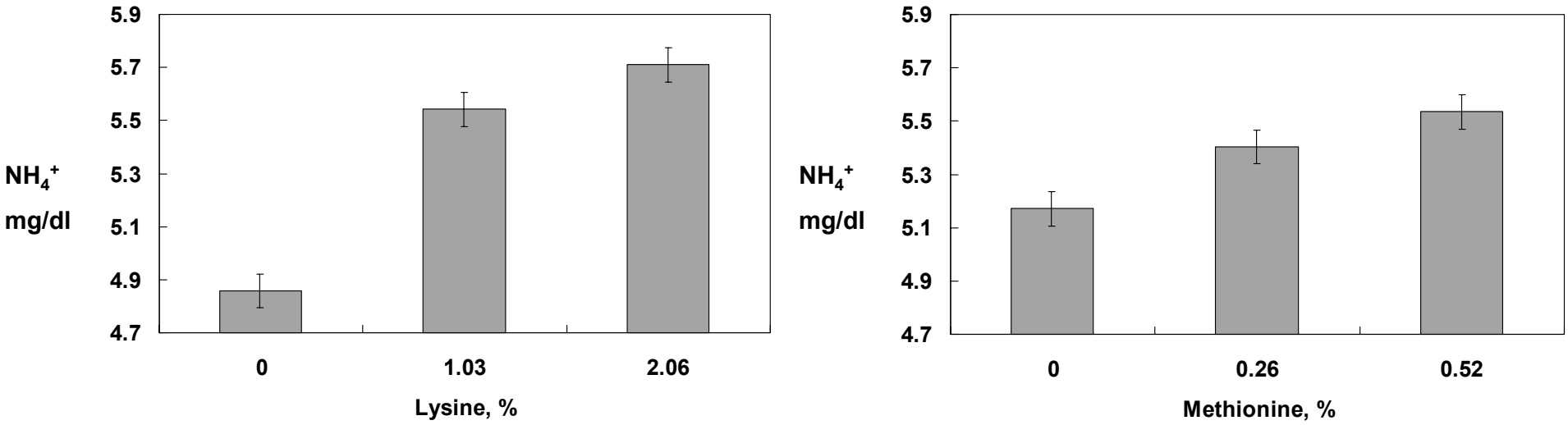


Figure 3-16. Comparison of least squares means for concentration of $NH_4^+$ of diets with or without supplemental lysine or methionine.

suggesting that less energy was captured from fermentation (Bateman et al., 2002). Therefore, ruminal fermentation in the continuous system was limited at higher concentration of Lys addition but was not limited with those concentrations of Met in the present study.

### 3.3.4 Microbial Nitrogen Synthesis

Microbial N synthesis was calculated from the proportion of bacterial pellet N to fermentor effluent pellet N (Table 3-9). The formula for calculating percentage of bacterial N synthesis is: [{% purine in effluent pellet ÷ (% purine in bacterial pellet ÷ N in bacterial pellet)} ÷ N in effluent pellet] × 100. There were no effects of the interaction of Lys and Met ($P > 0.05$) (Figure 3-17) nor Lys ($P > 0.05$) or Met ($P > 0.05$) supplementation (Figure 3-18) observed in the synthesis of microbial N. However, there was a high standard error observed for the percentages of bacterial N in effluent pellet among treatments. This was because bacterial and effluent pellets were harvested from replicate 2 only. Therefore, the synthesis of microbial N was calculated from one replicate only which resulted in high standard errors. If data of bacterial N synthesis from two replicates were both included, more observations would have been available to decrease the standard error and therefore, power to detect treatment differences would have been increased.

As compared to the control diet, diet 2 and 8 had a similar percentage of the total N that was of bacterial origin. It is unclear why the other diets appear to have a reduction in bacterial N capture. However, it is possible that the other combinations of AA used were toxic to the ruminal microbes. It is also possible that the other combinations of AA tested resulted in a lower degradability of feed proteins, which was observed as a decrease in bacterial N.

## 3.4 Summary and Conclusions

Time of sampling had no effect on mean concentrations of total VFA and $NH_4^+$ and proportions of individual VFA. Supplementation of Met and Lys had impacts on mean

Table 3-9. Least squares means for bacterial N synthesis of the continuous culture system fed diets with or without supplemental Met and Lys from replicate 2.

| | Experimental diets | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Diet1 | Diet2 | Diet3 | Diet4 | Diet5 | Diet6 | Diet7 | Diet8 | Diet9 | | | | |
| Lys | 0 | 1.03 | 2.06 | 0 | 1.03 | 2.06 | 0 | 1.03 | 2.06 | | *P*-value | | |
| Item Met | 0 | 0 | 0 | 0.26 | 0.26 | 0.26 | 0.52 | 0.52 | 0.52 | SEM | Lys | Met | Lys*Met |
| Bacterial pellet, | | | | | | | | | | | | | |
| DM, % | 8.21 | 6.59 | 10.20 | 9.20 | 9.12 | 10.84 | 8.58 | 8.04 | 9.57 | 1.46 | 0.2042 | 0.5147 | 0.9471 |
| Ash, % DM | 10.48 | 7.63 | 12.83 | 13.97 | 9.85 | 13.15 | 10.47 | 10.34 | 13.22 | 2.00 | 0.1180 | 0.4975 | 0.8069 |
| Purine, % | 7.14 | 6.94 | 9.50 | 9.84 | 8.62 | 9.20 | 7.64 | 7.58 | 9.92 | 1.48 | 0.3373 | 0.5457 | 0.8416 |
| N, % DM | 10.2 | 10.4 | 9.9 | 9.9 | 10.3 | 9.5 | 10.2 | 10.5 | 10.0 | 0.32 | 0.1265 | 0.4119 | 0.9950 |
| Effluent pellet, | | | | | | | | | | | | | |
| DM, % | 13.77 | 13.65 | 14.08 | 13.54 | 13.55 | 14.61 | 14.06 | 13.41 | 13.35 | 0.73 | 0.7328 | 0.8790 | 0.8121 |
| Ash, % DM | 21.79 | 21.68 | 20.74 | 21.36 | 20.59 | 22.05 | 20.34 | 23.07 | 21.65 | 1.25 | 0.8354 | 0.9351 | 0.5622 |
| Purine, % | 1.10 | 1.22 | 1.04 | 1.04 | 1.30 | 1.26 | 1.08 | 1.06 | 1.26 | 0.13 | 0.4562 | 0.7163 | 0.5373 |
| N, % DM | 4.27$^{b}$ | 4.27$^{b}$ | 4.49$^{ab}$ | 4.40$^{ab}$ | 4.42$^{ab}$ | 4.80$^{a}$ | 4.55$^{ab}$ | 4.42$^{ab}$ | 4.14$^{b}$ | 0.16 | 0.7180 | 0.2931 | 0.1598 |
| Bacterial N, % of total N in effluent[1] | 42.16 | 42.90 | 24.14 | 23.28 | 35.13 | 28.05 | 32.40 | 39.66 | 30.58 | 8.27 | 0.2755 | 0.5369 | 0.7332 |

[1] % Bacterial N synthesis = [{% purine in effluent pellet ÷ (% purine in bacterial pellet ÷ N in bacterial pellet)} ÷ N in effluent pellet] × 100.

[a, b]: means in the same row with different superscripts are significantly different ($P < 0.05$).

