AMINO ACID SUPPLY IN LACTATING DAIRY CATTLE

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Abstract

Amino acid flow from the rumen was quantified and key aspects of rumen fermentation were evaluated in lactating dairy cattle. Rumen bacteria and protozoa were analyzed for amino acid (**AA**) composition and digestibility, and values were incorporated into the Cornell Net Carbohydrate and Protein System (**CNCPS**). Two in vivo studies were performed to evaluate the effects of a commercial fermentation byproduct on nutrient digestion, microbial growth, omasal flow of nutrients, and milk production in lactating dairy cattle. The objectives of the first portion of the first study (Chapter 2) was to evaluate nutrient digestion in the rumen and total tract, along with omasal flows of N. Inclusion of fermentation byproduct decreased dietary N degradation in the rumen, with no detrimental effects on microbial N flows. The second part of the experiment (Chapter 3) involved measurment of microbial growth and turnover, partitioning of protozoa and bacterial N, and determination of omasal AA flow. Cows fed the fermentation byproduct had increased flows of non-microbial AA at the omasal canal. Comparison of the observed results against model predicted values indicated that the rumen sub-model of CNCPS v.7 was able to provide accurate estimations of total microbial N. The second trial (Chapter 4) was performed to evaluate the effects of the fermentation byproduct on milk production in groups of lactating cows. Cows fed fermentation byproduct had increased energy-corrected milk yield and protein yield compared with cows fed regular and rumen protected soybean meal. In Chapter 5, two methods not previously applied to microbial samples were used to characterize the AA profile and digestibility of bacterial and protozoal protein. The values obtained, along with other updates to protozoal growth and turnover parameters from Chapter 3 were

incorporated into the CNCPS. A comparison of model predicted vs. observed value from a larger literature dataset was used to evaluate improvements in the model's predictive capability. Overall, the use of omasal sampling, improved isolation of protozoa, and more accurate determination of post ruminal flows of digestible AA allowed for a more complete understanding of microbial protein synthesis and more accurate model predictions of post ruminal AA supply.

BIOGRAPHICAL SKETCH

Samuel Fessenden grew up on a family dairy farm in King Ferry, NY. His early years were spent developing a passion for the care of animals and learning about agriculture through the examples and lessons of his parents and extended family. He attended Southern Cayuga Central School from 1995-2007. Also living in King Ferry at the time were Dr. Michael Van Amburgh and Dr. Danny Fox, who encouraged Sam to study Animal Science at Cornell University. Sam enrolled in the fall of 2007, and quickly became involved in research in Mike's lab. Most of his knowledge of chemistry was learned through lab work and interactions with Dr. Debbie Ross and Dr. Pete Van Soest. While at Cornell, his interest in applied dairy nutrition was greatly influenced by experiences in Dairy Fellows, the Dairy Science Club, and Alpha Gamma Rho Fraternity. After graduation with a B.S. with Distinction in Research, Sam was accepted into a M.S. degree program at the University of Minnesota in the laboratory of Dr. Marshall Stern. Marshall and Sam shared an interest in fermentation of all types; Marshall's lab often had several active experiments utilizing yeast to ferment sugars for production of (mostly) human edible products. While in Minnesota, Sam met his significant other, Brenda. Sam's M.S. thesis was titled: Effects of bismuth subsalicylate and beta extract of hops (*Humulus lupulus*) on in vitro fermentation with ruminal microbes. His work with continuous culture fermenters helped him gain a better understanding of the complexities of the rumen, and fostered a deeper interest in quantitative animal nutrition. In 2013 he moved back to Ithaca, NY to pursue his doctorate, with special interest in the interaction between rumen microbiology and applied dairy nutrition, and the continued development of the Cornell Net Carbohydrate and Protein System.

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Dr. Debbie Ross taught me almost everything I know about laboratory work. Her perseverance and patience is much appreciated, especially during the great race to the finish. In so many ways, this dissertation is a reflection of her hard work and seemingly endless patience.

Members of my committee have also dedicated much time and expertise aiding in my training. Thank you to Dr. Thomas Overton for always challenging me to think outside my specific area of interest; Dr. Daryl Nydam for re-shaping the way I think about application of scientific research to dairy farms; Dr. Timothy Hackmann for helping me think about microbiology in an applied manner; and Dr. Elliot Block for providing a unique blend of industry and academic perspective to our work. I am also very grateful for the financial support of my coursework and research provided by Arm and Hammer Animal Nutrition.

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CHAPTER 1: Literature Review

INTRODUCTION AND OVERVIEW

In ruminants, symbiosis between microorganisms and host is a complex and unique relationship. The pre-gastric location and unique physiologic aspects of the rumen allow the animal to utilize substrate not readily digested by non-ruminants. Due to limited oxygen and readily available nitrogen sources, strictly anaerobic bacteria capable of digesting complex carbohydrates (such as cellulose) are able to flourish in the rumen. These microorganisms also provide high quality microbial protein to the small intestine, allowing the animal to survive and even reproduce in poor nutritional situations relative to non-ruminant animals. However, microbial competition and recycling of nutrients in rumen fermentation can result in energetic inefficiencies and loss of otherwise usable and valuable nutrients to the environment. The high cost of protein, coupled with environmental concerns regarding excess nutrient excretion has lead researchers to balance diets closer to the requirements of the animal. Mathematical models are useful tools to aid in ration balancing, as requirements and supply can be calculated and optimized. Development of models with mechanistic components have allowed for a more complete understanding of the ruminant animal, and have helped guide research to quantitative, applicable outcomes. When modeling the ruminant animal, a mechanistic understanding of the symbiosis between microorganism and host is needed to provide robust predictions of performance across a variety of production situations. This review will first serve to give an overview of microbial ecology, with particular emphasis on microbial catabolic and anabolic processes. The second section will focus on quantitative aspects of rumen microbial metabolism, and their application in model development.

PART 1: RUMEN MICROBIAL ECOLOGY

Characterization of Rumen Microbial Populations

The rumen hosts a large and diverse population of microorganisms. Initial efforts to describe microbial populations in the rumen were based on morphologic traits, substrate utilization, end-product accumulation and simple biochemical tests (Hungate, 1966). The efforts put forth by early researchers, while extremely important, lacked any true genetic component and thus provided limited insight into true diversity of rumen microbial ecology. The information available on individual rumen bacterial species is largely limited to strains that have been successfully cultivated in the laboratory, which can lead to severe underestimation of the true diversity (Amann et al., 1995; Shin et al., 2004). In 1996, only 22 predominant species of rumen bacteria were classified, at a time when *Bergey's Manual of Systematic Bacteriology* recognized approximately 5,000 bacterial species in total (Krause and Russell, 1996). Later, Mackie et al. (2002) indicated that about 200 species have been successfully cultured. Now, culture independent techniques have more recently provided researchers with tools to accurately describe the diversity of the rumen community (Firkins and Yu, 2006; Deng et al., 2008). Current estimates put total microbial operational taxonomic units to over 3,000 in the rumen alone (Denman and McSweeney, 2015). While these genome sequencing techniques does not identify species, these methods have shown a light on the true microbial diversity of the rumen. The relative cost and ease of next-generation sequencing has resulted in a tremendous number of papers describing the microbial diversity in various environments. However, the degree to which these approaches improve animal productivity will depend on the ability of researchers to use the tools to gain insight into the functional aspects of the ecosystem; not simply describe the diversity and subsequent association with disease states or poor productivity. A more

mechanistic understanding of the metabolic aspects of the major microbial groups in the ecosystem is needed to make appreciable gains in animal health and productivity.

An excellent example of the difference between qualitative and quantitative analysis is in the use of small subunit ribosomal RNA gene sequencing. While DNA based methods have provided vastly more knowledge about the diversity of many natural environments (Tringe et al., 2005), metagenomic approaches alone do not provide sufficient insight into the functionality of the microorganisms present in the community. The relative ease of DNA extraction and use of high-throughput sequencing has allowed the generation of an enormous number of papers, however many of them lack true functional connections. Metagenomic approaches have proved to be useful in the discovery of novel gene coding regions for lignocellulose degradation in the rumen (Hess et al., 2011). More functional analysis includes the other 'omics' such as transcriptomic, proteomic, and metabolomic approaches. Advancements in extraction and analysis of environmental samples will likely lead to more metatranscripomics and metaproteonomic based work (Zhou and Thompson, 2002). Proteomic work has been extremely successful in antibacterial drug discovery (Brötz-Oesterhelt et al., 2005), and techniques using LC-MS have demonstrated functional differences at a metabolic level in humans.

While various omic based work might drive future understanding of microbial metabolism, the underpinning of our current knowledge of metabolism is still firmly rooted in the study of cultivatable organisms. Much of this work in the ~20 species that have been extensively studied is limited further to relatively few strains, often in pure or co-culture (Russell and Dombrowski, 1980; Russell et al., 1988a; Van Kessel and Russell, 1996). Most mechanistic models of rumen fermentation, including the Cornell Net Carbohydrate and Protein System (**CNCPS**), rely on metabolic pathway data in pure and co-culture of select strains. In vitro fermentation with mixed

rumen microbes has been used to determine kinetics of digestion, particularly for fiber (Van Soest, 2015), and more recently has been adapted to evaluate antimicrobial activity of compounds (Castillejos et al., 2005; Foskolos et al., 2015), particularly toward methanogens (Bodas et al., 2012) . While this technique allows for a large number of compounds to be identified, information regarding the specific mode of action is rarely available or investigated (Burt, 2004), thereby limiting the utility of the data for modeling exercises. However, the studies that provide insight into important relationships between microbial communities and substrate use have proved to be exceedingly important in model development (Argyle and Baldwin, 1989, Mourino et al., 2001; Denton et al., 2015; Diaz et al., 2014). The development of a dynamic version of the CNCPS rumen sub-model (Higgs, 2014) has taken into consideration studies that apply a quantitative approach to describe protozoal and bacterial interactions, especially regarding microbial protein degradation and synthesis. In such a system, elements such as predation and competition for substrate (Denton et al., 2015; Diaz et al., 2014) can be modeled using an intuitive approach. Still, parameterization of such models is often difficult (Baldwin et al., 1987; Dijkstra et al., 2002) and simplification of microbial descriptions is needed until sufficient data exist to allow for more complexity that leads to improved predictions. The CNCPS v.7 incorporates bacteria and protozoa in a mechanistic manner, with sub-populations based on substrate utilization, such as structural and non-structural carbohydrate-degrading bacteria.

From a quantitative perspective, descriptions of bacteria typically found in ruminant studies are often based on crude isolation technique, as is the case with liquid associated bacteria (**LAB**) and solid associated bacteria (**SAB**) fractions. Perhaps incorrectly, LAB are often assumed to be non-structural carbohydrate digesters, while SAB are typically considered to be fibrolytic

organisms, although this separation is not definitive. Detachment procedures used to obtain SAB typically only have 50-60% efficiency (Whitehouse et al., 1994) and few researchers have sought to ensure that the genetic diversity or composition of the detached isolates is comparable to the population of interest (Martínez et al., 2009). Alternatively, it is also likely that the LAB portion also contains a significant number of fibrolytic organisms that are simply not attached to substrate at the time of separation (Russell, 2002). From a nutritional point of view, general categorization of the various microbial species is based on type of substrate utilized and/or end-products generated. Non-structural and structural carbohydrate fermenters, proteolytic and methanogenic classifications are most commonly found in the literature, although many microorganisms fall into several categories if classified on this artificial division.

Protozoa are the most conspicuous of the rumen microbes. Their role in the rumen is complex and not fully understood (Williams and Coleman, 1997). When animals are kept in isolation from birth, colonization of protozoa can be prevented, thus demonstrating that protozoa are not essential to rumen function (Bird and Leng, 1984). The beneficial or detrimental role of protozoa also seems to be dependent on diet. Detrimental effects might be realized in low rumen N situations where sequestration of engulfed bacteria can limit microbial output (Newbold and Hillman, 1990). Some methanogenic archaea associate closely with ciliated protozoa (Sharp et al., 1998) and high populations of methanogens can contribute to energy loss through methane production. Beneficial aspects of rumen protozoa could be through modulation of rumen pH by sequestration of engulfed sugar and starch granules. Coleman (1992) demonstrated a tremendous ability of *Entodinium* spp. to take up starch, and others have seen beneficial modulating effects on fermentation pH when ciliated protozoa were present in cattle fed feedlot diets (Towne et al., 1990, Nagaraja et al., 1992). This effect on pH is largely thought to aid in digestion of fiber in

the rumen (Williams and Coleman, 1997). Much of the starch is used for reserve carbohydrate synthesis, a process which can actually be seen as an increase in opacity under a light microscope (Figure 1.1).

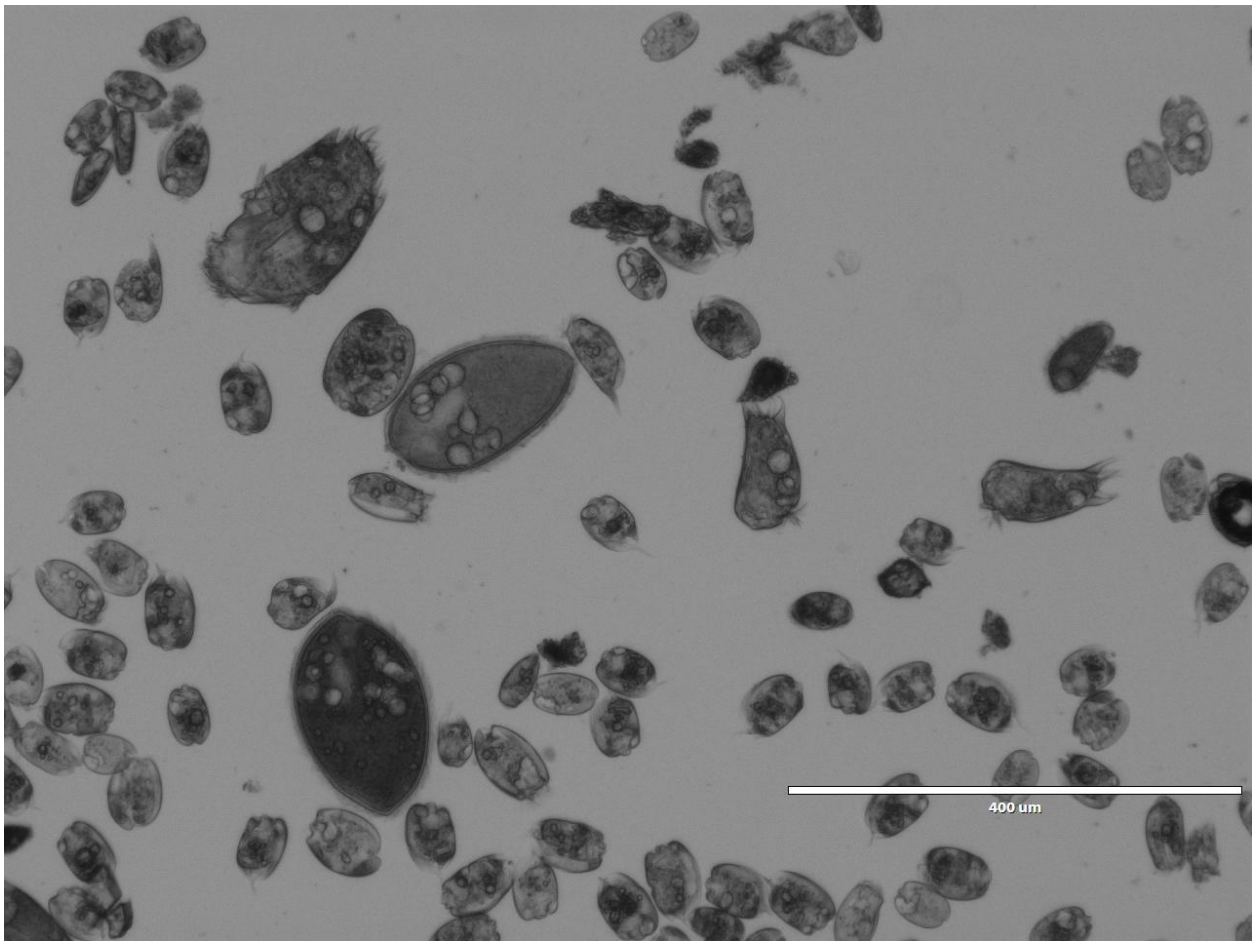


Figure 1.1. Mixed rumen protozoa from a lactating dairy cow. Differences in opacity between protozoa is likely due to reserve carbohydrate synthesis. Photo taken by S. Fessenden in the lab of T. Hackmann.