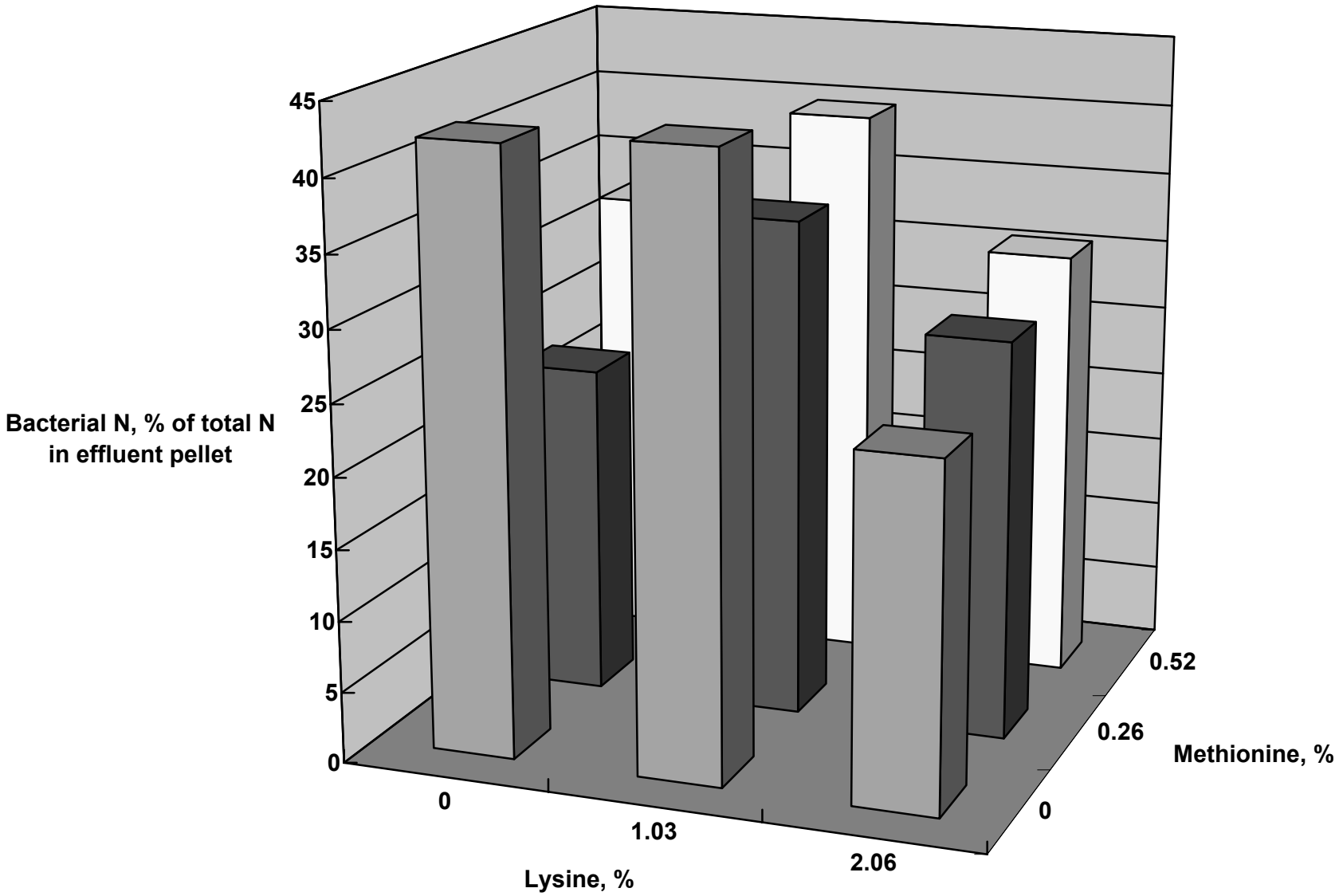


Figure 3-17. Least squares means for percentage of bacterial N in effluent pellet of diets with or without supplemental lysine and methionine (SEM=8.2749).

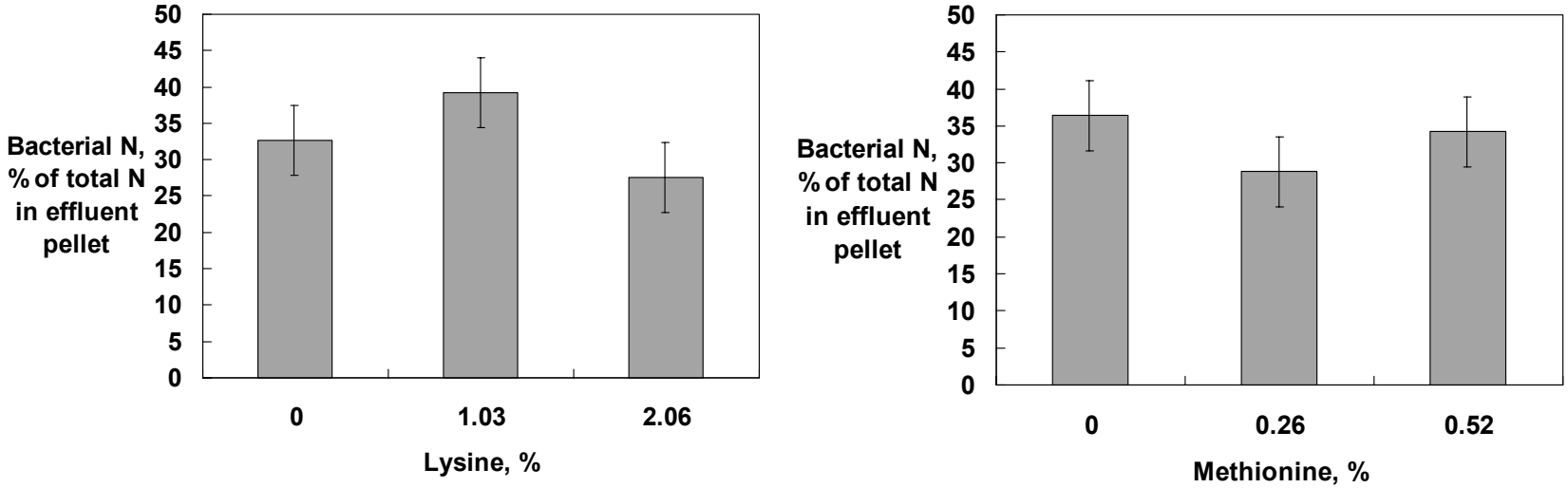


Figure 3-18. Comparison of least squares means for percentage of bacterial N in effluent pellet of diets with or without supplemental lysine or methionine.

concentrations of total VFA and $NH_4^+$, proportions of acetate, propionate, butyrate, isovalerate, valerate, and the ratio of acetate to propionate. Percentages of microbial N in effluent pellets were not affected by supplementing Lys and Met.

The hypothesized ideal concentrations of Lys and Met (diet 5) did not result in the greatest concentration of total VFA. The combination of 0.52% Met and 1.03% Lys (diet 8) resulted in the highest mean concentration of total VFA, second high mean concentration of $NH_4^+$, and the lowest ratio of acetate to propionate among treatments with no decrease in the microbial CP production.

In conclusion, supplementing 0.52% Met and 1.03% Lys to an alfalfa hay-concentrate based diet (1:1 ratio) may supply the ruminal microorganisms an optimal level of Met and Lys to improve ruminal fermentation in the continuous culture system.

# CHAPTER 4. EFECTS OF FREE METHIONINE AND LYSINE ON PERFORMANCE AND RUMINAL FERMENTATION OF LATE LACTATION HOLSTEIN COWS

## 4.1 Objective

This experiment was conducted to study the effects of free Met and Lys supplementation on ruminal fermentation and milk production response of lactating dairy cows. The objective was to determine if the optimal concentrations of free Met and Lys obtained from the previous in vitro experiment would have a similar impact on in vivo ruminal fermentation and the milk production responses of Holstein cows.

## 4.2 Materials and Methods

### 4.2.1 Dietary Treatments and Animal Care

Sixteen Holstein cows in late lactation (mean DIM=223) were paired by their current milk production (average 20.4 kg/d), parity, and days in milk (DIM). Details of the DHI records for the control and treatment groups are shown in Table 4-1. Cows from each pair were randomly assigned to either the control or treatment diet. The control diet had a 60:40 forage to concentrate ratio (DM basis), and was based on corn silage and alfalfa hay with a commercial protein pellet, whole cottonseed, and ground corn as concentrates. The treatment diet was the control diet supplemented with Met and Lys at 0.29% and 2.27% (%DM), respectively. Therefore, diets were not isonitrogenous. Proportions of each ingredient for the control and treatment diets are listed in Table 4-2. Methionine was provided as DL-Met (Rhodimet$^{TM}$ NP99; Rhône-Poulenc Animal Nutrition; Antony Cedex, France), and Lys was provided as L-Lys-HCl (L-Lys-HCl; Biokyowa Inc; Cape Girardeau, MO.). Treatment diets were offered as total mixed rations (TMR). Prior to the start of data collection, all cows were trained to use the Calan gate feeding system (America Calan; Northwood, NH) and were allowed to adapt to the control diet for 8 d. After all cows were consuming their diet and had ample time for adaptation to the feeding system, the treatment

Table 4-1. Mean DHI records for the control and treatment groups prior to the experiment.

| | Control | Treatment |
|---|---|---|
| Number of cows | 8 | 8 |
| Number of first lactation | 3 | 3 |
| Average lactation number | 2.4 | 2.1 |
| Average DIM | 223 | 222 |
| Average milk, kg/d | 21.6 | 19.2 |

Table 4-2. Composition of the control and treatment diets.

| | Experimental diets | |
|---|---|---|
| Ingredient | Control | Treatment |
| | % of DM | |
| Corn silage | 48.59 | 47.35 |
| Protein concentrate[1] | 15.39 | 15.00 |
| Alfalfa hay | 12.41 | 12.09 |
| Cottonseed | 11.07 | 10.78 |
| Corn | 10.82 | 10.54 |
| Sodium bicarbonate | 0.74 | 0.72 |
| Limestone | 0.49 | 0.48 |
| Trace mineral salt[2] | 0.49 | 0.48 |
| Lysine | 0 | 2.27 |
| Methionine | 0 | 0.29 |

[1] 22.16% corn, 56.16% soybean meal, 10.85% dolomitic limestone, 5.42% mono-calcium phosphate, 5.42% trace mineral salt.

[2] 99% DM, 60.60% Cl, 39.34% Na, 70 mg/kg Co, 400 mg/kg Cu, 70 mg/kg I, 1750 mg/kg Fe, 2800 mg/kg Mn, 0 mg/kg Se, 3500 mg/kg Zn.

group was supplemented with DL-Met and L-Lys-HCl. The AA in the treatment group were mixed with the TMR. Cows were milked twice daily at 0600 h and 1530 h. Cows were fed twice daily at 0700 h and 1500 h and were allowed access to feed except while being milked. All cows were handled and housed under protocols approved by the Institutional Animal Care and Use Committee of the LSU Agricultural Center.

### 4.2.2 Sample Collection and Analytical Procedures

The entire experiment was a 36 d trial that included 8 d of adaptation (pre-experimental period) followed by a 28 d experimental period. During the experimental period, as-fed intake and milk production were recorded daily and milk was sampled at each milking and sent to the Louisiana DHIA lab (Baton Rouge, LA) for analyses of percentage of milk fat and protein, and somatic cell counts (SCC). Milk yield was corrected by 4% milk fat and energy. The formula for 4% fat corrected milk (4% FCM) is: 0.4 x milk yield + 15 x fat yield, and the formula for energy corrected milk (ECM) is: 0.3246 x milk yield + 12.86 x fat yield + 7.04 x protein yield. Milk production efficiency was calculated as the kg of milk produced per kg dry matter intake (DMI), 4% FCM efficiency was calculated as the kg of 4% FCM produced per kg DMI, and ECM efficiency was calculated as the kg of ECM produced per kg DMI. Somatic cell count score (SCCS) was calculated as: 3 + (LN (SCC ÷ 100) ÷ 0.693147). The TMR of the control and treatment groups were sampled daily at each feeding, and feed ingredients were sampled weekly to analyze for total N, ADIN, ash, DM (AOAC, 1980), and ADF (Van Soest et al., 1991). Total N was tested in order to calculate the CP content. The CP content was calculated as: N × 6.25. Acid detergent insoluble nitrogen was tested in order to separate the available CP from the bound CP. Bound CP was calculated as: ADIN × 6.25. The content of available CP was calculated as: CP – bound CP. Ruminal fluid from each cow was collected via stomach tube prior to (d –2), at the midpoint (d 15), and at the end (d 28) of the experiment. Ruminal fluid was transported to the

laboratory immediately after collection and pH was measured. A 4 ml sub-sample was then pipetted and prepared for VFA analysis. The remainder of the sub-sample was then acidified by adding 1 ml of 20 % (vol/vol) $H_3PO_4$ and stored frozen (-20 $^oC$) for subsequent $NH_4^+$ analysis. Procedures for analyses of VFA and $NH_4^+$ were discussed in the previous chapter. Body weight for each cow was recorded prior to (d –2) and at the end (d 28) of the experiment.

### 4.2.3 Experimental Design and Statistical Analysis

Because production response data were in a time series, the effects of supplementing Met and Lys to the diet on DMI, organic matter intake (OMI), milk yield, milk production efficiency, 4% FCM efficiency, ECM efficiency, yield and percentages of milk components, and SCCS were analyzed as repeated measurements using a mixed model that included terms of treatment, day, and the interaction of treatment and day. Cow was included as a random term that was used to test the main effect of treatment. Day was modeled as a repeated term that was assumed to be correlated within cow nested within the main effect of treatment (Littell et al., 1998). Data of production responses collected in the pre-experimental period were used as a covariate for statistical analysis.

The effects of supplementing Met and Lys to the diet on pH, concentrations of total VFA and $NH_4^+$, and proportions of individual VFA were also analyzed as repeated measurements using a mixed model that included terms of treatment, period, and the interaction of treatment and period. Cow was included as a random term that was used to test the main effect of treatment. Period was modeled as a repeated term that was assumed to be correlated within cow nested within the main effect of treatment. Data of ruminal performance collected in the pre-experimental period were used as a covariate for statistical analysis.

Chemical analyses of experimental diets including the control and treatment diets, and body weight changes of the control and treatment groups were analyzed using ANOVA. All data are

presented as least squares means. All calculations were completed using SAS (SAS Institute Inc., 1990). Significance was declared at $P < 0.05$.

### 4.3 Results and Discussion

In this study, experimental diets for the control and treatment groups were formulated based on NRC (2001) for a 545-kg, 30 month-old cow at 280 days in milk, producing 28 kg of 3.7% fat milk per day. The predicted CP profile of the control diet was 14.9% CP with 10% RDP and 4.9% RUP. The predicted CP profile of the treatment diet was 17% CP with 12.2% RDP and 4.8% RUP. The predicted $NE_l$ for the control diet (0.73 Mcal/lbs DM) was similar to the treatment diet (0.72 Mcal/lbs DM).

The concentrations of Met and Lys evaluated in this experiment were chosen based on the results from replicate 1 of the in vitro study reported previously. Treatment concentrations for Met and Lys used in this study were converted to 100% DM basis from diet 6 in the previous in vitro study. The average DMI for each cow of the treatment group in this study was 13.18 kg/d. Therefore, daily supplementation of DL-Met was 38.22 g/cow/d and L-Lys-HCl was 299.19 g/cow/d.

#### 4.3.1 Chemical Analyses of Ingredients and Experimental Diets

Chemical analyses of the control and treatment diets are presented in Table 4-3. The treatment diet had greater total N content ($P < 0.01$) and ADF ($P < 0.01$) than the control diet as expected when AA were added to the diet. The actual CP contents of the control (13.38% and 14.9%, for the actual and predicted CP content, respectively) and treatment (15.59% and 17%, for the actual and predicted CP content, respectively) diets were both lower than the predicted contents. The treatment diet supplied more available CP ($P < 0.01$) than the control diet but there were no differences ($P > 0.05$) in the contents of DM, OM, ADIN, or ash between the treatment and control diets.

Table 4-3. Least squares means for chemical analysis of the experimental diets with or without supplemental Met and Lys.

| | Experimental diets | | | |
|---|---|---|---|---|
| Item | Control | Treatment | SEM | *P*-value |
| DM, % | 44.30 | 44.69 | 0.34 | 0.4105 |
| OM, % | 40.40 | 40.78 | 0.35 | 0.4707 |
| | % of DM | | | |
| N | 2.14 | 2.49 | 0.04 | < 0.0001 |
| CP[1] | 13.38 | 15.59 | 0.26 | < 0.0001 |
| ADIN[2] | 0.39 | 0.38 | 0.01 | 0.5259 |
| Bound CP[3] | 2.46 | 2.39 | 0.08 | 0.5259 |
| Available CP[4] | 10.92 | 13.20 | 0.28 | < 0.0001 |
| ADF | 32.84 | 30.30 | 0.59 | 0.0029 |
| Ash | 8.81 | 8.74 | 0.32 | 0.8807 |

[1] CP = N × 6.25.

[2] ADIN = acid detergent insoluble nitrogen.

[3] Bound CP = ADIN × 6.25.

[4] Available CP = CP – Bound CP.

### 4.3.2 Lactational Performances

Least squares means for lactational performances of cows fed diets with or without AA supplementation are presented in Table 4-4. There were no treatment effects ($P > 0.05$) observed for any production measure. These results are in agreement with Harrison et al. (2000), who reported no significant differences of DMI or components of milk from cows fed diets with or without supplemental L-Lys and RP-Met. These data also agree with Koudele et al. (1999), who reported no effect of Lys supplementation on milk production or milk components from high-producing cows fed diets supplemented with 50 g L-Lys/cow/d plus 15 g RP-Met/cow/d.

Both the control and treatment groups had numerically lower DMI (mean DMI=13.92 kg/d) and milk yield (mean=18.73 kg/d) compared to the DMI (21 kg/d) and milk yield (28 kg/d) predicted from NRC (2001). Two possible reasons to cause depressions of the DMI and milk yield included weather conditions and lack of adaptation to Calan gates. The Louisiana Office of State Climatology reported that the average temperature in Baton Rouge was 27.2 $^{o}$ C and the relative humidity (RH) was 80.5% during the time that this study was conducted from August to September 2002. The calculated mean temperature-humidity index (THI; West, 1994) was 78.53 indicating that both the control and treatment groups were experiencing mild (THI=72 to 79) to moderate (THI=80 to 89) heat stress (Armstrong, 1994) during the experimental period. Reduced milk yield was reported by Johnson (1987) when the mean daily THI was above 72, and reduced milk yield and intake of total digestible nutrients (TDN) were reported by Johnson et al. (1963) when the mean daily THI was in the range of 71 to 81. Therefore, depressed DMI and milk yield should have been expected in this study under hot weather condition. Failure in using Calan gates should be also considered as one of the possible reasons that may have caused depression of voluntary DMI. All experimental cows were allowed to adapt to the Calan gates for 8 days;

Table 4-4. Least squares means for lactational performances and body weight gains of cows fed diets with or without supplemental Met and Lys.

| | Experimental diets | | | |
|---|---|---|---|---|
| Parameter | Control | Treatment | SEM | *P*-value |
| DMI, kg/d | 14.66 | 13.18 | 0.73 | 0.1760 |
| OMI, kg/d | 13.29 | 11.97 | 0.63 | 0.1809 |
| Milk yield, kg/d | 18.41 | 19.05 | 0.61 | 0.4702 |
| 4% FCM[1], kg/d | 18.31 | 19.26 | 0.63 | 0.3085 |
| ECM[2], kg/d | 19.23 | 20.20 | 0.67 | 0.3318 |
| 4% FCM efficiency[3] | 1.33 | 1.41 | 0.10 | 0.5444 |
| ECM efficiency[4] | 1.40 | 1.48 | 0.10 | 0.5576 |
| Milk efficiency[5] | 1.27 | 1.33 | 0.09 | 0.6590 |
| Body weight gain[6], kg | 9.20 | 21.42 | 5.95 | 0.1687 |
| Fat, | | | | |
| % | 3.96 | 4.05 | 0.05 | 0.2523 |
| kg/d | 0.73 | 0.78 | 0.03 | 0.2305 |
| Protein, | | | | |
| % | 3.01 | 3.02 | 0.03 | 0.7442 |
| kg/d | 0.55 | 0.57 | 0.02 | 0.4499 |
| SCCS[7] | 3.54 | 3.69 | 0.12 | 0.4000 |

[1] 4% FCM = 0.4 x milk yield + 15 x fat yield.

[2] ECM = 0.3246 x milk yield + 12.86 x fat yield + 7.04 x protein yield.

[3] 4% FCM efficiency = kg 4% FCM production ÷ kg DMI.

[4] ECM efficiency = kg ECM production ÷ kg DMI.

[5] Efficiency = kg milk production ÷ kg DMI.

[6] Total gain or loss of body weight over the 28 d experimental period.

[7] SCCS = 3 + (LN (SCC ÷ 100) ÷ 0.693147).

however, 50% of the cows in this study could not achieve their maximal DMI without human assistance for opening the Calan gates.

Although there was no impact of AA supplementation on DMI and milk yield ($P > 0.05$), the treatment group had numerically lower DMI and greater milk yield and, therefore, resulted in numerically higher milk production efficiencies. There was an interaction of AA treatment and day observed in the DMI ($P < 0.0001$), OMI ($P < 0.0001$), 4% FCM efficiency ($P = 0.0018$), and ECM efficiency ($P = 0.0015$) indicating that the treatment group maintained lower DMI and OMI, and greater 4% FCM (Figure 4-1) and ECM (Figure 4-2) efficiencies than the control group at the conclusion of the experiment even though there were no differences observed at the beginning of the study.