The role of fungi is also not completely understood (Williams and Newbold, 1990). It is often postulated that fungal sporangium attachment to fiber and penetration of rhizoids into the plant cuticle aids in the access of fibrolytic organisms into the fiber matrix (Tomme et al., 1995). Fungal populations also express cellulase of their own, but viability and function of these enzymes in the rumen is uncertain because enzymes might be rapidly degraded by proteolytic enzymes in the extracellular space (Nsereko et al., 2000). The difficulty in culturing has limited metabolic studies, and RNA techniques for estimating contribution to total microbial N pools and flows are prone to errors (Raskin et al., 1997). More recent work using qPCR with rDNA has estimated the contribution of fungal N as 0.5% of total microbial N (Denman and McSweeney, 2006).

When viewed relative to substrate utilization, bacteria and protozoa are the populations of major importance from a quantitative modeling perspective as they represent the primary microbial species in the rumen. For the purposes of the first section of this review, a description of the degradation of general substrate categories will be discussed, and specific species of microbes will be addressed when appropriate. Carbohydrate hydrolysis by specific types of bacteria will be described, followed by a review of rumen proteolysis and microbial protein synthesis. Nitrogen recycling, bacterial and protozoa interactions, and the concept of stimulation of microbial protein synthesis with peptides and AA will be discussed.

Carbohydrate Degradation in the Rumen

Many types of carbohydrate enter the rumen through the diet. The classification of carbohydrate is largely dependent on focus of the classifier. Plant physiologists typically emphasize biosynthesis, while animal nutritionists emphasize biodegradation (Van Soest, 1994). In the following sections, major nutritionally relevant categories will be discussed in relation to

their rate of degradation in the rumen. Three general categories are simple sugars (mono and disaccharides), plant storage compounds (starch and fructans), and structural carbohydrates (principally pectin, hemicellulose, and cellulose). Nutritional relevance of each carbohydrate category has led to the creation of various fractionation schemes for carbohydrate, especially in regard to the fiber portion. The generally accepted scheme currently in use is the detergent fiber system, developed by Van Soest et al. (1991). Other proposed systems have attempted to isolate more chemically uniform feed fractions and relate these fractions to specific animal performance (Prosky et al., 1988; Theander et al., 1995). However, application of the Lucas test indicates that the alternative systems do not represent nutritionally significant or uniform fractions (Lucas, 1964; Van Soest, 1967).

Ruminal digestion of carbohydrate is initiated by extracellular hydrolysis of polysaccharide to form smaller saccharides (Martin, 1994). Intracellular fermentation of these compounds typically forms pyruvate as an intermediate product, which is then metabolized to volatile fatty acids (Russell and Hespell, 1981). Due to the general lack of oxygen for final electron disposal, oxidation reactions must be accompanied by a simultaneous reduction (redox). Therefore, ruminal fermentation of carbohydrate is often described as an exergonic process that results in accumulation of incompletely oxidized end products. For a nutritionist, extent and rate of digestion of the more complex polysaccharides are of most interest. Non-structural carbohydrates in the plant are largely used as an energy store for the growing seed. The physiochemical structure of the seed reflects biological function; protection of the embryo until germination, as demonstrated by the protein encapsulation of starch by the plant. Physical processing, along with chemical composition and endosperm type have a great deal of influence on the site and extent of starch degradation (Oba and Allen, 2003). There are many rumen microbes that utilize starch as

a principal energy source. *Streptococcus bovis* is one of the most prolific colonizers of starch granules (McAllister et al., 1990b), and appears to use cell associated amylases. A major end-product of fermentation in this species includes lactate, which can lower culture pH rapidly (Russell and Dombrowski, 1980). The rod shaped *Succinimonas amyloytica* and *Ruminobacter amylophilus* degrade starch to succinate, and seem to be more prevalent when mixed diets of grain and hay are fed (Stewart et al., 1997). *Ruminococcus bromii* has also been detected in the rumen (Klieve et al., 2007) and has been shown to colonize resistant starch quite effectively (Ze et al., 2012). *Prevotella* spp. is one of the most numerous and ubiquitous groups of bacteria in the rumen. Their preferred polymeric substrates included starch and a number of cell wall polysaccharides, excluding cellulose (Cotta, 1992). The principal end products include acetate and succinate. *Provotella* spp. is also known to have an important role in the fermentation of peptides (Wallace et al., 1993).

The amount of starch degradation in the rumen also has great implications on the digestion of other materials, such as fiber (Poore et al., 1993; Beckman and Weiss, 2005). Rapid fermentation of a large starch pool in the rumen can lead to a decrease in ruminal pH due to fermentation acid production rate exceeding the buffering capacity of the rumen (Mertens, 1997). Bacterial attachment to substrate is highly sensitive to low pH, and even temporarily increase in pH during mastication might be enough to promote attachment of fiber degrading microbes, as this process occurs within 10 minutes of ingestion (Koike et al., 2003). While low pH is generally known to reduce fiber degradation (Calsamiglia et al., 2002), the microbes that are attached to substrate might be able to tolerate transient dips in pH (Mourino et al., 2001). Locally higher pH might be maintained in the micro-environment associated with the cellulosome used by *Ruminococcus* spp. Bacteria attached to substrate are likely more capable with dealing with a lower pH

environment, as energy required to maintain cell integrity is more readily available. Given these considerations, it is important to understand how the chemical and structural aspects of starch affect animal performance.

Cellulose is the most abundant biopolymer on earth, and forms the main bulk of forages in ruminant nutrition. As such, cellulose degradation is of great interest. Structural carbohydrate digestion is largely influenced by physical factors in the complex matrix of cellulose, phenolic compounds and proteins bound in the plant cell wall. Structure of the cell wall and degree of cross-linking between phenolic compounds and hemicellulose varies according to type of plant (Chesson and Forsberg, 1997). Legumes exhibit a higher rate of digestion compared with grasses, however extent of digestion can be lower depending on many factors including growing environment, variety and maturity (Van Soest, 1994). Neutral detergent fiber from plant parts not associated with structural components (e.g. alfalfa leaves) demonstrates highly fermentable characteristics resulting from a lack of vascular tissue and cross-linking of lignin and hemicellulose.

Compared to their aerobic counterparts, cellulolytic anaerobes degrade structural carbohydrate in very specialized way. Many cellulolytic organisms maintain an intimate association with the fiber matrix through a multi-enzyme complex (Bayer et al., 2004). Binding proteins and processive cellulases work to degrade the matrix, releasing individual sugars and cellobiose. Free, extracellular cellulases typical found in aerobic systems are rarely found in anaerobic bacteria. Indeed, *Fibrobacter succinogenes* contains a coding region only for endocellulases, not exocellulase or processive cellulases, suggesting most degradation of the polymer occurs in the periplasmic space (Wilson, 2009; Ransom-Jones et al., 2012). Therefore, the access to substrate is likely a rate determining step in cellulose degradation both in the rumen

(Fields et al., 2000), and in cellulosic ethanol fermentations (Monlau et al., 2012). The *Ruminococcus* spp. namely *R. albus* and *R. flavefaciens* are largely recognized as the most active bacteria involved in fiber breakdown, and each use extensive extracellular cellulase complexes (Chesson et al., 1986, Morrison et al., 1990). *Butyrivibrio fibrosolvens* is another moderately fibrolytic species that is responsible for much of the butyrate produced in the rumen. This species is recognized to ferment a wide variety of sugars, and likely plays a key role in hemicellulose degradation (Hespell and Cotta, 1995). Strains of *B. fibrosolvens* have also been shown to have amylolytic and proteolytic capabilities (McAllister et al., 1990a; Attwood and Reilly, 1995).The physical breakdown of the plant structure ultimately has profound influences on passage from the rumen, which in turn determines extent of digestion and subsequent energy availability of the diet, along with rumen fill and intake behavior of the animal. Much work has been performed in this area, and it continues to be a particularly active topic (Mertens, 1997; Huhtanen et al., 2006; Raffrenato, 2011).

Protein Degradation by Rumen Microbes

The first step in dietary protein degradation is hydrolysis by membrane bound proteases (Brock et al., 1982). This step is generally considered to occur rapidly in the rumen; however precise measurement of the rate of degradation has eluded researchers for decades. For many, differences in solubility and in sacco disappearance is key (Ørskov and McDonald, 1979; Michalet-Doreau and Ould-Bah, 1992). Subsequent research has shown that other properties, such as tertiary structure and cross-linking are also important (Nugent et al., 1983; Wallace et al., 1997). Rumen bacteria are the most active at protein breakdown, with several key species exhibiting high proteolytic activity. *Ruminobacter amylophilus* and *Prevotella* spp., are most commonly associated with high levels of cell associated protein breakdown (Blackburn and

Hobson, 1962; Wallace, 1994), while *B. fibrosolvens* shows a much higher level of extracellular protease expression (Cotta and Hespell, 1986), likely allowing it to access hemicellulose in the chemically diverse fiber matrix. Relative activity of each strain is likely dependent on diet (Nolan and Dobos, 2005). *Streptococcus bovis*, one of the fastest growing anaerobic bacteria, also exhibits proteolytic activity, and its acid tolerance can allow it to become dominant in diets with high levels of soluble carbohydrates (Russell and Robinson, 1984). The proteolytic activity of some common bacteria seems to show a negative feedback mechanism. Cotta and Hespell (1986) demonstrated a 70-80 % reduction in proteolytic activity in *B. fibrosolvens* when higher levels of AA and ammonium chloride were included in the culture. The distribution of proteases present in rumen fluid seems to be largely dependent on diet (Wallace and Brammall, 1985; Wallace et al., 1997) and complete hydrolysis requires the cleaving of many diverse chemical bonds. The presence of feedback mechanisms and variation in specific activity of certain proteases can provide evidence for possible reduction of dietary protein breakdown when soluble AA and N are fed, although response at the whole animal level has not been well studied. This could be an appropriate area for functional proteomic work.

Cilate protozoa have been known to be proteolytic, especially toward endogenous and bacterial proteins. The types of proteases present differ from those of bacteria cultures, with higher thiol and carboxyl types present (Coleman, 1983). Interestingly, protozoa seem to prefer larger, even insoluble types of protein containing materials. Brock et al. (1982) demonstrated a proteolytic activity one-tenth that of rumen bacteria when provided with soluble sources. Protozoa exhibit a voracious appetite for more complex forms of protein, and much of their AA requirement likely comes from the engulfment of bacteria, chloroplasts, and feed particulate matter (Wallace et al., 1997). The predation and recycling of bacterial protein to NPN

compounds can account for a relatively large portion of the microbial N pool; with estimates of up to 50% of the microbial protein is degraded to NPN in the rumen (Wells and Russell, 1996; Oldick et al., 2000).

After protein hydrolysis, peptides and amino acids are transported into the cell where they can be incorporated into microbial protein or further degraded to ammonia. Preference of rumen bacteria for amino acids *vs*. peptides has been well studied in individual populations (Wallace et al., 1997) however mixed cultures of bacteria show inconsistent results. Peptide degradation is most limited in peptides blocked at the N-terminus, or with glycine or proline at the N-terminal end (Wallace, 1996). Once free AA and short peptides are hydrolyzed, amino acid uptake by most growing ruminal microbes is rapid (Argyle and Baldwin, 1989), while some populations lack transport mechanisms to move excess amino acids into extracellular medium. Therefore, export of nitrogen as ammonia is crucial to avoid cellular death when using peptides and amino acids as energy sources (Tamminga, 1979; Russell et al., 1988b). This process has been the target of many attempts to manipulate N efficiency in the rumen. Russell et al. (1988a) isolated obligate AA fermenters in the rumen, and concluded the activity of this group can account for a disproportionate amount of the ammonia produced in the rumen (Rychlik and Russell, 2000).

Ammonia has long been recognized as an important nitrogen source for rumen microbes (Lewis et al., 1957). Satter and Slyter (1974) performed a series of in vitro and in vivo experiments on ammonia requirements by rumen microbes. They determined that concentrations of 2 to 5 mg/100 mL of rumen fluid were needed to optimize microbial fermentation (Satter and Slyter, 1974; Satter and Roffler, 1975; Slyter et al., 1979). The rumen ammonia pool is generally small, and its rate of turnover can be quite rapid. Ammonia N that is fixed into amino acids by bacteria and subsequently degraded again to ammonia has been considered a wasteful process, as

net energy is lost in the conversions. Most of the ammonia N that is not assimilated by microbes diffuses into the portal blood supply for eventual conversion to urea.

Protozoa have historically been considered to play a major role in ammonia production through bacterial predation (Wallace and McPherson, 1987; Williams and Coleman, 1997) and many have concluded that defaunation would benefit overall efficiency of microbial protein synthesis. Eugène et al. (2004) performed a meta-analysis of the effect of defaunation on rumen, showing that feed conversion efficiency, ADG, microbial protein synthesis and flow, and ruminal N dynamics all improved with defaunated animals. This was generally consistent with the earlier work of Jouany (1996). In many of the studies with defaunated animals, rumen ammonia N decreases relative to faunated animals, typically leading authors to attribute the effect to the absence of protozoal proteolytic and deaminative activities (Hristov and Jouany, 2005). Hackmann and Firkins (2015) postulated a second explanation, whereby defaunation allowed for increased numbers of ammonia-assimilating bacteria, thereby decreasing the available pool through overall increased uptake. Indeed, bacterial lysis still occurs in defaunated animals (Koenig et al., 2000) resulting in up to 50% of the microbial outflow consisting of intra-ruminally recycled N. As mentioned before, bacteria also generally demonstrate up to 10 times greater proteolytic activity than protozoa when provided with soluble protein sources (Brock et al. 1982). These alternative explanations demonstrate the risk associated with looking only at pool size (e.g. concentration) when the turnover of the pool is more relevant to the biologic mechanism of interest. Similar quantitative aspects of intra-ruminal N turnover were summarized by Wells and Russell (1996). These authors concluded that liquid dilution rate has one of the strongest effects on turnover of the microbial N pool. This is important as it pertains to models of rumen microbial growth. Assuming a static turnover value measured at lower levels of intake

and production might result in inaccurate values for microbial growth when attempting to simulate situations that differ considerably from the studies originally used to parameterize the model.