Least squares means for body weight of the control and treatment groups were numerically increased after the experiment as expected. Although, there was no AA treatment effect (P = 0.1687) observed on body weight gain between the control and treatment groups in this study, the numerically greater weight gain found in the treatment group (21.42 kg vs. 9.2 kg) suggests that AA supplementation may have favored energy partition to body tissue rather than production of milk and milk contents for cow in late lactation (Samuelson et al., 2001).

Compared to the general curves of the average DMI, milk production, and body weight change for dairy cattle at various feeding periods (Figure 4-3), the trends of the actual DMI for the control and treatment groups in the present study (Figure 4-4) were both increasing while the average DMI should be declining at week 32 to 36. This increase in the DMI of both groups suggests that DMI for both groups were improved during the experiment even though the actual DMI for both groups were lower than the predicted DMI. Actual milk yield for the control and treatment groups were both declining (Figure 4-5), and these were in agreement with the general curve of the average milk production (Figure 4-3). The AA treatment had no effect ($P > 0.05$) on

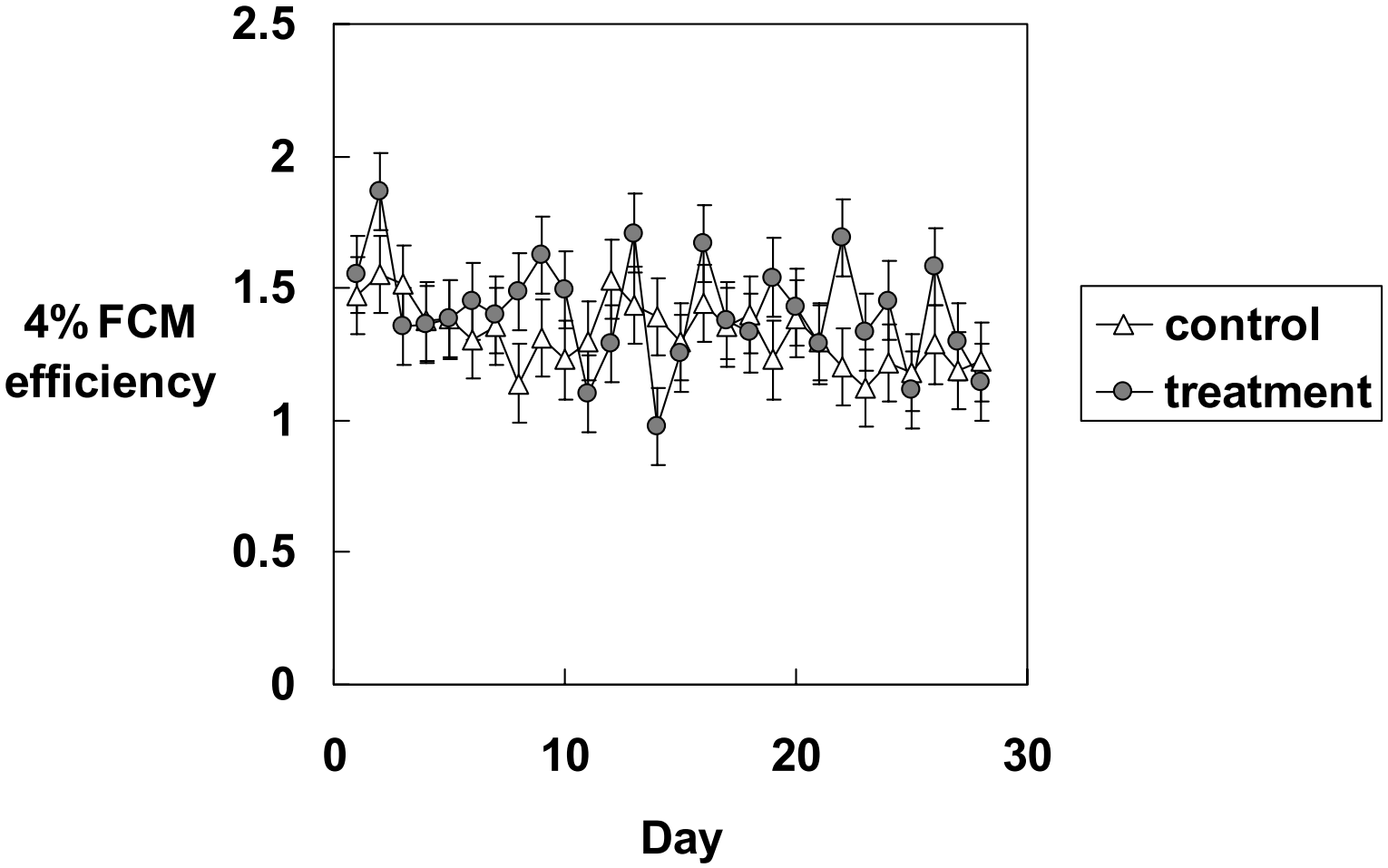


Figure 4-1. Least squares means for 4% FCM efficiency of cows fed diets with (●) or without (△) supplemental lysine and methionine.

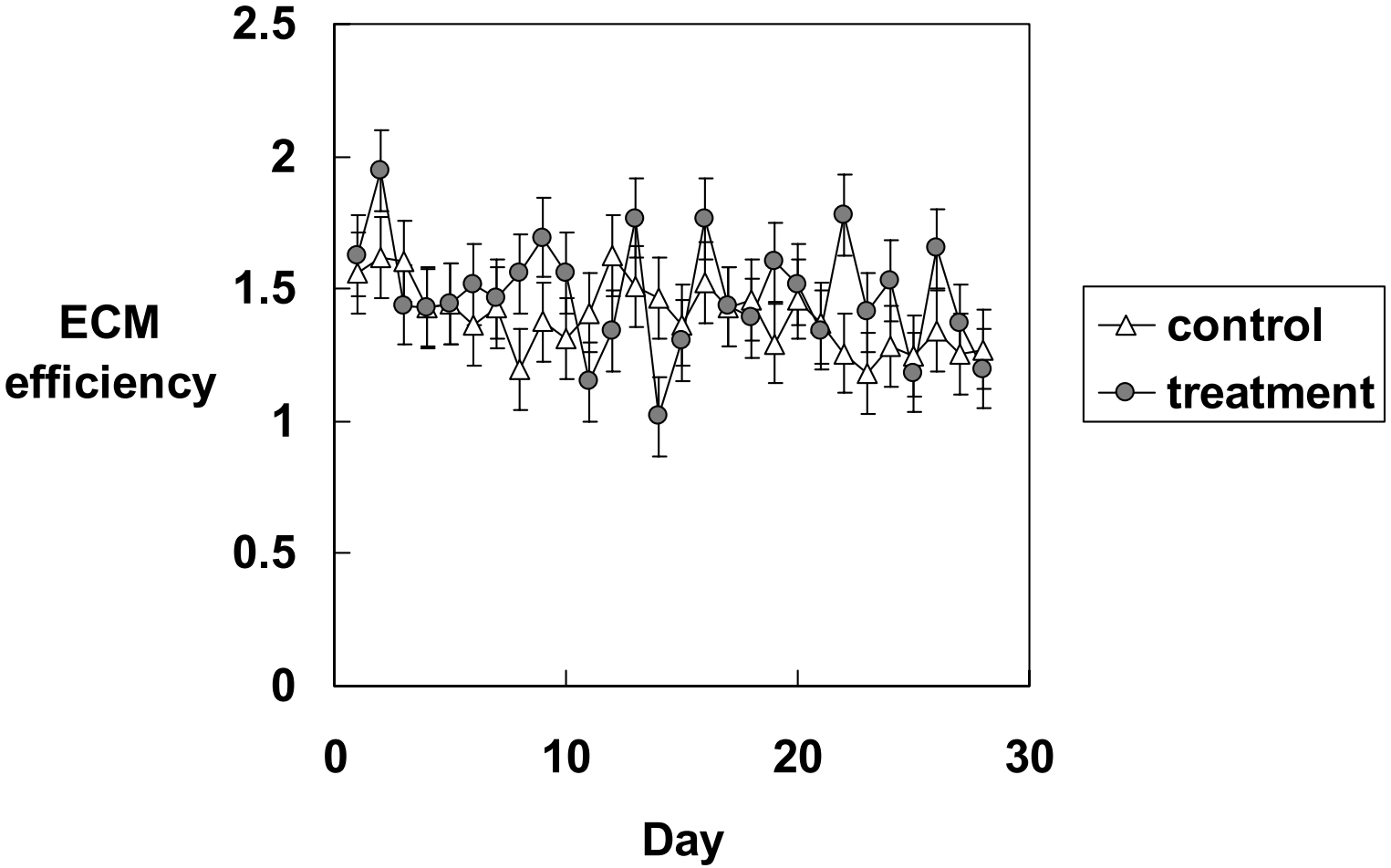


Figure 4-2. Least squares means for ECM efficiency of cows fed diets with (●) or without (△) supplemental lysine and methionine.