Microbial Protein Synthesis

Fermentation of hexose provides the energy basis for microbial protein synthesis. Even the more simple forms of N can be assimilated by rumen microorganisms to synthesize microbial protein. This capability was understood well before Hungate began describing rumen bacteria. A. I. Virtanen was one of the earlier researchers in bacterial biochemistry. It is notable that his initial work on phosphorylation of hexose at the start of glycolysis would win the Nobel Prize in chemistry in 1945. This was no trivial feat for the era considering he is flanked in 1944 by Otto Hahn, the discoverer of nuclear fission, and James Sumner in 1946, a Cornell researcher who was the first person to isolate an enzyme (urease) in pure form. Virtanen's later work would demonstrate the importance of rumen microbial protein synthesis to the ruminant host in a series of studies using purified diets, where the only true protein available to the animal was provided from microbial protein synthesis (Virtanen, 1966).

Microbial protein synthesis requires N, which is supplied to the rumen in several forms. Nutritionally relevant N containing compounds in the feed include amino N, mainly provided through feed proteins, peptides and amino acids, and non-amino N in compounds such as ammonia and urea. Other forms of are present in feed, such as nitrates and N_2 gas. Nitrates have been investigated as possible N sources and even terminal electron acceptors for anaerobic respiration (Jones, 1972), however it was determined that the rate of reduction was not sufficient to prevent nitrite accumulation. Although an appreciable amount of nitrogen gas is introduced via feed ingestion and diffusion from blood, research has indicated that approximately 0.1 mg of

N is fixed per kg of rumen contents, even in low N diets (Li Pun and Satter, 1975). Nitrogen is also supplied to the rumen via endogenously recycled N in the form of urea and endogenous amino N mainly associated with epithelial cell turnover. While this endogenous portion does not represent a net supply, it does provide some modulation of available N for microbial growth (Nolan and Dobos, 2005).

Since protein synthesis is an energy dependent event, synchronization of rumen available carbohydrate and protein would seem like an important way to optimize rumen microbial protein output. Readily fermentable carbohydrate sources are more effective than fiber at promoting microbial uptake of degraded protein (Stern and Hoover, 1979). In vivo studies demonstrated that infusion of readily fermentable carbohydrate sources decreases NH_3-N concentrations by increasing N use by rumen microbes (Casper and Schingoethe, 1989, Cameron et al., 1991). A decrease in NH_3-N concentrations can be explained in two ways: 1) The increase in energy from carbohydrate metabolism allows for higher growth rates and more assimilation of ammonia, and 2) non-fibrolytic bacteria can incorporate amino acids and peptides directly, thereby reducing NH_3-N production (Russell, 2002). Production responses to synchronization of dietary fermentable N and carbohydrate substrate is difficult to accurately study, because many types of bacteria utilize amino acids and carbohydrates as energy sources (Bach et al., 2005). In contrast, recent work has shown that asynchronous dietary supply of N may not be detrimental. Reynolds and Kristensen (2008) demonstrated that oscillation of rumen available N levels (asynchronous supply) increased retained nitrogen, probably due to modulating effects of recycled nitrogen. Isotope studies have shown that recycled NH_3-N can account for 23 to 95% of microbial incorporated nitrogen (Nolan and Dobos, 2005; Firkins et al., 2007; Recktenwald, 2010). This large range in incorporation offers a tempting way to increase the N economy of the ruminant

though up-regulation of recycled urea N in place of rapidly degradable feed N. Unfortunately, regulation of recycled urea N is poorly understood. Some earlier studies have demonstrated that urea N transport appears to be regulated by mass action (Houpt, 1970), however the presence of urea transporters in the gut epithelium support the concept that urea-transport is tightly regulated (Marini et al., 2006). Several candidate mechanisms have been explored including UT-B, a major urea transporter that is well distributed in the rumen epithelia (Stewart and Smith, 2005). Several studies have been unable to demonstrate up-regulation of UT-B activity in response to dietary N manipulation (Marini et al., 2004; Røjen et al., 2011). More recently UT-B activity has been shown to be more sensitive to short-chain fatty acids and pH fluctuations in the rumen than plasma urea N concentration (Lu et al., 2015).

Directly manipulating urea transfer at an organ level as a way to increase urea N recycling will remain elusive until a more mechanistic understanding of urea transport is achieved. However, it is quite possible to achieve a greater amount of urea N recycling though decreasing dietary N, but care must be taken to maintain adequate rumen N balance to avoid prolonged limitation of microbial growth. This is not necessarily contradictory to the fact that cattle can exist on extremely low N diets, it would just appear that the regulatory mechanisms seem insufficient to maximize milk or meat output in such situations. Ruminant ecosystems in the wild are well adapted to fluctuations in substrate availability, as demand for elevated amount of AA associated with late gestation and lactation is well timed with seasonal availability of substrate.

Assimilation of the simple N compounds such as ammonia into AA follows occurs mainly through the synthesis of Ala, Glu, and Gln (Morrison and Mackie, 1997). Figure 1.2 displays the major AA biosynthetic pathways, as described in Hackmann and Firkins (2015). It has been commonly thought that NH_3-N is the sole source of N for AA synthesis in cellulose degrading

microbes. Bryant and Robinson (1963) incubated three common types of cellulolytic bacteria with a wide variety of N compounds including ammonium sulfate, urea, purines, and 17 different AA. The authors reported that that no other N source stimulated growth as strongly as ammonium sulfate, and cautioned the fact that ^{15}N studies were needed to determine the origin of microbial N. The results from this study did not demonstrate NH_3-N is the exclusive N source. Nonetheless, the perhaps incorrect interpretation that fiber fermenting bacteria utilize only NH_3-N was subsequently used by Satter and Slyter (1974) to bolster the importance of maintaining 2 to 5 mg/dL concentration of ammonia--a recommendation that strongly persists today. The issues of focusing solely on concentration of nutrients have been previously discussed here and in the literature with regard to volatile fatty acids (Hall et al., 2015).

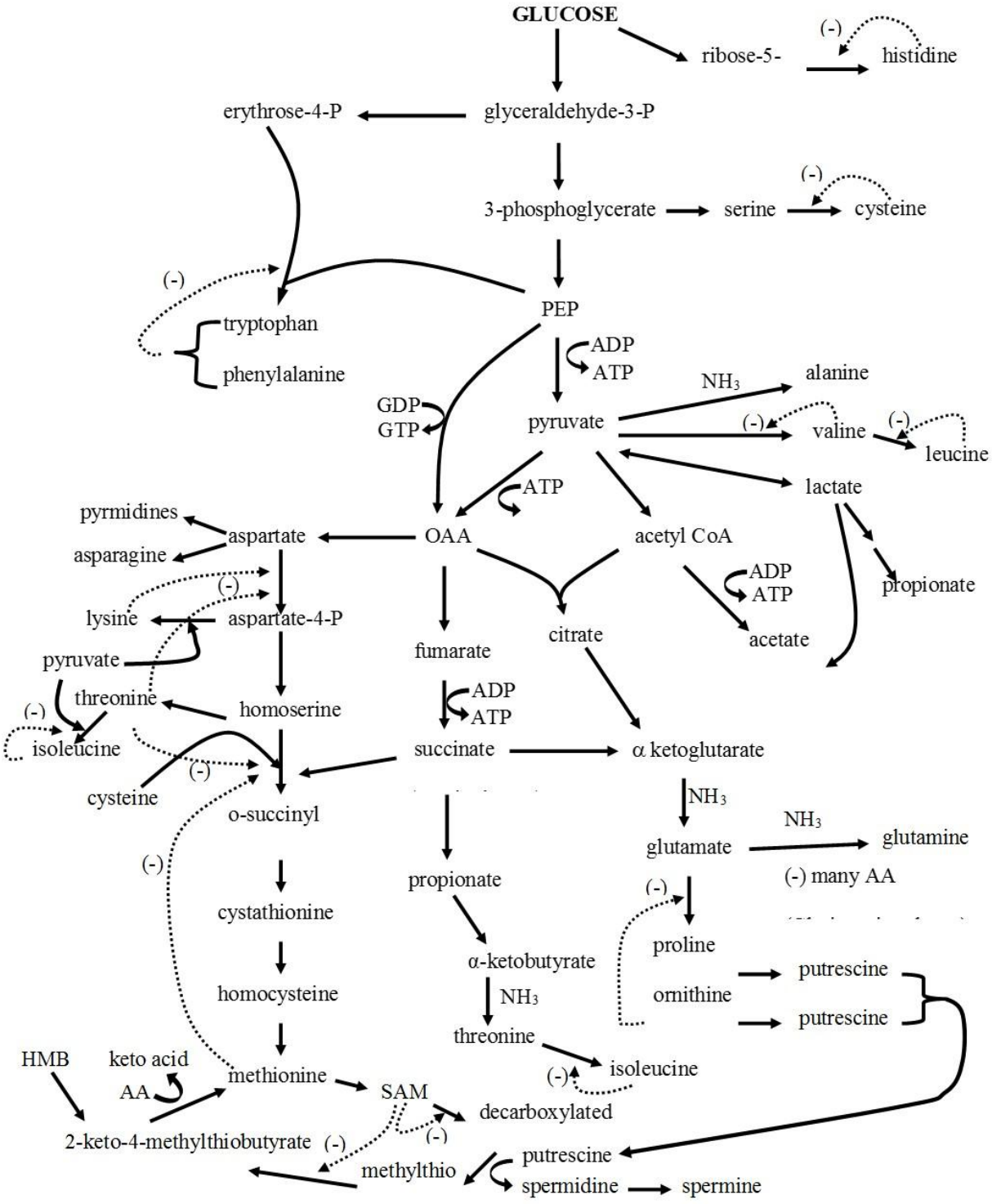


Figure 1.2. Amino acid biosynthetic pathways in various bacteria, primarily Escherichia coli. Reproduced from Hackmann and Firkins (2015).

Role of AA and Peptides in the Rumen

In vitro studies with ^{14}C and ^{15}N have provided evidence against the claim that fiber degrading bacteria utilize NH_3-N only. The most common species studied include *R. albus, R. flavefaciens* and *F. succinogenes*, as they are considered to be the more dominant fibrolytic bacteria in the rumen. Atasoglu et al. (2001) demonstrated that high amino acid and peptide concentrations resulted in reduced ^{15}N enrichment, leading to an estimate of approximately 50% of the cellular N coming from non-ammonia N (**NAN**) sources. This was consistent with other work using ^{14}C labeled amino acids and peptides (Ling and Armstead, 1995). In vivo experiments using ^{15}N show lower enrichment of the solids associated bacteria, which is often attributed to preference for NAN. Alternatively, this observation might be due to methodological artifacts during the separation process, or incomplete equilibrium of the ^{15}N ammonia pool in the microenvironment associated with the biofilm (Atasoglu et al., 2001). Studies demonstrating a beneficial effect of preformed AA on fiber degradation in vivo rarely are able to determine if the effect is through direct incorporation of amino N, or the supply of required branched-chain carbon skeletons. Practically, when concentrations of amino-N in vitro are closer to physiologic levels, >80% of microbial N was reported to be from the ammonia pool (Atasoglu et al., 2001). This could arguably be functionally similar to 100% (as is assumed by current models) when considerations are taken for the relative rate of protein synthesis by slower growing cellulolytic bacteria vs. NFC bacteria and protozoa.

Supply of preformed amino acids and the effect on microbial populations has been an active area of research for years. Pure, co, and mixed culture data have been the principal method to investigate possible stimulatory outcomes. Argyle and Baldwin (1989) demonstrated that peptides are more stimulatory than free AA. In relation to ammonia, Russell (1993)

demonstrated that *S. bovis* grew almost twice as fast when the ammonia based culture medium was supplemented with amino acids. In another more recent study *R. albus* has demonstrated ability to take up peptides, but no transport mechanism was present for free AA (Kim et al., 2014). This is in line with earlier work using mixed cultures, where peptides seemed to be taken up much more rapidly than mixtures of free AA (Prins et al., 1979). In vivo work has been more varied. When Soto et al. (1994) infused amino acid or peptides into the rumen of sheep, no changes were observed, however diet fermentability and intakes were low. Due to this, the authors suggested that enhanced growth would only occur when energy (ie, carbohydrate availability) was not limiting. This was confirmed in a later study by Chikunya et al. (1996). In more recent studies measuring omasal flows of soluble NAN fractions, no changes were observed in rumen microbial output and digestion of OM when proteins of varying protein solubility were fed (Choi et al., 2002b; Reynal et al., 2007). However, responses in the Choi et al. study were more consistent with fermentable energy limitation, while the Reynal et al. study demonstrated higher flows of soluble NAN to the omasum.

Peptide and amino acid uptake is also altered by cell membrane fluidity and permeability, which can be influenced by feed components and bioactive compounds such as ionophores. These hydrophobic molecules have a monovalent functional group capable of binding a proton, along with an ion binding site, where monovalent (Na^{+} and K^{+}) or divalent cations (Ca^{++} and Mg^{++}) are bound. Some ionophores can bind several types of cations, although many have selectivity for specific ions (Pressman, 1976). Once dissociated into a membrane, the molecule can effectively shuttle protons across the membrane if the appropriate ion gradient is present. The direction of ion and proton movement is dictated by relative concentrations of the ion gradient in accordance with the Nerst equation (Hegarty and Gerdes, 1999).

Ionophores can be particularly effective at disrupting microbial cellular processes because many microorganisms maintain a higher concentration of potassium inside their cells and they expel sodium and protons. Because the sodium gradient is larger than the potassium gradient, efflux of potassium results in a net increase of protons in the cell with ionophore treatment. In addition, intracellular pH declines as cells utilize membrane bound ATPase in an attempt to re-establish the protonmotive force. Energy levels quickly decline and the cell reaches a de-energized state that suppresses growth (Russell, 2002).

The secondary mode of action of bioactive compounds is centered on membrane disruption leading to cell suppression or death. Disruption typically starts with a compound forming bonds with specific elements of the lipid bi-layer, such as cholesterol or membrane bound proteins (Lohner and Blondelle, 2005). These bonds change the fluidity of the membrane, and might limit the function of membrane bound proteins important to metabolism and growth, especially transport proteins (Keukens et al., 1995). The cell is then unable to conduct normal cell processes or adapt to changing environmental conditions. Some potent bactericidal compounds will disrupt the membrane in such a way causing rupture, lyses and cell death. Plant saponins are believed to work in this fashion (Makkar et al., 1995; Wang et al., 1998).