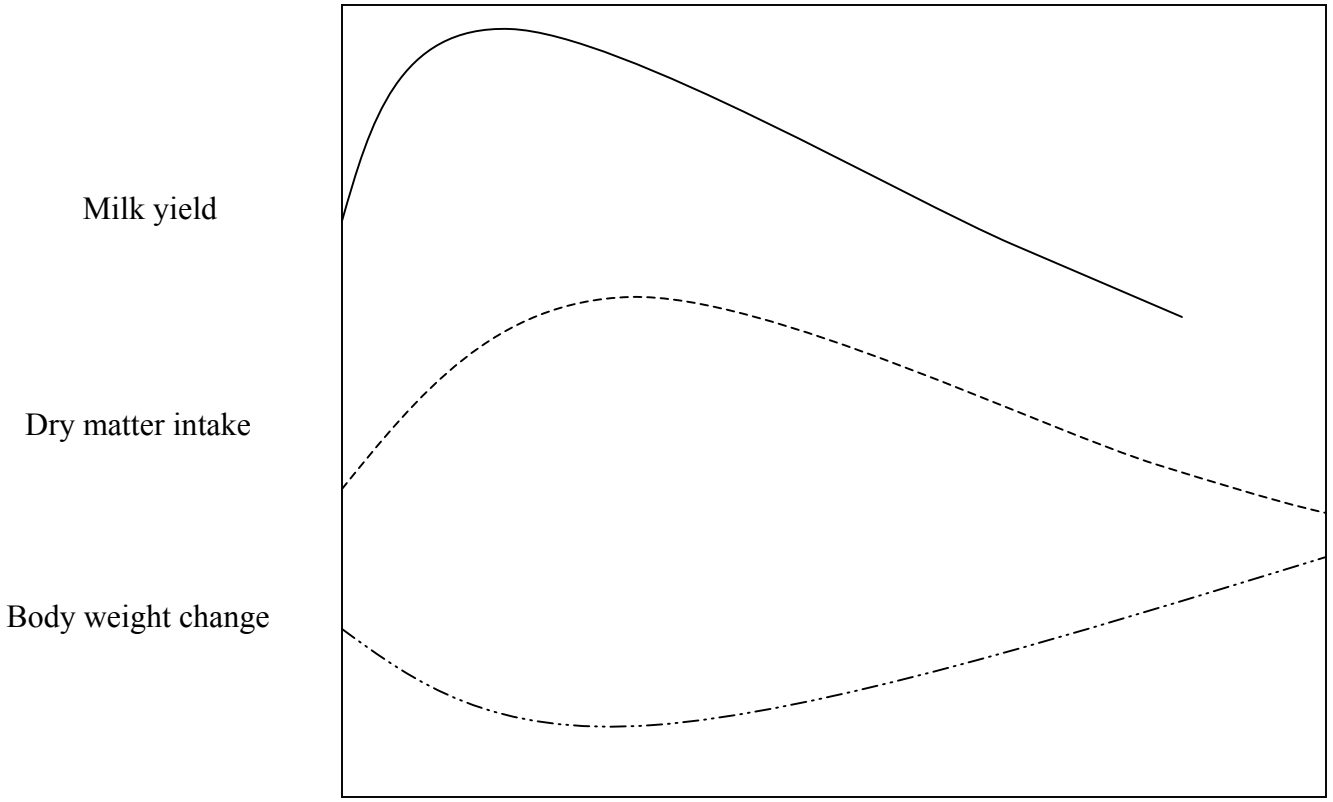


Figure 4-3. Representative curves of milk yield, DMI, and body weight change for cows at various feeding periods.

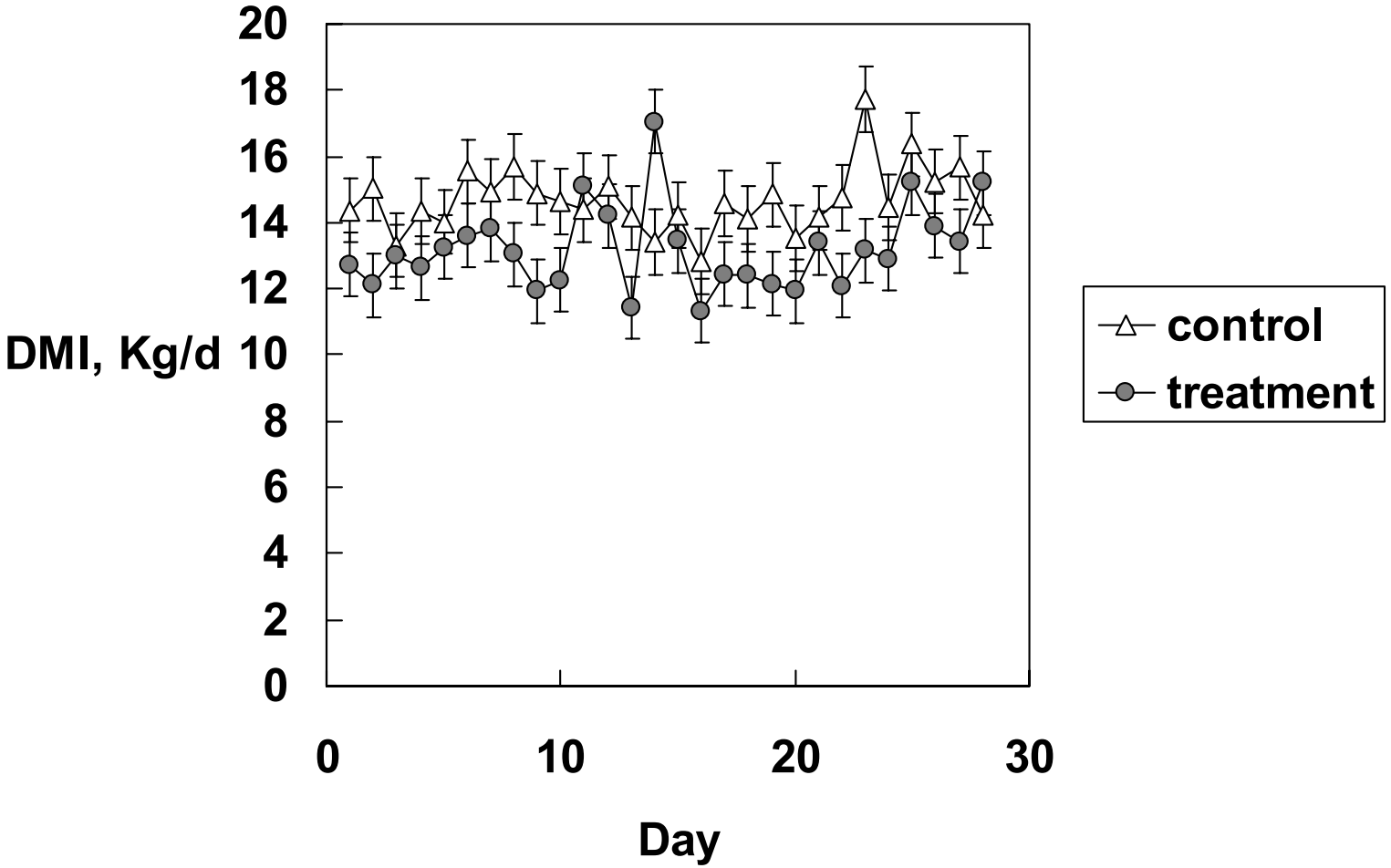


Figure 4-4. Least squares means for DMI of cows fed diets with (●) or without (△) supplemental lysine and methionine.

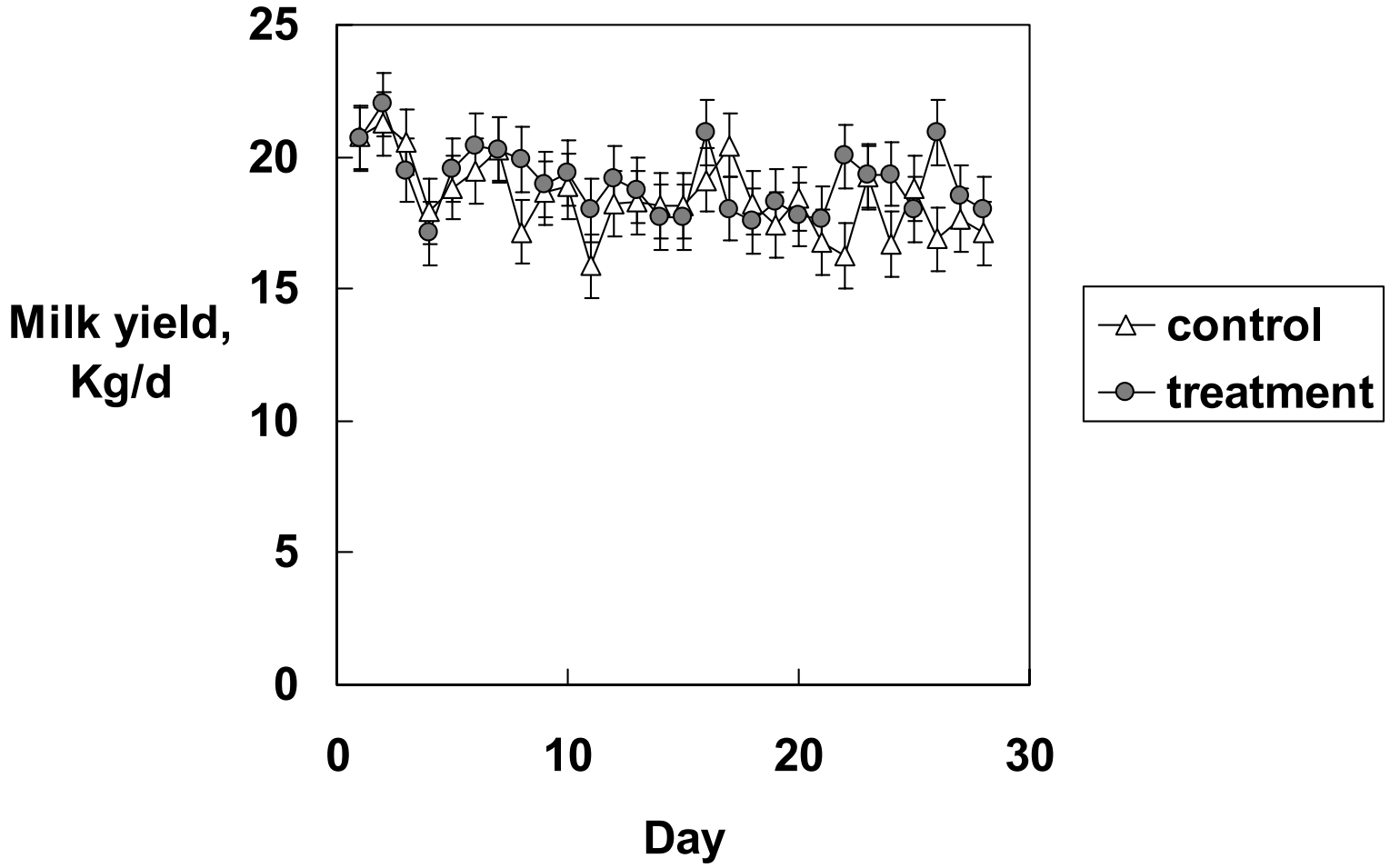


Figure 4-5. Least squares means for milk yield of cows fed diets with (●) or without (△) supplemental lysine and methionine.