The efficacy of many antimicrobial compounds is not typically a broad spectrum phenomenon, as certain species can develop resistance mechanisms to maintain membrane integrity. In the case of ionophores, Gram-negative bacteria exhibit more resistance than cells that lack an outer protective membrane, although this is not always the case. Some studies have shown that Gram-negative bacteria can be equally sensitive (Chen and Wolin, 1979). More recent work has demonstrated that most of these bacteria actually contain a Gram-positive cell wall structure (Nagaraja et al., 1997). Pure culture studies have demonstrated that ionophore

resistance (specifically to monensin) is largely mediated by the presence of carbohydrates on the outer surface of the cells, as is the case with monensin-resistant *P. bryantii* $B_1$4 (Callaway and Russell, 1999). Bacteria can develop these structures quickly and resistance is present even when two separate ionophores are alternated in a feeding regime (cross-resistance) (Newbold et al., 1992; Nikaido, 1998). Rumen microbes have also been shown to change membrane fatty acid content to alter permeability (Di Pasqua et al., 2006). These defense mechanisms, coupled with disruption of sodium gradient dependent transport mechanisms central to the uptake of peptides and amino acids might explain the protein sparing effect of monensin (Russell and Strobel, 1989; Recktenwald et al., 2014). Ultimately, the response of rumen microbes to soluble AA and peptides will likely be determined by energy availability, presence of membrane altering compounds, and relative needs of specific AA and/or keto-acids by key groups of rumen microbes.

Rumen protozoa have traditionally been thought to derive most AA from preformed bacterial and feed protein. However, evidence does exist for de-novo synthesis of amino acids by rumen protozoa. When ^{14}C labeled monosaccharides or acetate were provided, 5 to 35% of the label was found in TCA perceptible protein, indicating possible synthesis of AA in isotrichid protozoa (Williams and Harfoot, 1976), although bacterial contamination cannot be ruled out. More recently, Newbold et al. (2005) cloned a glutamate dehydrogenase in entodiniomorphid protozoa that was likely acquired via horizontal gene transfer from bacteria (Ricard et al., 2006). This relatively high K_m system utilizing ammonia and α-ketoglutarate is widely used by other rumen microorganisms, and suggests that there are adequate mechanisms for ammonia assimilation in protozoa. The degree to which this assimilation is quantitatively important has not yet been determined. Engulfment of bacterial and feed N is still likely the largest contributor to protozoal

N. Lysine is known to be synthesized from DAP (Onodera, 1986) although this N can still be considered bacterial origin.

Free AA and Peptides from a Commercial Feedstuff

The concept of possible stimulation of rumen microbes by amino acids and peptides has led to the development of feedstuffs that contain high amounts of soluble amino-N. One such product is Fermenten™ a commercial feedstuff marketed by Arm and Hammer Animal Nutrition (Princeton, NJ). This product is made from a waste stream of commercial amino acid production, such as Lys and Glu. These AA are produced using selected microorganisms that over-express the AA of interest. The cultures are processed to remove the targeted AA, leaving a waste stream of soluble AA and short peptides which is then sprayed onto a carrier such as wheat middlings. This is dried and marketed as a feed product for cattle. Fermenten has a concentration of 82 g/kg of amino acids and peptides up to 10 AA in length (Lean et al., 2005). Research experiments have sought to demonstrate the efficacy of this fermentation byproduct in stimulating microbial rumen populations. A meta-analysis of in-vitro data from continuous culture fermenters demonstrated an almost 16% increase in microbial nitrogen output vs. a control with no fermentation byproduct addition (Lean et al., 2005). In vivo work has been more varied, with some studies showing limited effect on rumen metabolism and cattle performance (Broderick et al., 2000), or effects mediated by other dietary components, such as sugar (Penner et al., 2009). Of interest to the experimental outcomes is that the fermenter data used purines as a microbial marker, and inclusion of a fermentation byproduct like Fermenten likely also introduced additional dietary purines relative to control. Incomplete degradation of these purines would lead to an overestimation of microbial protein flow in fermenters with the fermentation byproduct. Purine concentration of the diets, bacteria, or fermenter effluent was not reported in the Lean et

al. (2005) paper, so it is difficult to determine the quantitative importance of this possible overestimation; however an estimation can be made: Lean et al. (2005) reported a 0.271 g/d increase in microbial N flow/d. Using the mean purine concentration of 952 mg/g of microbial N reported by Illg and Stern (1994), the flow would correspond to 258 mg of additional purines flowing from the fermenters fed fermentation byproduct. Using the weighted average of 3.6 g/d of fermentation byproduct inclusion in the diet at 8.4 % of feedstuff DM as N as reported by Lean et al. (2005), it can be calculated that 0.3 g of N was provided via byproduct.

Amino acid fermentations are typically performed using *E. coli*, and purine content can be estimated to be 10% of cell DM under commercial growth conditions (Neidhart, 1996). At 8.5% nitrogen content, purines would account for 1,136 mg of purine/g of microbial N. Assuming 75% of the N in the final byproduct was of microbial origin, the purine content of the byproduct could be estimated at 72.2 mg/g of DM. With 3.6 g/d inclusion of byproduct, 260 mg more purines could be attributed to dietary difference alone. Therefore, this possible overestimation bias could account for the entire observed treatment under the described assumptions. As such, future studies investigating microbial fermentation byproducts should utilize ^{15}N as a microbial marker to avoid the bias associated with undegraded feed purines.

Overall, the ruminal degradation of substrate and incorporation of N compounds into microbial protein is dictated by a large variety of factors. The majority of the well-studied populations are responsible for much of the substrate degradation in the rumen. Populations that fulfill specific niches can have disproportionately large influences on the ecosystem, and manipulation of rumen fermentation often target these species. The following sections of this review will focus on methods to quantify rumen digestion and microbial metabolism.

PART 2 QUANTITATIVE ASPECTS OF RUMEN FERMENTATION

Omasal Sampling Technique

Abomasal and duodenal fistulas have traditionally been used to sample post-ruminal digesta flows. This data has historically provided the information needed to more completely understand ruminal digestion and metabolism. However, cannulation of post ruminal sites in animals is an invasive procedure and many complications can occur (Harmon and Richards, 1997). Omasal sampling though cannulas have been performed since the 1960's (Oyaert and Bouckaert, 1961), although this technique still required intensive surgical techniques. Spot sampling of omasal contents thought a rumen cannula as described by Punia et al. (1988) avoided the need for post-ruminal cannulation, however re-insertion and placement was needed at each desired sampling time. Huhtanen et al. (1997) described a device that, once inserted into the omasum, would allow for repeated sampling over a longer time period without negative effects on intake. This method was then adapted by the University of Wisconsin researchers for a series of studies on omasal flows of nutrients (Reynal and Broderick, 2005). The technique involves a specialized sampling tube (Figure 1.3) passed thought a rumen cannula, with the open end of the tube residing at the omasal orifice. This technique has been validated against duodenal sampling (Ahvenjärvi et al., 2000; Ipharraguerre et al., 2007) and these evaluations demonstrated that when combined with a triple marker method (France and Siddons, 1986), the technique can allow for fairly small coefficients of variation in measurement of ruminal digestion variables. Significant bias in organic matter digestion was reduced when compared to abomasal sampling due to the contributions of endogenous secretions (Ahvenjärvi et al., 2000).

Figure 1.3. Sampling device used to obtain digesta sample from the omasal orifice in lactating dairy cattle. The tube is passed thought a rumen cannula, and the open end of the tube is located in the omasal orifice. The weight (125 mL bottle filled with sand and sealed) is passed thought the orifice to retain the device. Alternating suction and pressure is used to withdraw a sample.

Triple Marker System

The choice of digestion markers can also have a strong influence on the measurement of post-ruminal nutrient flow. Methods relying on single markers typically must use assumptions such as homogeneous digesta flow, no marker migration or concentration in specific phases, along with other ideal marker behavior (Faichney, 1975). The use of multiple phases and markers allows for reconstitution of true digesta flow, and limited assumptions related to marker behavior are necessary. The analysis by Ahvenjärvi et al. (2003) indicated that a triple marker system utilizing Yb, Co, and iNDF would result in the most accurate and precise flows. As such, much of the recent omasal sampling performed in the last 20 years has utilized ruminal infusion of Yb and Co markers into the rumen, while iNDF is associated with the feed. A depiction of a typical experimental setup is demonstrated in Figure 1.4. When combined with omasal sampling, Ipharraguerre et al. (2007) notated noted unrealistic and highly variable data when using duodenal flows and a triple marker system of Co-EDTA, $YbCl_3$, and iNDF, likely due to Yb marker migration in the acid conditions of the abomasum. These issues were not seen with omasal data in the same cows. In a series of companion publications, researchers in Finland and Wisconsin provided a meta-analysis of data using omasal sampling and triple marker systems (Broderick et al., 2010; Huhtanen et al., 2010). These papers provided useful data from which to build and evaluate models of rumen fermentation. The Broderick et al. (2010) paper demonstrated the NRC (2001) overestimated RUP by 21%, and underestimated microbial-N flow by 26%. This series of studies also provided much needed data for evaluation of the CNCPS, by which post ruminal N and AA flows could be compared to model predictions (Higgs, 2014; Van Amburgh et al., 2015). This technique has also been adopted by the Van Amburgh lab group (Figure 1.4) for studies of omasal nutrient flows.

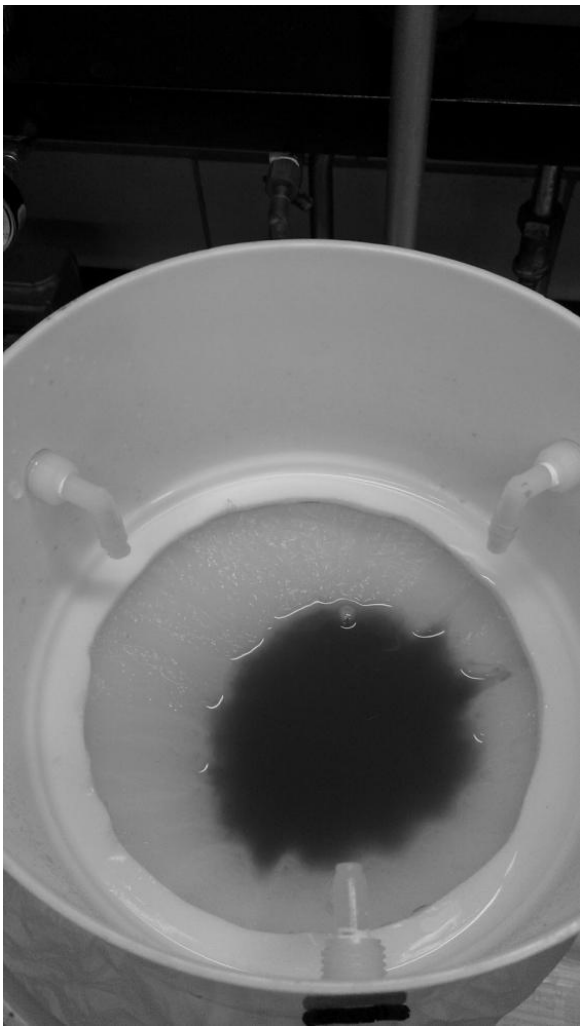
Figure 1.4. Experimental setup with ruminal infusion of CoEDTA and $YbCl_3$ for a study utilizing the triple marker technique. Peristaltic infusion pumps are located above the cows.

Isolation of Protozoa from the Omasum

Several different procedures have been performed to isolate protozoa from rumen fluid. The most common issues to address in the isolation protocol are feed particle and bacterial contamination (Volden et al., 1999). The typical isolation procedure relies on filtration and/or centrifugation to isolate biomass that is assumed to be representative of protozoa. One of the early studies of microbial composition isolated protozoa only through repeated centrifugation (Czerkawski, 1976). For large scale separations, Storm and Ørskov (1983) used a large filtration and separation system to examine microbial biomass from animals coming into abattoirs, however feed and bacterial contamination was likely high. To address this, researchers began using flocculation and sedimentation to remove large feed particles, followed by centrifugation and filtration on nylon cloth to wash away bacteria (Williams and Yarlett, 1982; Williams and Strachan, 1984; Martin et al., 1994). Glucose was used to enhance flocculation, although this likely altered microbial composition as a result of competition for growth substrate. Since robust flocculation is needed for proper feed removal, anaerobic technique is also important. For a protozoal isolation to be representative of the population in the rumen, techniques must strive to be rapid, have limited addition of any growth promoting substances, and avoid lysis of microbial cells. Many of the previously reported studies have suffered from weaknesses in one or more of these areas.

More recent work with microbial populations has necessitated the development of a rapid technique to isolate mixed protozoa cultures with viability enough to culture. The techniques are described in the paper by Denton et al. (2015); and might provide useful data when combined with the omasal sampling technique. The procedure uses a combination of flocculation, sedimentation, and filtration to recover much of the protozoa in a sample in a form that has high

viability, low feed contamination, and no addition of substrate that is known to appreciably change cell composition. Strained rumen fluid is briefly incubated in separatory funnels to allow for removal of large feed particles, followed by filtration on a nylon cloth, all under anaerobic conditions. Figure 1.5 shows two key pieces of apparatus used in the separation procedure.



A **B**

Figure 1.5. Apparatus for isolation of protozoal samples. Strained fluid is incubated in a separatory funnel (A) and floating feed particles are removed. A layer of sedimented protozoa can be seen near the stopcock. Clarified fluid, including the sediment layer is then filtered on nylon cloth under constant CO_2 gassing (B) to isolate protozoal biomass. Gassing is used to preserve viability if needed for the experiment.

Development and Evaluation of the CNCPS

Techniques that utilize quantitative measures of nutrients and microbial biomass are important for developing and evaluating complex mathematical models of rumen fermentation. Evaluating the components of the model separately is critical to determining the proper area to focus research efforts. An example of this process is found in the development arch of the CNCPS, especially version 6 forward. Updated fractionation schemes and passage rates (Seo et al., 2006; Lanzas et al., 2007a; Lanzas et al., 2007b) necessitated the need to evaluate the ability of the model to predict ME and MP limiting milk. Three published studies were used to evaluate the ability of the model to predict with several combinations of new calculations and sub-routines (Tylutki et al., 2008). Model bias was reduced considerably from v. 5. The authors concluded that significant gains in accuracy and precision were achieved; all while improving user experience thought an object oriented programming approach. However, the discussion indicated that model evaluations against a different dataset investigating diets with varied rumen N and MP limitations (Recktenwald, 2007) indicated more varied results. The evaluation showed poor ability to predict first limiting milk production (r^2=0.29), with strong systematic bias. Overall accuracy was good in MP deficient diets, but the model was imprecise. This indicated that improvements needed to be made in the ruminal sub-model that would require more mechanistic description of microbial metabolism, nitrogen recycling, and rumen protein degradation. Version 6.1, described by Van Amburgh et al. (2010) revisited rates and pool calculations for protein, among other improvements. Previously, soluble protein pools were assigned to a solids passage rate, and the kd of the pool was typically between 150-300% per hour, effectively allowing for almost complete degradation in the rumen. Several studies have demonstrated that soluble true protein can provide 5-15% of the MP supply (Choi et al., 2002a;

Choi et al., 2002b; Volden et al., 2002; Reynal et al., 2007). The update assigned the soluble protein pool to the liquid passage rate, and decreased the rates of degradation to more realistic numbers. Additional data from Ross (2013) allowed for changes to the soluble pool fractionation scheme, correcting a large overestimation of NPN pools in many common feedstuffs such as soybean meal and alfalfa silage. The subsequent evaluation of the new model against a dataset provided by Van Amburgh et al. (2010) demonstrated improvements enough to allow consistent formulation of diets below 16% CP on commercial farms. The ability to more accurately predict ME and MP adequacy was a key step toward formulating rations balanced for amino acids, as concluded by an independent evaluation by Pacheco et al. (2012).