productions and percentages of milk fat and protein for cows fed diets with or without AA supplementation. Although there were no differences in percentages of milk fat and milk protein between the control and treatment groups, the treatment group had numerically greater percentages of milk fat and milk protein. There was a tendency for increasing the percentage of milk fat for both the control and treatment groups (Figure 4-6) and this tendency agrees with the general curve of the change in milk fat percentage. The trend line of milk protein percent for the treatment group (Figure 4-7) showed a tendency for increasing the percentage of milk protein during the experimental period and resulted in a numerically higher milk protein percent than the control group. At about day 8 to day 11 during the experimental period, the milk protein % for the treatment group passed over the control group and kept increasing throughout the reminder of the experimental period. The SCCS for both groups was not affected by AA supplementation ($P > 0.1$), and indicated that all cows were experiencing a slight to moderate degree of mastitis during the experiment.

In this study, there were no AA treatment effects observed on lactational performance and body weight changes. Although the treatment group had numerically lower DMI, the yield of milk and production of milk fat and milk protein of the treatment group were numerically greater than the control group. This is in agreement with the study by Harrison et al. (2000) who reported numerically lower DMI but numerically greater yield of milk and production of milk fat and protein when cows were fed diets supplemented with Met and Lys. However, Carnerio et al. (1998c) reported that feed intake tended to increase when Met was provided either in drinking water or in the feed. Koudele et al. (1999) reported that the percentage of milk protein for cows receiving Met and Lys supplementation tended to be greater than cows receiving no AA supplementation, and numerically greater yield of milk and percentage of milk fat were also observed for cows supplemented with AA. Because there was no significant improvement

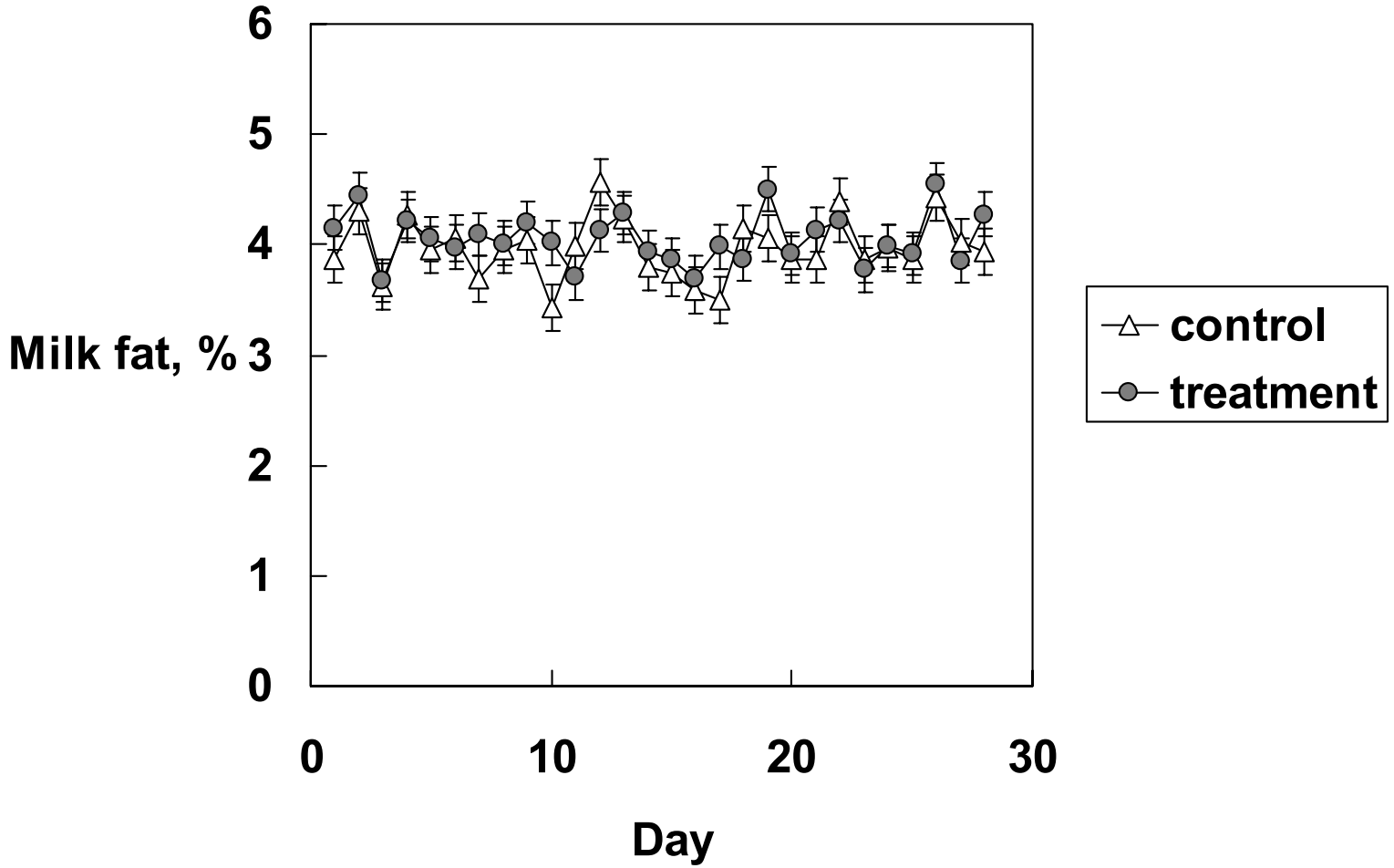


Figure 4-6. Least squares means for milk fat percentage of cows fed diets with (●) or without (△) supplemental lysine and methionine.

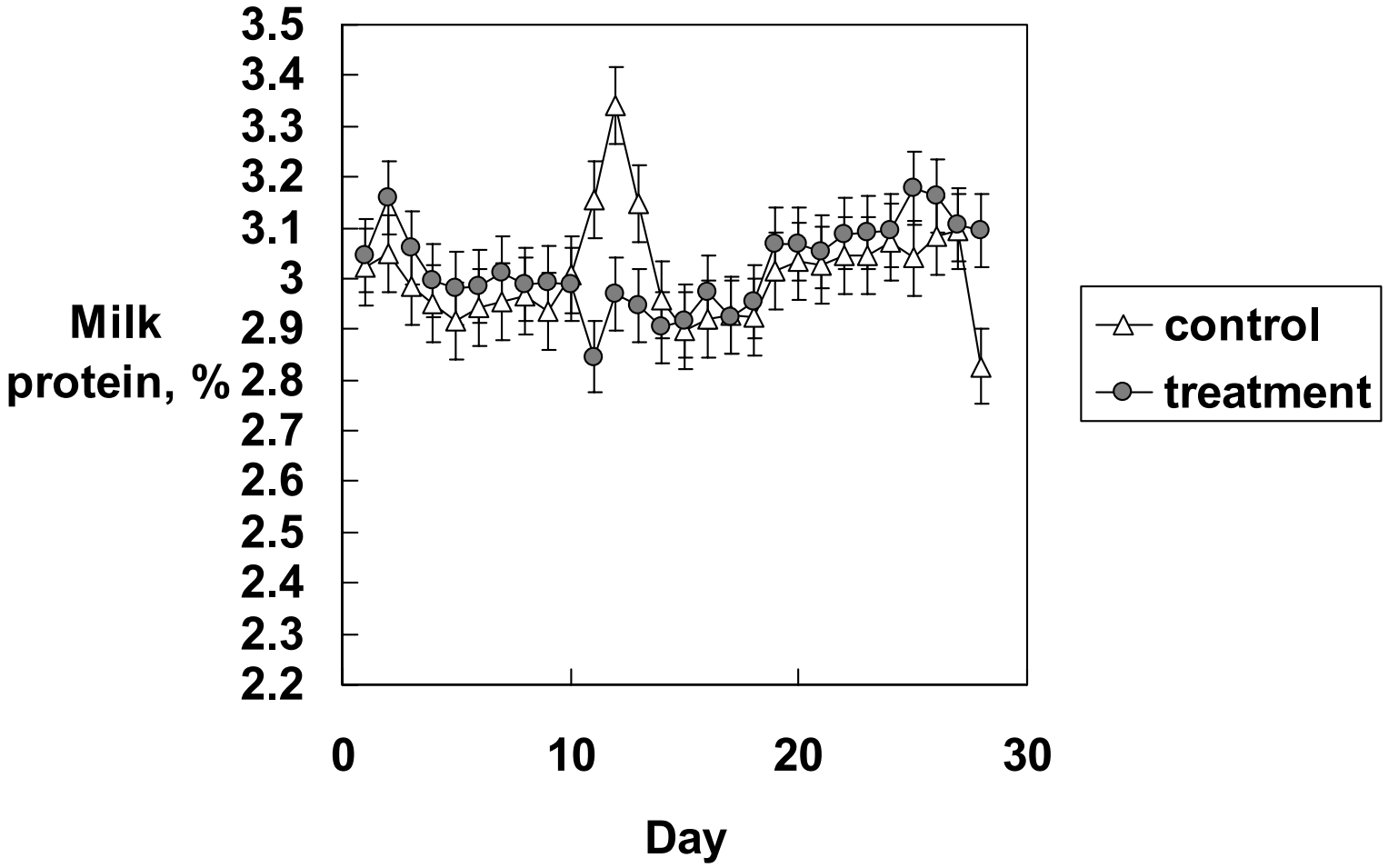


Figure 4-7. Least squares means for milk protein percentage of cows fed diets with (●) or without (△) supplemental lysine and methionine.