The CNCPS uses a factorial approach to calculate AA adequacy, and as such, the supply side of the model requires some quantitative understanding of three principal AA flows: Undegraded feed AA, microbial AA, and endogenous AA. In the initial publications describing the CNCPS, O'Connor et al. (1993) acknowledged that improvements in the supply model would require more information on the amino acid profile and availability of all three flows. The list for improvement as perceived by O'Connor et al. (1993) is reproduced here:

1. More research is needed to define quantitatively the components of metabolic fecal protein and the amino acid composition of these components to predict more accurately the amino acids required for metabolic fecal protein losses.
2. The amino acid content of tissue protein may need to be further defined based on the various components of empty body protein.
3. Research is needed to determine the amino acid content of the various products of the conceptus to describe more accurately amino acids required for gestation.

4. Additional research is needed to determine more accurate estimates of efficiencies of utilization of absorbed amino acids for specific physiological functions, especially because these efficiencies have a larger effect on requirements for absorbed amino acids (NRC, 1985).
5. Further research on the amino acid content of soluble and insoluble available dietary protein escaping ruminal degradation for various natural and by-product feedstuffs is required to increase the accuracy of predicting available amino acids supplied by specific cattle diets.
6. Additional research is necessary to determine accurate estimates of the amino acid composition of bacterial cell wall and non-cell wall protein fractions.
7. More research needs to be conducted concerning the true digestibility of bacterial cell wall and non- cell wall protein fractions.

The areas outlined in this roadmap have seen a considerable amount of excellent research since 1993 that has improved our understanding of AA dynamics in the ruminant. By focusing research through model development, researchers have been able to provide quantitative, mechanistic information to improve animal performance. Version 6.5 of the model (Higgs et al., 2015, Van Amburgh et al., 2015) specifically incorporated new data pertaining mostly to items 1-4 on the list, however it was quickly realized in the development of version 6.5 that further advances in understanding of endogenous and microbial AA flows would require a re-working of the rumen sub-model. Therefore, as part of the dissertation work of Dr. Ryan Higgs, the model was moved into a dynamic platform to more accurately describe rumen N and AA dynamics, with special interest in endogenous AA flows, microbial AA metabolism including protozoa, and digestibility of protein in the small intestine (Higgs, 2014). This re-working of the model allowed

for the evaluation of predicted individual essential AA flows against measured values from a more contemporary dataset of lactating cows at greater levels of intake and production. In this evaluation, many EAA were predicted with reasonable accuracy and precision, however Lys flow in particular was over-predicted relative to the measured values. In the discussion of Higgs (2014) it was suggested that the partition of microbial and non-microbial flow in these studies was responsible for some bias, and that microbial composition of the AA might not be well understood. Limited reporting of methods, cattle and feed characteristics, and results also present a difficulty to fully characterize the model inputs. Field use of the model often has better input characterization than data obtained from the literature, thus literature evaluations can at times undervalue the model's predictive value. Evaluation against fewer, better characterized studies can provide useful information when combined with larger dataset evaluation. Due to the variety of markers, sampling sites, and feed chemistry data availability, literature datasets of post-ruminal AA flow might contain significant bias. This can be seen in an evaluation of version 6.1 of the CNCPS by Pacheco et al. (2012) where characterization of feedstuffs was incomplete, and likely contribute to lower reported model performance than is realized in the field.

SUMMARY AND OBJECTIVES

Overall, rumen microbial ecology is complex, and attempts to understand the system must be done with a specific end in mind. Some of the largest advances in our understanding of the symbiotic relationship have come when researchers had specific, quantitative goals in mind. Omasal sampling, protozoa isolation, and model development are excellent examples of researchers seeking to apply research outcomes in a productive manner. As such, the remainder of this dissertation will attempt to demonstrate how the combination of these techniques can help drive knowledge and model development forward.

Therefore, the specific objectives of the work described by this dissertation are to:

1) Provide better understanding of the role of free amino acids and peptides from a commercially available fermentation byproduct in lactating dairy cattle fed nitrogen-efficient diets.
2) Utilize omasal sampling, an improved microbial isolation technique, and more robust measurements of AA composition to better characterize the omasal flow of AA from microbial populations, particularly protozoa.
3) Evaluate the ability of a new, dynamic version of the CNCPS with updated microbial AA characteristics to predict N and AA flow against a literature dataset.

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CHAPTER 2: Effects of commercial fermentation byproduct or urea on milk production, rumen metabolism, and omasal flow of nutrients in lactating dairy cattle

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ABSTRACT: Eight ruminally cannulated multiparous Holstein cows averaging 60 ± 10 DIM and 637 ± 38 kg of BW were assigned to one of two treatment sequences in a switchback design. Treatment diets contained (dry matter basis) 44% corn silage, 13% alfalfa silage, 12% ground corn, and 31% protein premix containing either a control mix of urea and wheat middlings (**CON**) or a commercial fermentation byproduct meal at 3% diet inclusion rate (**EXP**). Diets were formulated to be iso-nitrogenous and iso-caloric, with similar levels of neutral detergent fiber (**NDF**) and starch. The trial consisted of three 28 d experimental periods, where each period consisted of 21 d of diet adaptation and 7 d of data and sample collection. Digestion markers were infused continuously during the sampling period and composited omasal samples were used to calculate nutrient flows. The EXP diet provided 18 g/d more soluble AA and peptides vs. the CON diet. Energy corrected milk yield was 41.7 and 43.1 kg/d for CON and EXP, respectively with no treatment differences in milk fat and protein yield or content. Ammonia N concentration and pool size in the rumen was greater in cows fed the EXP diet. No differences were observed in rumen or total tract dry matter, organic matter, or NDF digestibility. Ruminal degradation of feed N was 15% lower in cows fed EXP diets, resulting in differences in omasal flow of N fractions. Cows fed EXP diets digested more NDF per unit of N degraded in the rumen,

indicating possible beneficial effects on cellulolytic microbial populations. Results demonstrate a fermentation byproduct meal had a sparing effect on degradable feed protein in N efficient diets, but did not increase microbial N flow from the rumen.

Keywords: omasal sampling, soluble protein, CNCPS, microbial protein synthesis, Fermenten

INTRODUCTION

Protein is one of the most expensive macronutrients in dairy cattle rations, and overfeeding degradable protein relative to supply results in excessive N losses to the environment (Huhtanen and Hristov, 2009). Efficient use of feed N can be achieved by first meeting the requirements of the rumen microbial population, followed by balancing diets to meet the amino acid requirements of the cow. To decrease competition for quality protein that could otherwise be fed to humans, dairy cattle can be fed byproducts of human food production, thereby converting waste product streams into highly valuable milk protein. One such byproduct of commercial amino acid production is Fermenten (Arm and Hammer Animal Nutrition, Princeton, NJ). Commercial AA production is performed using bacterial cultures, resulting in a waste stream with high amounts of soluble nitrogenous compounds. These compounds of bacterial origin are in the highly available form of ammonia, AA, small peptides and purines. Amino acids and peptides have been hypothesized to increase the flow of microbial protein from the rumen through stimulation of microbial protein synthesis (Cotta and Russell, 1982, Lean et al., 2005). Increased microbial N flow also reduces reliance on expensive rumen undegradable dietary protein sources commonly used to provide adequate AA to high producing dairy cattle. Previous research with varying sugar levels suggests that fermentation byproducts might only affect certain microbial populations (Penner et al., 2009).

Mathematical models such as the Cornell Net Carbohydrate and Protein System (**CNCPS**) (Higgs et al., 2015; Van Amburgh et al., 2015) have been successfully used to optimize rumen microbial output and meet animal nutrient requirements while reducing N losses to the environment (Tylutki et al., 2008). The framework of such models requires proper characterization of the metabolizable AA outflows from the rumen to support model development and evaluation. The omasal sampling technique developed by Huhtanen et al. (1997) and modified by Ahvenjärvi et al. (2000) provides useful data to assess the accuracy of model predictions of ruminal digestion and flow of AA to the small intestine.

The hypothesis of this study was that inclusion of a fermentation byproduct with soluble AA and peptides will increase post-ruminal non-ammonia N flow at the omasal canal. The specific objectives of this study were to 1) evaluate the effect of urea or soluble AA and peptides from a commercial fermentation byproduct on rumen digestion and omasal flows of nutrients, and 2) provide comparisons of model predicted vs. measured values for rumen N outflows.

MATERIALS AND METHODS

The experiment was conducted from April – July 2014 at the Cornell University Ruminant Center in Harford, NY. All animals involved in this experiment were cared for according to the guidelines of the Cornell University Animal Care and Use committee. The committee reviewed and approved the experiment and all procedures carried out in the study.

Animals and Experimental Design

Eight ruminally cannulated multiparous Holstein cows averaging (mean ± SD) 60 ± 10 DIM and 637 ± 38 kg of BW were enrolled in a 3 week pre-trial acclimation period where all animals were managed and housed in a tie-stall and individually fed a common diet. At the end of the 3 week period, animals were stratified by pre-trial milk production and randomly assigned to one of two treatment sequences in a switchback design. The trial consisted of three 28 d experimental periods, where each period contained 21 d for diet adaptation and 7 d of data and sample collection. All cows were injected on day 1 with bovine somatotropin (500 mg of Posilac, Elanco Animal Health, Greenfield, IN) and at 14 d intervals thereafter for the entire trial. Cattle were housed in individual tie-stalls with free access to water. Cows were milked 3 x daily at 06:00, 14:00, and 22:00 h through a parlor except during sampling periods, when cattle were milked in the tie-stalls. Milk yield was recorded and milk samples taken at each milking on day 21, 22, and 23 of each period and analyzed for fat, true protein, lactose, somatic cell count, total solids, and milk urea nitrogen at a commercial laboratory (DairyOne, Ithaca, NY). Body weights were measured weekly after the 14:00 h milking, and condition score was recorded weekly as the average of two trained scorers. Change in body weight was calculated as the difference between weights taken on day 28 of each period.

Treatment Administration and Sample Collection

Treatment diets contained (DM basis) 44% corn silage, 13% alfalfa silage, 12% ground corn, and 31% protein premix containing either a control mix of urea and wheat middlings (**CON**) or Fermenten (**EXP**) at 3% inclusion rate in the final diet (Table 2.1). Forages and other ingredients were analyzed for chemical composition for use in the CNCPS v. 6.5 using wet chemical methods by Cumberland Valley Analytical Services (Hagerstown, MD). Rumen degradable protein and NH_3-N balance for CON and EXP diets as predicted by the CNCPS v. 6.5 were 8.2 and 7.8 % of DM and 120 and 115% of NH_3-N requirement, respectively. The forage and corn grain portion of the diets were mixed daily as a single batch and delivered to the cattle housing facility, where the batch was split in half and either the CON or EXP protein premix was added to complete the treatment diets. Final mixing was done in a Super Data Ranger (American Calan Inc., Northwood, NH) and the resulting TMR was offered once daily at 07:00 h. Orts were collected and weights recorded at 06:00 h and feeding rate was adjusted daily to yield orts of 5 to 10 % of daily intake. Weekly samples of corn silage, alfalfa silage, corn grain, protein premixes, and TMR were analyzed for DM by drying at 60°C for 48 h and diets were adjusted to maintain intended formulation. Dried samples were ground through a 1-mm screen (Wiley no. 4 Mill, Arthur H. Thomas, Philadelphia, PA), composited by period and analyzed for nutrient composition (Tables 2.1 and 2.2). Intake of DM was calculated from DM determinations on TMR and orts. During sampling days, daily samples of TMR and orts were processed in the same manner as above, and equal parts DM from each sampling day were combined to create a sampling period composite for each cow within period.

Table 2.1. Chemical composition (mean ± SD)[1] of forages used in the experiment

Item	Corn silage	Alfalfa silage
DM, %	32.6 ± 0.7	33.7 ± 0.9
CP, % of DM	7.3 ± 0.4	21.8 ± 0.6
Soluble protein, % of CP	57.2 ± 2.7	61.3 ± 3.7
NDICP, % of CP	14.3 ± 1.2	10.7 ± 1.2
ADICP, % of CP	11.4 ± 0.3	8.8 ± 1.0
aNDFom, % of DM	40.0 ± 2.6	40.3 ± 2.0
30h uNDFom, % of aNDFom	46.2 ± 2.1	52.4 ± 3.0
120h uNDFom, % of aNDFom	29.6 ± 1.0	46.5 ± 2.7
240h uNDFom, % of aNDFom	25.1 ± 1.8	42.3 ± 2.6
ADF, % of DM	26.2 ± 2.2	34.2 ± 2.2
ADL, % of DM	3.2 ± 0.2	7.9 ± 0.6
Starch, % of DM	33.6 ± 1.8	1.0 ± 0.5
Ether extract, % of DM	3.5 ± 0.1	4.0 ± 0.3
Ash, % of DM	3.1 ± 0.1	11.0 ± 0.4

[1]Analyzed values from 3 period composite samples.

Table 2.2. Ingredient and nutrient composition (mean ± SD)[1] of experimental diets

	Diet	
Item	CON	EXP
Ingredient, % DM		
Corn silage	44.6	44.6
Alfalfa silage	12.0	12.0
Corn meal	12.0	12.0
Expeller soybean meal[2]	8.0	8.0
Soybean hulls	5.8	5.8
Citrus pulp, dry	3.3	3.3
Chocolate meal	2.4	2.4
Saturated fatty acid[3]	1.2	1.2
Molasses	0.9	0.9
Blood meal	1.7	1.7
Wheat middlings	4.8	3.2
Fermentation byproduct[4]	–	3.0
Calcium carbonate	–	0.7
Urea	0.4	–
Calcium sulfate, dihydrate	1.7	–
Sodium bicarbonate	0.33	0.40
Salt white	0.30	0.32
Magnesium oxide	0.17	0.17
Dicalcium phosphate	0.16	0.16
Supplemental methionine[5]	0.06	0.06
Vitamin and mineral mix[6]	0.18	0.18
Nutrient composition		
DM, %	44.5 ± 0.7	44.2 ± 0.8
OM, % of DM	93.9 ± 0.3	93.8 ± 0.6
CP, % of DM	15.9 ± 0.6	16.1 ± 0.5
RDP, % of DM[7]	8.4 ± 0.1	8.0 ± 0.1
Starch, % of DM	27.5 ± 1.1	27.8 ± 0.5
Sugars, % of DM	5.4 ± 0.4	5.3 ± 0.3
NFC, % of DM[7]	41.7 ± 0.2	41.8 ± 1.3
aNDFom, % of DM	30.9 ± 0.2	31.2 ± 0.2
ADF, % of DM	19.9 ± 1.5	19.7 ± 0.6
ADL, % of NDF	10.0 ± 0.9	10.0 ± 1.4
Ether extract, % of DM	5.0 ± 0.2	4.9 ± 0.2
ME, Mcal/kg[7]	2.5 ± 0.1	2.5 ± 0.1

[1]Analyzed values from 3 period composite samples.
[2]SOYPLUS (West Central Cooperative, Ralston, IA).
[3]ENERGY BOOSTER 100 (MSC Company, Dundee, IL).
[4]FERMENTEN (Church & Dwight, Inc., Princeton, NJ).
[5]SMARTAMINE M (Bluestar Adisseo Nutrition Group, Alpharetta, GA).
[6]Provided (per kg of diet DM): 44 mg of Zn, 32 mg of Mn, 10 mg of Cu, 1 mg of Co, 1 mg of I, 0.3 mg of Se, 5000 IU of vitamin A, 980 IU of vitamin B, and 25 IU of vitamin E.
[7]Calculated by the Cornell Net Carbohydrate and Protein System v. 6.5.