observed in lactational performances of cows supplemented with Met and Lys in this study, there appeared to be no Met or Lys deficiencies for milk production by cows in late lactation. This is in agreement with the study by Schwab et al. (1992). Although there were no significant improvements on production responses observed when supplementing Met and Lys to cows during late lactation in this study, changes in 4% FCM efficiency, ECM efficiency, and body weight gain in the AA treatment group after the 28 d study suggest that supplemental Met and Lys maybe improve the post-ruminal supply of metabolizable AA to cows in late lactation with positive energy balance. These cows apparently used this improvement for depositing body weight or fetal growth rather than production of milk and milk components.

### 4.3.3 Ruminal Fermentation Performance

Ruminal fluid from all experimental cows was collected via stomach tube at three times in the experimental period. The three times represented prior to (d –2), at the midpoint (d 15), and at the end (d 28) of the experiment. Least squares means for ruminal fermentation of cows fed diets with or without AA supplementation are the average values from the three sampling times and are presented in Table 4-5. Because of the contamination of ruminal fluid with salvia during the collection process, data of total VFA concentrations and ruminal pH may be erroneous.

There were significant differences in the concentration of $NH_4^+$ and the proportions of acetate, butyrate, and valerate in ruminal fluid. The concentration of $NH_4^+$ for the treatment group was greater ($P < 0.01$) than the control group as expected, and this is in agreement with studies from the previous in vitro experiment and by Cruz Soto et al. (1994) who reported increased $NH_4^+$ in ruminal fluid from sheep receiving AA infusion. Demeyer and Van Nevel (1986) speculated that only 0.2 mg/dl of ruminal ammonia was needed by ruminal microorganisms to allow microbial growth. The lowest concentration of $NH_4^+$ in this study was

Table 4-5. Least squares means for ruminal performances of cows fed diets with or without supplemental Met and Lys.

| | Experimental diets | | | |
|---|---|---|---|---|
| Parameter | Control | Treatment | SEM | *P*-value |
| pH | 6.32 | 6.18 | 0.07 | 0.1659 |
| $NH_4^+$, mg/dl | 0.95 | 1.31 | 0.06 | 0.0008 |
| | | | | |
| Total VFA, m*M* | 83.46 | 89.63 | 3.16 | 0.1968 |
| Acetate, % | 66.95 | 64.54 | 0.60 | 0.0130 |
| Propionate, % | 18.35 | 18.70 | 0.44 | 0.5760 |
| Isobutyrate, % | 1.22 | 1.30 | 0.07 | 0.4563 |
| Butyrate, % | 9.72 | 11.64 | 0.22 | < 0.0001 |
| Isovalerate, % | 2.21 | 2.47 | 0.10 | 0.0776 |
| Valerate, % | 1.54 | 1.35 | 0.06 | 0.0263 |
| Ratio[1] | 3.67 | 3.48 | 0.10 | 0.2199 |

[1] Ratio of acetate to propionate in ruminal fluid.

0.95 mg/dl from the control group suggesting that ruminal fermentation for both of the control and treatment groups should not have been limited by the $NH_4^+$ concentration in the rumen.

The proportion of butyrate was increased ($P < 0.01$), but the proportions of acetate ($P < 0.05$) and valerate ($P < 0.05$) were decreased while the pH, total VFA production, proportions of propionate, isobutyrate, and isovalerate, and the ratio of acetate to propionate were not affected by supplementing Met and Lys. Although there was no difference in the mean concentration of total VFA, the treatment group had numerically greater total VFA production than the control group, but this may have been confounded by salvia contamination.

Increased concentrations of ruminal $NH_4^+$, lower proportions of acetate and valerate, higher proportions of butyrate, numerically greater production of total VFA, and the numerically lower ratio of acetate to propionate for cows fed Met and Lys are in accordance with results obtained from the previous in vitro study (Table 3-6). These results also agree with the study by Cruz Soto et al. (1994) who reported greater production of total VFA, greater proportions of BCFA, and numerically lower proportion of acetate and greater proportion of propionate when sheep received a ruminal infusion of AA mixture. Even with possible contamination of saliva during the collecting process, results of the ruminal performances from this in vivo study confirmed results obtained from the previous in vitro study.

**4.4 Summary**

Supplementing Met and Lys to Holstein cows in late lactation had no impact on mean DMI, OMI, milk yield, production and percentages of milk components, SCCS, 4% FCM and ECM efficiencies, and body weight gain. Although DMI for the treatment group was numerically lower than the control group (13.18 kg/d vs. 14.66 kg/d, respectively), milk yield (19.05 kg/d vs.18.41 kg/d), % milk fat (4.05% vs.3.96%), and % milk protein (3.02% vs. 3.01%) for the treatment group were numerically greater than the control group and resulted in numerically greater 4%

FCM and ECM efficiencies. There was no AA effect observed on the mean body weight gain for both the control and treatment groups, but the mean body weight gain for the treatment group was numerically greater than the control group (21.42 kg vs. 9.20 kg, respectively). Ruminal concentration of $NH_4^+$, proportions of acetate, butyrate, and valerate were affected by supplementing Met and Lys. The concentration of ruminal $NH_4^+$ was increased by AA supplementation. The proportion of butyrate was increased, but proportions of acetate and valerate were decreased while the pH, total VFA, proportions of propionate, isobutyrate and isovalerate, and the ratio of acetate to propionate were not affected by supplementing Met and Lys.

## 4.5 Conclusions

Results of this in vivo study confirmed the results obtained from the previous in vitro study and indicate that positive responses of Met and Lys in vivo should be expected and profitable if correctly timed and supplemented at the proper concentrations. Data obtained from this study suggest that supplementing free Met and Lys to Holstein cows in late lactation altered ruminal fermentation, but these impacts may have favored energy partitioning to body tissue rather than eliciting significant responses of milk production and milk components. Possible reasons included: 1) cows were in late lactation, 2) a short experimental period was utilized, 3) a change in forage base from alfalfa hay to corn silage, and 4) a less than optimal combination of Met and Lys was used in this study. Based on numerically greater milk efficiency and body weight gain observed in the treatment group when cows were in late lactation with positive energy balance, improvements in lactation responses should be expected when the ideal concentrations of Met and Lys are supplemented to cows in peak lactation.

# CHAPTER 5. SUMMARY AND CONCLUSIONS

## 5.1 Summary

In the in vitro study, time of sampling had no effect on mean concentrations of total VFA and $NH_4^+$ and proportions of individual VFA. Supplementation of Met and Lys had impacts on mean concentrations of total VFA and $NH_4^+$, proportions of acetate, propionate, butyrate, isovalerate, valerate, and the ratio of acetate to propionate. Percentages of microbial N in effluent pellets were not affected by supplementing Lys and Met. The hypothesized ideal concentrations of 1.03% Lys and 0.26% Met did not result in the greatest concentration of total VFA. The combination of 0.52% Met and 1.03% Lys resulted in the highest mean concentration of total VFA, second highest mean concentration of $NH_4^+$, and the lowest ratio of acetate to propionate among treatments with no decrease in bacterial CP production.

In the in vivo study, supplementing Met and Lys to Holstein cows in late lactation had no impact on mean DMI, OMI, milk yield, productions and percentages of milk components, SCCS, 4% FCM and ECM efficiencies, and body weight gains. Although DMI for the treatment group was numerically lower than the control group (13.18 kg/d vs. 14.66 kg/d, respectively), milk yield (19.05 kg/d vs.18.41 kg/d), % milk fat (4.05% vs.3.96%), and % milk protein (3.02% vs. 3.01%) for the treatment group were numerically greater than the control group and resulted in numerically greater 4% FCM and ECM efficiencies. There was no AA effect observed on the mean body weight gain for both the control and treatment groups, but the mean body weight gain for the treatment group was numerically greater than the control group (21.42 kg vs. 9.20 kg, respectively). Ruminal concentration of $NH_4^+$, and proportions of acetate, butyrate, and valerate were affected by supplementing Met and Lys. The concentration of ruminal $NH_4^+$ was increased by AA supplementation. The proportion of butyrate was increased, but proportions of acetate and valerate were decreased while the pH, total VFA, proportions of propionate, isobutyrate and

isovalerate, and the ratio of acetate to propionate were not affected by supplementing Met and Lys.

## 5.2 Conclusions

In conclusion, supplementing 0.52% Met and 1.03% Lys to an alfalfa hay-concentrate based diet (1:1 ratio) may supply the ruminal microorganisms an optimal level of Met and Lys to improve ruminal fermentation in the continuous culture system.