Marker Infusion and Omasal Sampling

During the last week of each period, cows entered the infusion and omasal sampling phase. A triple marker system using CoEDTA (Udén et al., 1980), $YbCl_3$ (modified from Siddons et al., 1985), and undegraded NDFom (**uNDFom**) (Raffrenato, 2011) were used to quantify liquid, small particle, and large particle flow at the omasal canal, respectively. Cobalt-EDTA and $YbCl_3$ were dissolved in distilled water and continuously infused into the rumen at rates of 2.8 g/d Co and 3.4 g/d Yb in 2.75 L of solution/d. All animals received a 3 L priming dose of the Co and Yb solution immediately prior to infusion start. On d 21 of each period, cattle were fitted with an indwelling catheter (Micro-renathane tubing, Braintree Scientific Inc., Braintree, MA) in the jugular vein for infusion of the microbial marker. Double-labeled urea ($^{15}N^{15}N$-urea, 98% purity, Cambridge Isotope Laboratories Inc., Andover, MA) in sterile saline (9 g NaCl/L) was continuously infused a rate of 150 mL/d, providing 0.675 g/d of $^{15}N^{15}N$-urea. Before starting the infusion, samples of whole ruminal contents, feces, urine, plasma, and rumen microbes were taken for determination of ^{15}N background. All markers were infused continuously from 14:00 h on d 21 until 10:00 h on d 28 of each period via peristaltic pump (Masterflex, Cole-Parmer Instrument Company, LLC, Vernon Hills, IL). All cows had at least 72 h of continuous infusion to reach uniform marker distribution before any sampling occurred, as suggested by Broderick and Merchen (1992) and conducted previously in our laboratory (Marini and Van Amburgh, 2003; Recktenwald, 2010).

Omasal samples were obtained using the omasal sampling technique developed by Huhtanen et al. (1997) and adapted by Reynal and Broderick (2005). Samples of whole omasal contents were collected from the omasal canal during three 8 h intervals: at 16:00, 18:00, 20:00, and 22:00 h on d 24; at 00:00, 02:00, 04:00, and 06:00 h on d 26; and at 08:00, 10:00, 12:00, and

14:00 on d 27. Sample times were chosen to encompass every 2 h of the average 24 h cycle. During each 8 h interval, a 425 mL spot sample was obtained at the first 3 time points, while 675 mL were taken at the last time point. Spot samples were split into subsamples of 50 mL (x2), 125 mL, and 200 mL; with an additional 250 mL subsample at the last time point. One of the 50 mL samples (**OF**) was acidified with 1 mL of 50% H_2SO_4, combined within period, and stored at -20°C for subsequent NH_3-N and VFA analysis, while the other was processed and stored for a separate investigation of soluble non-ammonia N flows. The 125 mL subsamples were held on ice and combined within interval, yielding a 500 mL sample for bacterial isolation. The 200 mL samples were combined within period and stored at -20°C, yielding a 2.4 L composite for digestion phase separation. The additional 250 mL sample obtained at the end of each interval was immediately processed to isolate protozoa (**OP**) as described in Chapter 3 for investigation of microbial nitrogen and amino acid flows.

The bacterial isolations from each 8 h shift were combined within period to yield an omasal bacteria (**OB**) sample for each cow within period. Isolation was performed according to Whitehouse et al. (1994) with modifications. Briefly; whole omasal contents were filtered through 4 layers of cheesecloth and solids were rinsed once with saline, and the filtrate (A) was treated with formalin (0.1% v/v in final solution) and stored at 4°C. The solids retained on the cheesecloth were incubated for 1 h a 39°C in a 0.1% methylcellulose solution, mixed for 1 min at low speed (Omni Mixer, Omni International, Kennesaw, GA) to detach solids associated bacteria, and held at 4°C for 24h. The contents were then squeezed through 4 layers of cheesecloth and the filtrate (B) was treated with formalin (0.1% v/v in final solution). Filtrates A and B were then combined and centrifuged at 1,000 x *g* for 5 min at 4°C to remove small feed particles and protozoa. The supernatant was centrifuged at 15,000 x *g* for 20 min at 4°C and the

bacterial pellet, representing both solid and liquid associated bacteria, was collected and stored at −20°C until lyophilization and later analysis.

Spot fecal and rumen fluid samples were taken at the same time points as omasal spot samples. Fecal samples were composited by period and stored at −20°C, while rumen fluid (**RF**) was acidified with 50% H_2SO_4 and composited by period before storage at -20°C. On day 24 of each period, a sample of whole rumen contents was taken 4 hours after feeding for isolation of rumen microbes. Spot urine and blood samples were taken at the second time point of each interval. Blood samples were collected into tubes containing sodium heparin, centrifuged (3,000 × *g* for 20 min at 4°C), and plasma was harvested and stored at −20°C. Urine samples were immediately acidified to pH < 2 with 50% H_2SO_4 and stored at −20°C. On the last day of each period, rumen contents were evacuated, weighed, mixed, and a representative sample was obtained and stored at −20°C. Rumen contents were returned to the cow via the rumen cannula.

Sample Processing and Chemical Analysis

Sampling period TMR and orts composites were analyzed for DM at 105°C for 6 h and ash according to AOAC (2005), and for total N using a combustion assay (Leco FP-528 N Analyzer, Leco Corp., St. Joseph, MI). Composited TMR and orts samples were analyzed for aNDFom (Mertens, 2002), and uNDFom after 240 h of in vitro incubation with rumen fluid, according to Raffrenato (2011). The 2.4 L pooled omasal composites were thawed and separated into omasal large particle (**LP**), small particle (**SP**) and liquid phase (**LQ**) as described in Reynal and Broderick (2005). All phase samples were freeze dried and either ground through a 1 mm screen on a Wiley mill (LP) or homogenized with a mortar and pestle (SP and LQ) before analysis. Concentration of Co and Yb was determined by ICM-MS in all phase samples (Cornell University Nutrient Analysis Laboratory, Ithaca, NY) and the LP and SP phases were analyzed

for uNDFom. All omasal phases were analyzed for DM, OM, aNDFom and total N as described previously for feed samples to determine ruminal digestion and flow parameters. Concentrations of Yb, Co, and uNDFom in each phase were used to calculate the concentration of each nutrient in a sample theoretically representing omasal true digesta (**OTD**) (France and Siddons, 1986). Composite fecal samples were thawed, thoroughly mixed, and a subsample was dried for 72 h at 60°C in a forced air oven. Subsamples from rumen evacuations were freeze-dried and the dried feces and rumen contents were ground to pass a 1 mm screen on a Wiley mill. Dry matter, OM, total N, aNDFom and uNDFom was determined on the dried ground feces and rumen contents as described above for pool size and fecal excretion calculations. Ammonia N concentration was determined in RF and OF using the colorimetric method of Chaney and Marbach (1962). Urea N concentration was determined in plasma and urine using an enzymatic colorimetric assay based on a commercial kit (No. 640, Sigma-Aldrich, St. Louis, MO). Volatile fatty acid concentration in RF and OF was determined by HPLC (Agilent 1100 series HPLC, Agilent Technologies, Santa Clara, CA) using crotonic acid as an internal standard (Siegfried et al., 1984).

Samples of OB, OP, rumen contents and omasal digesta phases were analyzed for NAN and ^{15}N as follows: 20 μg of N from each sample was weighted into tin capsules and 10 μL of 72 m*M* K_2CO_3 were added and incubated at 60°C overnight to volatilize ammonia. Samples were then analyzed for NAN and ^{15}N using a Carlo Erba NC2500 elemental analyzer interfaced with an isotope ratio mass spectrometer (Cornell University Stable Isotope Laboratory, Ithaca, NY). Samples of rumen bacteria, protozoa, and contents for natural abundance of ^{15}N were prepared and analyzed separately in the same manner as the enriched samples.

Calculations

Total N entering the omasal canal was partitioned into three fractions: Ammonia N, microbial N, and non-ammonia non-microbial N (**NANMN**). Ammonia N flow was determined using the concentration of ammonia N in the OF sample and the flow of liquid determined using the triple marker system. Total non-ammonia N (**NAN**) flow was calculated as the difference between total N and ammonia N. Microbial N flow was determined using ^{15}N atom percent excess (**APE**) in OTD and ^{15}N APE of the OB and OP samples. The APE was calculated for digesta and microbial samples for each cow within period as follows:

^{15}N APE = (enriched ^{15}N-atom% - mean natural ^{15}N-atom%) / mean natural ^{15}N-atom%

Mean natural abundance of ^{15}N in rumen bacteria, protozoa, and contents was 0.3684 (SD ± 0.0002). The natural abundance of ^{15}N in rumen bacteria, protozoa, and contents was assumed to be representative of OB, OP, and OTD, respectively. Protozoa N flow was calculated using gravimetric determinations from omasal liquid and protozoa biomass flow (described in Chapter 3). The ^{15}N APE in protozoa and bacteria was then used to calculate total microbial N flow:

Omasal protozoa NAN flow (g/d) = OP DM flow (g/d) x OP NAN (g/g DM) x OP ^{15}N APE (g/g NAN)

Omasal bacteria NAN flow (g/d) = [OTD NAN flow (g/d) x OTD ^{15}N APE (g/g N)] – [OP NAN flow (g/d) x OP ^{15}N APE (g/g NAN)] / OB ^{15}N APE (g/g NAN)

Microbial NAN flow (g/d) = OP NAN flow (g/d) + OB NAN flow (g/d)

The NAN content (g/g DM) of the OB and OP samples was used to calculate the flow of total microbial biomass. Flow of NANMN was calculated as the difference between total NAN flow

and microbial NAN flow. Endogenous N flows were not determined in this study, as such all NANMN was assumed to be dietary in origin. Therefore, RUP flow was estimated by multiplying NANMN by 6.25. Rumen degradable protein supply was calculated as total N intake minus RUP flow. Apparent and true ruminal digestibility of DM, OM, aNDFom and N were calculated as follows:

Apparent digestibility = nutrient intake – omasal nutrient flow

True digestibility = nutrient intake – (omasal nutrient flow – microbial nutrient flow)

where all intakes and flows are grams per day. Apparent total tract digestibility of DM and OM was determined using the fecal composite with uNDFom as an internal marker. Rumen and total tract digestibility of aNDFom can be considered true digestibility, as the use of sodium sulfite in the aNDFom procedure reduces microbial contamination (Van Soest, 2015).

Statistical Analyses

All data were analyzed using SAS version 9.3 (SAS Institute Inc. Cary, NC). Diet chemical composition was analyzed using PROC GLM and means were compared using the LSMEAN statement. All other data were analyzed using the MIXED procedure of SAS version 9.3. Due to slight negative effect of omasal sampling procedure on intake, milk production and associated intake were determined as the mean of 3 days before the infusion period began, while omasal parameters and associated nutrient intake were determined from data collected during the omasal sampling period. All variables were analyzed according to the following model:

$$Y_{ijkl} = \mu + S_i + C_{j:i} + P_k + T_l + ST_{il} + \varepsilon_{ijkl}$$

where Y_{ijkl} = dependent variable, μ = overall mean, S_i = fixed effect of sequence i, $C_{j:i}$ = random effect of cow within sequence, P_k = fixed effect of period k, T_l = fixed effect of treatment l, ST_{il}

= fixed interaction effect of sequence i and treatment l, and ε_{ijkl} = residual error. Degrees of freedom were calculated using the Kenward-Roger option. Means were determined using the least squares means statement, and treatment means were compared using the PDIFF option. Statistical significance was considered at $P \leq 0.05$ and trends were considered at $0.05 < P \leq 0.10$.

RESULTS AND DISCUSSION

Diets, animal performance, and rumen concentration of metabolites

Corn silage fed during the trial averaged 40.0 % aNDFom, 26.2% ADF and 33.6% starch, while alfalfa silage averaged 40.3% aNDFom, 34.2% ADF, and 21.8% CP (Table 2.1). Experimental diets were formulated to be iso-nitrogenous and iso-energetic. Model predicted RDP was decreased in EXP diets compared with CON (8.4 vs. 8.0% of DM; $P < 0.01$) as determined by the CNCPS v. 6.5 and was intended in diet formulation. Concentration of aNDFom tended to be greater in EXP diets (30.9 vs. 31.2% of DM; $P = 0.08$). All other analyzed nutrients were not different between diets ($P > 0.05$; Table 2.2).

Body weight change over the trial followed typical patterns for peak lactation dairy cattle, and was not affected by treatment. Condition score similarly was not affected (data not shown); all cows averaged 2.25 ± 0.14 (mean ± SD) for the duration of the trial. Degradable N source had no effect on intake or daily milk, protein or fat production (Table 2.3). This trial was not specifically designed to assess effects on milk production however; numerical differences were present and similar in direction and magnitude as low sugar diets reported by Penner et al. (2009). Milk urea N and plasma urea N concentration increased ($P = 0.01$) in cows fed the EXP diet. The relationship between rumen NH_3-N and plasma urea N is displayed in Figure 2.1. The

slopes of the lines were not different between treatments (0.63 for both CON and EXP; $P = 0.67$) and were numerically similar to the results observed by Recktenwald et al. (2014).

Table 2.3. Effect of rumen available nitrogen source on dry matter intake, milk production, and animal performance

	Diet[1]			
Item[2]	CON	EXP	SEM	*P*
Dry matter intake, kg/d	25.5	26.4	0.9	0.34
Milk yield, kg/d	41.7	43.1	1.4	0.36
ECM, kg/d	41.7	43.1	1.9	0.48
Milk fat, %	3.53	3.50	0.11	0.77
Milk fat, kg/d	1.47	1.51	0.08	0.60
Milk true protein, %	2.85	2.86	0.07	0.86
Milk true protein, kg/d	1.19	1.22	0.06	0.55
Milk urea N, mg/dL	10.5	13.0	0.4	<0.01
Plasma urea N, mg/dL	8.7	11.0	0.7	0.01
Urine urea N, mg/dL	30.4	48.1	19.2	0.37
Feed efficiency[3]	1.64	1.64	0.06	0.97
Body weight change, kg/d	0.29	0.39	0.12	0.58

[1]CON = 3% of diet DM as urea control mix; EXP = 3% of diet DM as fermentation byproduct.
[2]Values calculated from data collected on d 19-21 of each experimental period.
[3]ECM/DMI.

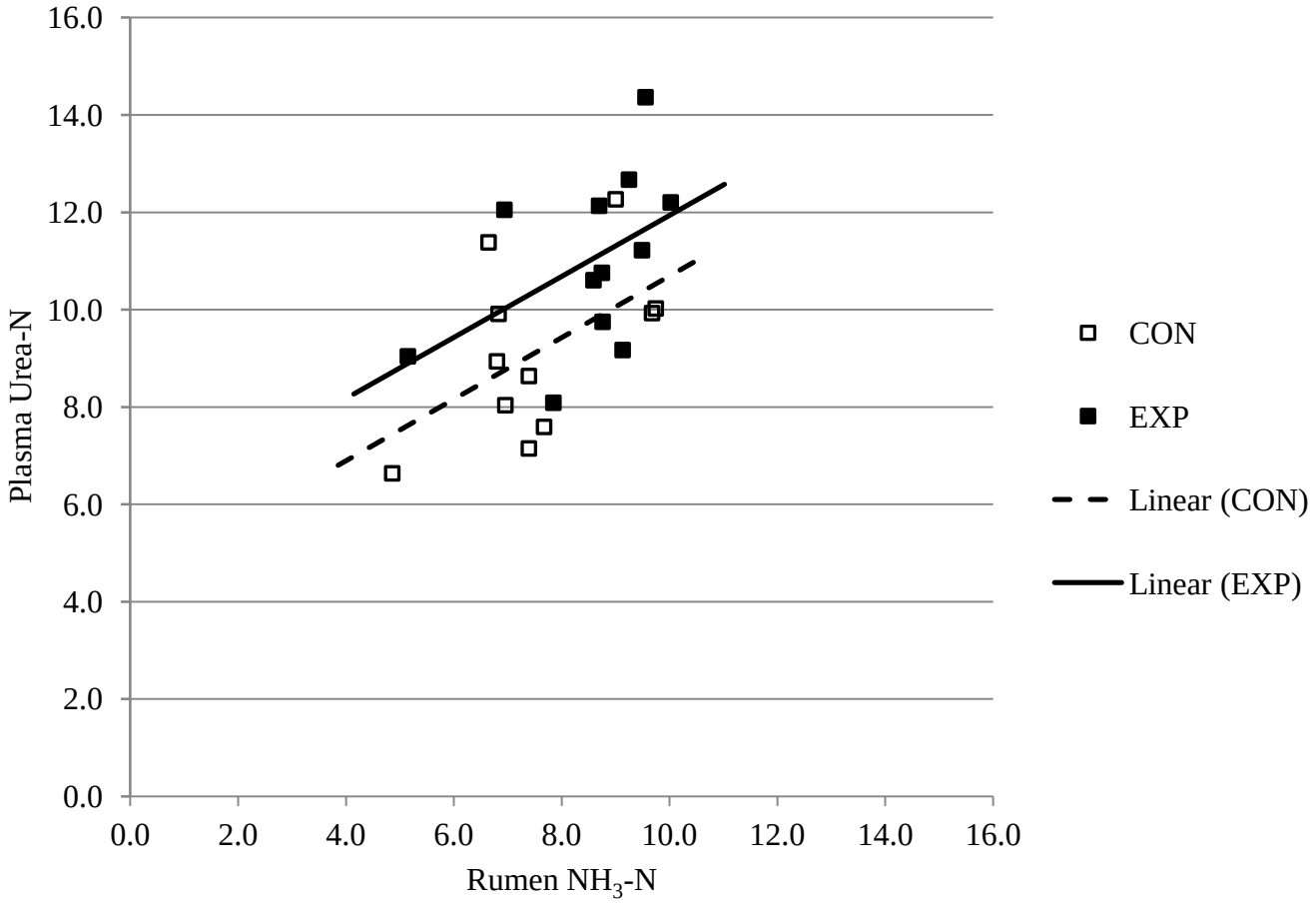

Figure 2.1. Relationship between rumen NH_3-N and plasma urea N in lactating dairy cows fed two different sources of rumen available N, where CON (□) = 3% of diet DM as urea control mix; EXP (■) = 3% of diet DM as fermentation byproduct meal. The equation representing relationship in cattle fed diet CON is $y = 0.6338x + 4.356$, $R^2 = 0.27$; the equation describing the relationship in cattle fed diet EXP is $y = 0.6274x + 5.663$, $R^2 = 0.22$.

Rumen NH_3-N pool size and concentration was increased ($P < 0.01$) in EXP cows (Table 2.4). Ammonia-N concentrations of CON diets were very close to the minimal optimal concentration of 5 mg NH_3-N/dL to support efficient microbial growth as recommended by Satter and Slyter (1974). This is consistent with the desired formulation of rumen available N in order to determine the effect of the fermentation byproduct on microbial N use. The compositing of samples done in the current experiment limit the ability to investigate temporal fluctuations in rumen NH_3-N concentrations; it is likely that both diets experienced some time below 5 mg/dL. Using VFA concentrations and ruminal digestibilities (Table 2.5) as an indicator, it would appear that any possible N limitations did not strongly affect microbial metabolism or their ability to degrade carbohydrate. No differences were seen in VFA concentration or pool size between degradable nitrogen sources.

Table 2.4. Effect of rumen available nitrogen source on rumen concentration and pool size[1] of ammonia N and volatile fatty acids (VFA)

	Diet[2]			
Item	CON	EXP	SEM	*P*
Ammonia N pool size, g	4.50	5.24	0.45	0.02
Ammonia N concentration, mg/dL	5.41	6.41	0.39	0.01
VFA pool size, mol				
Total VFA	8.05	8.12	0.31	0.81
Acetate (A)	5.23	5.30	0.16	0.71
Propionate (P)	1.87	1.87	0.14	0.95
Butyrate	0.73	0.73	0.03	0.97
Isobutyrate	0.02	0.02	0.00	0.87
Valerate	0.10	0.11	0.01	0.45
Isovalerate	0.09	0.10	0.01	0.56
Branched-chain VFA	0.12	0.12	0.01	0.76
A:P ratio, mol/mol	2.96	2.88	0.16	0.62
VFA concentration, m*M*				
Total VFA	97.3	99.3	3.0	0.48
Acetate	63.6	64.8	2.2	0.55
Propionate	22.1	23.0	1.0	0.55
Butyrate	8.9	9.0	0.4	0.69
Isobutyrate	0.3	0.3	0.1	0.77
Valerate	1.2	1.3	0.1	0.30
Isovalerate	1.1	1.2	0.1	0.53
Branched-chain VFA	1.4	1.5	0.2	0.78

[1]Nutrient concentration x rumen liquid volume measured from total rumen evacuation.
[2] CON = 3% of diet DM as urea control mix; EXP = 3% of diet DM as fermentation byproduct.

Table 2.5. Effect of rumen available nitrogen source on digestibility of DM, OM, and NDF

	Diet[1]			
Item[2]	CON	EXP	SEM	*P*
DM				
Intake, kg/d	23.8	23.9	0.7	0.91
Flow at omasal canal, kg/d	16.7	16.1	0.6	0.41
Apparently digested in the rumen, kg/d	7.1	7.9	0.4	0.15
Truly digested in the rumen, kg/d[3]	14.3	14.2	0.4	0.90
% of DM intake	60.3	59.6	1.4	0.72
Total tract apparent digestibility, %	68.6	68.2	0.5	0.47
OM				
Intake, kg/d	22.1	22.0	0.6	0.95
Flow at omasal canal, kg/d	13.4	12.8	0.5	0.39
Apparently digested in the rumen, kg/d	8.7	9.3	0.4	0.30
Truly digested in the rumen, kg/d[3]	15.0	14.9	0.4	0.77
% of OM intake	68.2	67.4	1.6	0.73
Total tract apparent digestibility, %	70.9	69.2	1.0	0.07
NDF				
Intake, kg/d	7.3	7.5	0.2	0.72
Flow at omasal canal, kg/d	5.1	5.0	0.2	0.70
Apparently digested in the rumen, kg/d	2.3	2.5	0.1	0.18
% of NDF intake	31.2	33.4	1.3	0.24
% of pdNDF intake	44.9	47.4	1.9	0.36
Total tract apparent digestibility, %				
% of NDF intake	41.0	40.8	1.0	0.89
% of pdNDF intake	59.0	57.8	1.3	0.49

[1]CON = 3% of diet DM as urea control mix; EXP = 3% of diet DM as fermentation byproduct.
[2]Values calculated from data collected on d 24-27 of each experimental period.
[3]Corrected for microbial and volatile fatty acid contribution to flows.

Rumen and total tract digestion of DM, OM, and NDF

Intake during the omasal sampling period was not different between diets ($P > 0.05$; Table 2.5). Slight disturbance of the cattle during sampling procedures might have reduced intakes during the omasal sampling period, therefore separate intakes are reported for the milk production data vs. the omasal sampling data (Tables 2.3 and 2.5, respectively). The average DM and OM digestibility in the experiment was 59.9 and 67.7 %, respectively, and were not different between treatments. Rumen aNDFom digestibility averaged 31.2 and 33.4 for diets CON and EXP, respectively ($P = 0.24$) when expressed as a percent of total aNDFom. Digestion of the potentially digestible pool was not different among treatments, and averaged 44.9 and 47.4 for diets CON and EXP, respectively ($P = 0.36$) True OM and DM digestion in the rumen was within the range reported by Huhtanen et al. (2010). Rumen aNDFom digestion was slightly lower than the mean determined by Huhtanen et al. (2010), however values observed in this study were similar those reported in studies performed with typical North American diets (Brito et al., 2006; 2007). The lack of response in aNDFom digestion to ruminal protein source has been observed previously when degradable protein sources were compared (Robinson, 1997; Brito et al., 2007). Arroquy et al. (2004) also reported no effect of RDP source on NDF or OM digestion in steers fed low quality forage. Apparent total tract OM digestibility tended to be lower in cows fed EXP diets (69.2 *vs*. 70.9 % for CON and EXP respectively; $P = 0.07$) although this is likely of limited biologic significance.

Omasal Nitrogen Flows and Ruminal N Digestibility

Nitrogen intake was similar between the two diets (Table 2.6). Compared to CON diets, the inclusion of the fermentation byproduct in EXP diets shifted 18 g/d N from the ammonia N pool to the soluble true protein pool, according to the CNCPS v. 6.5 protein fractionation scheme (Van Amburgh et al., 2013). Flow of NAN was not different between diets. Non-ammonia non-microbial N flow was numerically increased in cows fed the EXP diet (191 vs. 256 g/d for CON and EXP, respectively; $P = 0.09$) Microbial NAN flow as a percent of total flow was 69.9 and 61.5 % for CON and EXP, respectively ($P = 0.11$); absolute gram amounts were not different ($P = 0.31$). These values and slight decrease in flow associated with degradable protein source were similar to previous omasal studies investigating degradable protein supplementation in cows fed North American diets (Reynal et al., 2003; Brito et al., 2007).

A meta-analysis of continuous culture studies with fermentation byproducts has previously shown positive effects on microbial N flow from diets containing fermentation byproducts (Lean et al., 2005). Similar effects on microbial flows were not observed in the current study. The disparity between observations might be accounted for by the difference in diets, inclusion rates of the byproducts, and types of microbial markers used. Purines were used as a microbial marker in the Lean et al. (2005) study, and the fermentation byproducts might contain appreciable amounts of purines due to their microbial origin. Observed increases in microbial flows in that study might simply have been from an increased dietary supply of purines compared with the control diets. Previous in vivo work with several fermentation byproducts feeds also showed no significant difference from a urea control (Broderick et al., 2000), although microbial yield was also estimated from purine derivatives.

Table 2.6. Effect of rumen available nitrogen source on omasal nitrogen flow and digestibility

	Diet[1]			
Item[2]	CON	EXP	SEM	*P*
N intake, g/d	603	613	18	0.70
CNCPS fraction PA1	61	43	-	-
CNCPS fraction PA2	171	183	-	-
CNCPS fraction PB1	304	310	-	-
RDP Supply[3]				
g/d	2578	2230	117	0.05
% of DMI	10.9	9.4	0.6	0.07
Flow at omasal canal				
Total N, g/d	664	693	25	0.37
Total N flow predicted by CNCPS v. 6.5, g/d	664	674	-	-
Ammonia N, g/d	21.5	22.4	1.5	0.67
NAN				
g/d	642	670	25	0.38
% of N intake	106.6	109.1	3.4	0.58
NANMN				
g/d	191	256	26	0.09
% of N intake	31.3	41.7	3.5	0.05
RUP[4]				
g/d	1192	1601	159	0.09
% of DMI	5.0	6.7	0.6	0.04
RUP flow predicted by CNCPS v. 6.5, g/d	1784	1887	-	-
Microbial NAN				
g/d	450	409	28	0.31
% of total NAN	69.9	61.5	3.5	0.11
Microbial N flow predicted by CNCPS v. 6.5, g/d	351	352	-	-
Microbial efficiency				
g of microbial CP/kg of OTDR	28.9	26.1	1.7	0.26
True ruminal N digestibility, %	68.7	58.3	3.5	0.05
aNDFom digested/g of dietary CP degraded	0.97	1.23	0.1	0.02

[1]CON = 3% of diet DM as urea control mix; EXP = 3% of diet DM as fermentation byproduct.
[2]NANMN = non-ammonia non-microbial N, OTDR = organic matter truly digested in the rumen.
[3]Rumen degradable protein (RDP) supply = CP intake – RUP flow.
[4]Rumen undegradable protein (RUP) = NANMN x 6.25.

In the present study, the 65 gram difference in NANMN outflow was more than 3 times the 18 g difference in true soluble protein inflow associated with diet composition and intake. This indicates that the inclusion of the fermentation byproduct in EXP diets had an associative effect on degradation of other feedstuffs in the rumen. In effect, sparing of rumen degradable protein allowed feed true protein to escape the rumen and provide metabolizable protein to the cow. Thus, when using NANMN to calculate diet rumen undegraded protein concentration, diets contained 5.0 and 6.7 % of diet DM as RUP in CON and EXP diets, respectively ($P = 0.04$). True ruminal N digestibility was 15% lower in EXP diets (68.7 vs. 58.3 % for CON and EXP, respectively; $P = 0.05$). No differences were observed in efficiency of microbial CP synthesis / g of OM digested in the rumen. The observed degradable protein sparing effect resulted in more efficient microbial degradation of fiber (0.97 vs. 1.23 g of aNDFom digested / g of dietary CP degraded for CON and EXP diets, respectively; $P = 0.02$). These results indicate possible stimulation of fiber degrading populations in the rumen. Uptake of AA N by cellulolytic bacteria has previously been assumed to be minimal to non-existent, resulting in the assumption that NH_3-N is the sole source of N for microbial protein synthesis (Russell et al., 1992). More recent studies have clearly demonstrated stimulatory effects of AA N on cellulolytic populations (Atasoglu et al., 2001; Yang, 2002). Ruminal N degradation was decreased in cows fed the EXP diet, rumen NH_3-N pool size, concentration, and plasma urea N all increased relative to cows fed the CON diet. The dynamics of rumen proteolysis, amino acid uptake and disposal, and urea recycling are likely key to understanding this system. While urea N recycling was not determined in this study, it is possible that increased urea entry from the plasma pool allowed for elevated rumen NH_3-N levels (Marini and Van Amburgh, 2003; Valkeners et al., 2007). Additionally, even though total protein degradation was decreased, it is possible that soluble

protein and amino acids stimulated specific populations of bacteria with high affinity for such substrate (Russell et al., 1988). Previous in vitro work has demonstrated that peptide degradation and uptake is a rate limiting step (Broderick and Craig, 1989; Wallace et al., 1990), however elevated NH_3-N concentration typically follows greater protein degradation in batch cultures due to the closed nature of the system. In the rumen, solubilization of proteins and subsequent hydrolysis allows peptides and amino acids to enter and flow with the liquid pool, thus escaping further degradation in the rumen. In the current experiment, it is possible that hydrolysis of proteins might be enhanced and/or peptide and amino acid fermentation depressed through an unknown mechanism, resulting in increased undegraded feed N flow. The increased ammonia N pool size in the rumen likely results from both intra-ruminal recycling and urea influx from the plasma pool.