Results of the in vivo study confirmed the results obtained from the in vitro study and indicate that positive responses of Met and Lys in vivo should be expected and profitable if correctly timed and supplemented at the proper concentrations. Data from the in vivo study suggest that supplementing free Met and Lys to Holstein cows in late lactation altered ruminal fermentation, but these impacts may have favored energy partitioning to body tissue rather than eliciting significant responses of milk production and milk components. Possible reasons included: 1) cows were in late lactation, 2) a short experimental period was utilized, 3) a change in forage base from alfalfa hay to corn silage, and 4) a less than optimal combination of Met and Lys was used in this study. Based on numerically greater milk efficiency and body weight gain observed in the treatment group when cows were in late lactation with positive energy balance, improvements in lactation responses should be expected when the ideal concentrations of Met and Lys are supplemented to cows in peak lactation.

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## APPENDIX A. ANALYSIS OF VOLATILE FATTY ACIDS IN RUMINAL FLUID

Based on preparation produceres described in Grigsby et al., 1992. J. Anim. Sci. 70:1941-1949, and temperature gradient program described in Bateman et al., 2002. Prof. Anim. Sci. 18:363-367.

**Reagents**

1) 25% (wt/vol) metaphosphoric acid (fluka #79615) acid solution containing 2 g/l of 2-ethyl butyric acid (216.5 µl 2-EB to 100 ml m-phos acid solution; Aldrich #10,995-9).
2) VFA standard
   a) Add the following volumes of acids to a 100-ml volumetric flask and fill to volume with $dH_2O$. Store in refrigerator when not in use.

| MW | Acid | Volume (µl) | Conc (g/l) | Conc (m*M*) |
|---|---|---|---|---|
| 60.06 | Acetic | 330 | 3.46 | 57.62 |
| 74.08 | Propionic | 400 | 3.97 | 53.59 |
| 88.10 | Isobutyric | 30 | 0.29 | 3.29 |
| 88.10 | Butyric | 160 | 1.53 | 17.37 |
| 102.13 | Isovaleric | 40 | 0.375 | 3.67 |
| 102.13 | n-Valeric | 50 | 0.471 | 4.61 |

**Sample and Standard Preparation**

1) Centrifuge strained ruminal fluid at 30,000 x g for 20 min (this step may be skipped).
2) Mix 4 ml of rumen fluid supernatant with 1 ml of m-phosphoric acid solution containing 2 - EB.
3) Allow to stand in ice bath for 30 min (this step may be skipped)
4) Centrifuge at 30,000 x g for 20 min.
5) Remove supernatant for GC analysis
6) To insure that standard is prepared in the same manner as the samples, treat the mixed sample from step A-2 above as a sample.

Remember to correct for the dilution factor from the m-phos solution when calculating the final VFA concentrations (4 ml fluid mixed wth 1 ml acid provides a correction factor of 1.25).

For use on Shimadzu GC, samples should be in 2 ml autosampler vials. The optimal vials that we have used are ordered from ColeParmer. They are Target autosampler vials (#A98810-00). These are a screw cap vial so you also need caps and the septa color is important. The autosampler recognizes white as the color of the septa. The caps for these vials are #A98801-23

**Temperature Gradient Program**

1) The column temperature at the beginning of the program is 115 $^{o}$C and is held there for 0.1 min.
2) It is then increased at a rate of 10 $^{o}$C / min to 150 $^{o}$C and held there for 0.1 min.

3) It is then further increased at a rate of 11 $^{o}$C / min to 170 $^{o}$C and held there for 1 min.
4) The injector of the chromatograph is held at 250 $^{o}$C and the detector is held at 275 $^{o}$C.
5) Peak detection is by a flame ionization detector that uses a $H_2$/ air flame.
6) Helium is used as the carrier gas with a split less injection at a flow rate of 60 ml/min.

## APPENDIX B. PHENOL-HYPOCHLORITE ASSAY FOR AMMONIA

Adapted from Broderick and Kang. J. Dairy Sci. (1980) 63:64.

**CAUTION**: wear gloves and protective clothing when mixing these reagents or running this assay. Phenol is a cancer-causing agent and will burn the skin. WEAR GLOVES. This procedure allows for the use of repipets or pipetors. After reading, all waste material should be treated as hazardous waste and contained in bottles. All tubes and or cuvettes must be rinsed before discarding.

**Phenol reagent**

Dissolve 0.15 g of sodium nitroferricyanide (sodium nitropursside) in 1.5 l of distilled $H_2O$ ($dH_2O$. Add 33 ml (90% w/v) phenol (measured in graduated cylinder) and mix throughly. Bring solution to final volume of 3 l by addition of $dH_2O$ and store in brown glass bottle.
Phenol needed is 29.7 g. Use goggles when measuring phenol and be careful. Phenol can cause burns when it comes into contact with skin.

**Hypochlorite reagent**

Dissolve 15 g of sodium hydorxide in approximately 2 l of d $H_2O$. Add 113.6 g of disodium phosphate heptahydrate ($Na_2HPO_4 \bullet 7H_2O$) to this solution using mild heating and mixing. After the disodium phosphate has mixed allow the solution to cool. After cooling, add 150 ml of commercial bleach (5.25% sodium hypochlorite, you only need 131.25 ml if using 6% bleach) and mix throughly. Bring solution to 3 l by adding $dH_2O$. Filter solution through #1 filter paper and store in polyethylene bottle protected from light.

**Ammonia standard solution**

A stock solution of 100 m*M* (170 mg / dl) ammonia can be prepared by dilution 0.6607 g of ammonium sulfate (dry overnight before use) to 100 ml with 0.1N HCl.

Working standards can then be made from the stock solution. Dilute 1 ml of stock solution per m*M* concentration desired in working standard to 100 ml total using $dH_2O$.

**Procedure**

1) Samples of ruminal fluid will need to be diluted with dH2O prior to analysis to bring the concentration of NH3 into the working range of this assay. Therefore, mix 0.5 ml of clarified ruminal fluid with 4.5 ml of dH2O and use these "samples" for the reaction.
2) Add 0.05 ml (50 μl) of sample or standard into test tube (use $dH_2O$ for blanks).
3) Mix sample with 2.5 ml phenol reagent. Add phenol to all tubes then mix on vortex.
4) Add 2.0 ml hypochlorite reagent to all tubes and mix on vortex. Place in 95°C water bath for 5 min. Place marbles on top of each tube before inserting in water bath to prevent condensation from falling into the tubes.
5) After cooling, samples can be read on a spectrophotometer at 630 nm wave -length.
6) Dispose of all waste material in accordance with the hazardous waste regulations of your institution. **This means that the PHENOL can not be discarded in the municipal sewer without proper authorization.**

## APPENDIX C. PURINE ASSAY

Adapted from Zinn and Owens, Can. Anim. Sci. 66: 157-166 (Mar. 1986).

**Reagents:** - amount per sample
1) $HClO_4$ (70%) -2.5ml
2) 0.2 M $NH_4H_2PO_4$ (23g/L) -9ml
3) 0.0285 M $NH_4H_2PO_4$ (143ml of 0.2 M $NH_4H_2PO_4$/L) –17.5ml
4) 0.5 N HCl (41.85ml reagent grade HCl/L) –10ml
5) pH 2 water (add $H_2SO_4$ to $dH_2O$)
6) 0.4 M AgNO3 (6.9g /100ml) -0.5ml

**Procedures:**
1) Weigh 0.5g dried, ground sample into 25-ml screw-cap culture tube. (Hydrolysis may be incomplete if sample is wet.)
2) Add 2.5 ml $HClO_4$ (70%), tightly cap tube and incubate in 90-95$^{o}$C water bath for 1 hour (sample will appear charred). Break pellet for more complete extraction prior to step 3.
3) Add 17.5ml buffer (0.0285 M $NH_4H_2PO_4$). Mix. Re-insert tubes into 90-95$^{o}$C water bath for 10-15 minutes. Filter through Whatman no. 4 filter paper. Filtrate should have pH near 2 if the buffer has enough buffering capacity for the sample.
4) Transfer 0.5ml filtrate to 15-ml centrifuge tube, add 0.5ml $AgNO_3$ (0.4M), 9ml buffer (0.2 M $NH_4H_2PO_4$), and allow to stand in the dark a minimum of 30 minutes. Precision may be increased by allowing samples to stand overnight at 5 $^{o}$C.
5) Centrifuge and decant supernatant liquid (being careful to not disturb the pellet).
6) Wash pellet with pH 2 $dH_2O$ (adjusted to pH 2 with $H_2SO_4$).
7) Repeat step 5.
8) Add 10ml of 0.5 N HCl; vortex until thoroughly mixed.
9) Cover tube with marble and incubate in 90-95$^{o}$C water bath for 30 minutes.
10) Read absorbance of supernatant fluid at 260nm.

**Standards:**
Yeast RNA (0.5g) carried through total procedure diluted 1:20 after step 7.

## VITA

Yi-Hua Chung, also known as Ruby Yi-Hua Chung, was born on August 28, 1977, in Yang-Mei City, Tao-Yuan County, Taiwan. After graduating from Pan-Chiao Senior High School in spring, 1996, Ruby began her undergraduate studies at Chinese Culture University, Taipei, Taiwan, in fall at the same year and chose animal science as her major. After four years' studying and living on the beautiful mountain of Yang-Ming Shan, Ruby received her degree of Bachelor of Science and started to plan her journey of studying abroad. On the first of August, 2000, Ruby arrived in Baton Rouge with only two pieces of luggage and began a whole new chapter in her life. Ruby entered the master's program in the Department of Dairy Science of Louisiana State and A&M University. During her graduate studies at LSU, she joined the dairy nutrition research group as a graduate research assistant under the guidance of Dr. H. Gale Bateman, II. In August of 2003, Ruby will receive her degree of Master of Science and right after finishing her studies at LSU, Ruby will be moving up to Pennsylvania to join Dr. G. A. Varga's research group and starting her doctoral program at the Department of Dairy and Animal Science of the Pennsylvania State University.