The sparing effect on degradable peptides and amino acids also presents a key opportunity to utilize fermentation byproduct meal in conjunction with less expensive homegrown forages and protein feedstuffs such as alfalfa silage and untreated soybean meal. In such diets, overfeeding of degradable protein is common, as supply of metabolizable protein can be insufficient even at high levels of dietary crude protein. Future studies might investigate the ability of targeted feeding of degradable protein sources with the fermentation byproduct to increase the income over feed cost of nitrogen efficient rations.

CNCPS-Predicted vs. Observed N Flows

Cattle characteristics, diet composition and intakes were entered into the CNCPS v. 6.5, and the model was used to predict total omasal N flow, microbial N flow, and rumen undegraded protein flow. Due to individual animal variation and the limited number of independent observations, observed vs. predicted flow comparisons are on a numerical basis only (Table 2.6).

Observed RUP was calculated as NANMN x 6.25. This makes the assumption that all NANMN is of feed origin, neglecting any contribution of non-^{15}N endogenous N contributions (Lapierre et al., 2008). Total omasal N flow was well predicted by the model, while microbial N flow appeared to be under-predicted. Alternatively, recent evaluations of CNCPS v6.5 (Van Amburgh et al., 2015) against an omasal study showed good agreement between predicted and observed microbial N flows. Prediction of RUP flow was similar to observed values for EXP diets, while CON diets RUP flow was over-predicted, indicating the model is not accounting for protein sparing effect of fermentation byproducts. Within the structure of the model, microbial populations are stimulated when peptide balance is positive (Russell et al., 1992), however the assigned rates of degradation of many feedstuffs results in high peptide balance in most simulations. Updates to the feed library (Higgs et al., 2015) and model (Van Amburgh et al., 2010; Van Amburgh et al., 2015) have sought to correct this; however the current structure of the rumen sub-model in v. 6.5 has limited the ability to describe microbial N dynamics in a more mechanistic way, especially the interactions and associative affects between bacteria and protozoa and that most likely biases the predictions of microbial flow.

CONCLUSIONS

Degradable protein source, when provided as urea or soluble AA and peptides altered omasal flow of N fractions. A dietary difference of an additional 18 grams of soluble AA N resulted in 65 grams more NANMN flow from the omasum. This corresponds to a 15% decrease in ruminal protein degradation. Neutral detergent fiber was more efficiently digested in the rumen when expressed per gram of protein degraded, indicating possible stimulation of cellulose degrading microbes when providing soluble peptides and AA. The inclusion of Fermenten in the EXP diets did not increase total microbial N flow as has previously been observed in vitro; however NANMN flow as a percent of intake was increased. Results from this study demonstrate that stimulation of microbial populations does not always result in increased microbial protein flows post-ruminally. Subsequent research will focus on the effect of Fermenten on bacterial vs. protozoal N flows, along with omasal AA flows and digestibility.

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CHAPTER 3: Rumen digestion kinetics, microbial yield, and omasal flows of non-microbial, bacterial and protozoal amino acids in lactating dairy cattle fed fermentation byproduct or urea as a soluble nitrogen source.

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ABSTRACT: Eight ruminally cannulated multiparous Holstein cows averaging 60 ± 10 days in milk and 637 ± 38 kg of body weight were randomly assigned to one of two treatment sequences in a switchback design. Treatment diets contained (dry matter basis) 44% corn silage, 13% alfalfa silage, 12% ground corn, and 31% protein premix containing either a control mix of urea and wheat middlings (**CON**) or a commercial fermentation byproduct meal at 3% diet inclusion rate (**EXP**). The trial consisted of three 28 d experimental periods, where each period consisted of 21 d of diet adaptation and 7 d of data and sample collection. Digestion markers were infused continuously during the sampling period and composited omasal samples were used to calculate protozoal, bacterial, and non-microbial omasal flow of amino acids. Rumen pool sizes and omasal flows were used to determine digestion parameters, including fractional rates of carbohydrate digestion, microbial growth, and yield of microbial cells per gram of degraded substrate. Fermentation byproduct inclusion in EXP diets increased microbial N and amino acid N content in microbes relative to microbes from CON cows fed the urea control. Microbial amino acid profile did not differ between diets. Daily omasal flows of AA were increased in EXP cows as a result of decreased degradation of feed protein. The inclusion of the fermentation

byproduct increased non-microbial AA flow in cows fed EXP vs. CON. Average protozoa contribution to microbial N flow was 16.8 %, yet protozoa accounted for 21 % of the microbial EAA flow, with a range of 8 to 46 % for individual AA. Cows in this study maintained an average rumen pool size of 320 g of microbial N and bacterial and protozoal pools were estimated at 4 different theoretical levels of selective protozoa retention. Fractional growth rate of all microbes was estimated to be 0.069 h^{-1}, with a yield (g microbes) of 0.44 g / g of carbohydrate degraded. Results indicated that fermentation byproduct can increase omasal flow of AA while maintaining adequate rumen N available for microbial growth and protein synthesis. Simulations from a new dynamic version of the Cornell Net Carbohydrate and Protein System indicated strong agreement between predicted and observed values, with some areas key for improvement in AA flow and bacterial vs. protozoal N partitioning.

Keywords: rumen protozoa, amino acids, microbial growth, CNCPS, Fermenten

INTRODUCTION

Byproducts of human food production have successfully been used to improve the sustainability of the dairy industry (VandeHaar and St-Pierre, 2006). Efficient and effective use of byproduct feeds requires adequate knowledge of the fermentation characteristics of the feed. Fermenten (Church & Dwight, Inc., Princeton, NJ) is a commercially available fermentation byproduct feed derived from glutamic acid production and contains high amounts of rumen available nitrogen compounds in the form of soluble AA and small peptides. Lean et al. (2005) reported that Fermenten might increase the flow of microbial protein from continuous fermenters with rumen microbes through stimulation of microbial protein synthesis (Cotta and Russell, 1982); however production responses in vivo have been inconsistent (Broderick et al., 2000, Penner et al., 2009). Research in our lab (Chapter 2) demonstrated that fermentation byproduct

decreased dietary protein degradation in the rumen by approximately 15%, indicating a possible sparing effect of degradable protein. The results from this study warranted further investigation into possible effects on omasal AA flow, the partition between microbial and non-microbial fractions, and the effects on microbial growth and digestion parameters.

Mathematical models such as the Cornell Net Carbohydrate and Protein system (**CNCPS**) (Higgs et al., 2015, Van Amburgh et al., 2015) have been successfully used to optimize rumen microbial output and meet animal nutrient requirements while reducing N losses to the environment (Tylutki et al., 2008). A new, dynamic version of the CNCPS was developed (Higgs, 2014) that describes rumen degradation of substrates with mechanistic representations of growth of bacteria and protozoa and includes interactions among protozoa and bacteria such as predation and intra-ruminal microbial N turnover. Evaluations of this model indicated a strong ability to predict the partitioning between microbial and non-microbial nitrogen flows; however the partitioning between protozoa and bacteria along with individual AA predictions might require further refinement. As with most model development, evaluations of the rumen sub-model with independent data can be helpful for determining areas for improvement.

The hypothesis of this study was that the decreased ruminal protein degradation associated with fermentation byproduct inclusion would increase AA flow at the omasal canal, with limited effects on microbial growth and turnover. The objectives of this study were to 1) evaluate the effect of urea or soluble AA and peptides from a commercial fermentation byproduct on omasal flows of non-microbial, bacterial, and protozoal flows of AA, and 2) provide comparisons of model predicted vs. measured values for rumen microbial digestion and growth parameters.

MATERIALS AND METHODS

The experiment was conducted from April – July 2014 at the Cornell University Ruminant Center in Harford, NY. All animals involved in this experiment were cared for according to the guidelines of the Cornell University Animal Care and Use committee. The committee reviewed and approved the experiment and all procedures carried out in the study.

Animals, Treatments and Experimental Design

Eight ruminally cannulated multiparous Holstein cows averaging (mean ± SD) 60 ± 10 d in milk and 637 ± 38 kg of body weight were stratified by pre-trial milk production and randomly assigned to one of two treatment sequences in a switchback trial with three 28 d periods. Each period contained 21 d for diet adaptation and 7 d of data and sample collection. All cows were allowed a 3 week pre-trial acclimation period where animals were managed and housed in a tie-stall and individually fed a common diet. Details of the cattle housing, milking, and feeding management are described in Chapter 2. Treatment diets contained (DM basis) 44% corn silage, 13% alfalfa silage, 12% ground corn, and 31% protein premix containing either a control mix of urea and wheat middlings (**CON**) or Fermenten (**EXP**) at 3% inclusion rate in the final diet (Table 3.1). Rumen degradable protein and NH_3-N balance for CON and EXP diets as predicted by CNCPS v6.5 (Van Amburgh et al., 2015) were 8.2 and 7.8 % of DM and 120 and 115% of NH_3-N requirement, respectively.

Table 3.1. Ingredient and nutrient composition (mean ± SD)[1] of experimental diets

	Diet	
Item	CON	EXP
Ingredient, % DM		
Corn silage	44.6	44.6
Alfalfa silage	12.0	12.0
Corn meal	12.0	12.0
Expeller soybean meal[2]	8.0	8.0
Soybean hulls	5.8	5.8
Citrus pulp, dry	3.3	3.3
Chocolate meal	2.4	2.4
Saturated fatty acid[3]	1.2	1.2
Molasses	0.9	0.9
Blood meal	1.7	1.7
Wheat middlings	4.8	3.2
Fermentation byproduct[4]	–	3.0
Calcium carbonate	–	0.7
Urea	0.4	–
Calcium sulfate, dihydrate	1.7	–
Sodium bicarbonate	0.33	0.40
Salt white	0.30	0.32
Magnesium oxide	0.17	0.17
Dicalcium phosphate	0.16	0.16
Supplemental methionine[5]	0.06	0.06
Vitamin and mineral mix[6]	0.18	0.18
Nutrient composition		
DM, %	44.5 ± 0.7	44.2 ± 0.8
OM, % of DM	93.9 ± 0.3	93.8 ± 0.6
CP, % of DM	15.9 ± 0.6	16.1 ± 0.5
RDP, % of DM[7]	8.4 ± 0.1	8.0 ± 0.1
Starch, % of DM	27.5 ± 1.1	27.8 ± 0.5
Sugars, % of DM	5.4 ± 0.4	5.3 ± 0.3
NFC, % of DM[7]	41.7 ± 0.2	41.8 ± 1.3
aNDFom, % of DM	30.9 ± 0.2	31.2 ± 0.2
ADF, % of DM	19.9 ± 1.5	19.7 ± 0.6
ADL, % of NDF	10.0 ± 0.9	10.0 ± 1.4
Ether extract, % of DM	5.0 ± 0.2	4.9 ± 0.2
ME, Mcal/kg[7]	2.5 ± 0.1	2.5 ± 0.1

[1]Analyzed values from 3 period composite samples. Table is reproduced from Chapter 2
[2]SOYPLUS (West Central Cooperative, Ralston, IA).
[3]ENERGY BOOSTER 100 (MSC Company, Dundee, IL).
[4]FERMENTEN (Church & Dwight, Inc., Princeton, NJ).
[5]SMARTAMINE M (Bluestar Adisseo Nutrition Group, Alpharetta, GA).
[6]Provided (per kg of diet DM): 44 mg of Zn, 32 mg of Mn, 10 mg of Cu, 1 mg of Co, 1 mg of I, 0.3 mg of Se, 5000 IU of vitamin A, 980 IU of vitamin B, and 25 IU of vitamin E.
[7]Calculated by the Cornell Net Carbohydrate and Protein System v. 6.5.

Sample Collection and Processing

Digesta flow leaving the rumen was quantified using the omasal sampling technique developed by Huhtanen et al. (1997) and adapted by Reynal and Broderick (2005). A triple marker system using CoEDTA (Udén et al., 1980), $YbCl_3$ (modified from Siddons et al., 1985), and undegraded aNDFom (**uNDFom**) were used to quantify liquid, small particle, and large particle flow at the omasal canal, respectively. Double-labeled urea ($^{15}N^{15}N$-urea, 98% purity, Cambridge Isotope Laboratories Inc., Andover, MA) was infused into the jugular vein for use as a microbial marker following the method used for studies on urea recycling (Lobley et al., 2000). Details on marker preparation and infusion are reported in Chapter 2.

Samples of whole omasal contents were collected from the omasal canal during three 8 hour intervals: at 16:00, 18:00, 20:00, and 22:00 h on d 24; at 00:00, 02:00, 04:00, and 06:00 h on d 26; and at 08:00, 10:00, 12:00, and 14:00 on d 27. Sample times were chosen to encompass every 2 h of the average 24 h cycle. During each 8 h interval, a 425 mL spot sample was obtained at the first 3 time points, while 675 mL were taken at the last time point. Spot samples were split into subsamples of 50 mL (x2), 125 mL, and 200 mL; with an additional 250 mL subsample at the last time point. The 50 mL subsamples were used for a separate study of nutrient flows (Chapter 2). The 125 mL subsamples were held on ice and combined within interval, yielding a 500 mL sample for bacterial isolation. The 200 mL samples were combined within period and stored at -20°C, yielding a 2.4 L composite for digestion phase separation. The additional 250 mL sample obtained at the end of each interval was strained through 2 layers of cheesecloth and immediately processed to isolate protozoa.

The 2.4 L pooled omasal composites were thawed and separated into omasal large particle (**LP**), small particle (**SP**) and liquid phase (**LQ**) as described previously (Chapter 2).

Concentrations of Yb, Co, and uNDFom in each phase were used to calculate the concentration of each nutrient in a theoretical sample representing omasal true digesta (**OTD**) (France and Siddons, 1986). All phase samples were freeze dried and either ground through a 1 mm screen on a Wiley mill (LP) or homogenized with a mortar and pestle (SP and LQ) before analysis. On the last day of each period, rumen contents were evacuated, weighed, mixed, and a representative sample was obtained for pool size determinations and stored at −20°C prior to lyophilization and determination of rumen nutrient pool sizes.

The bacterial isolations from each 8 h sampling period were combined within period to yield and omasal bacteria sample for each cow within period. Microbial isolation was performed according to Whitehouse et al. (1994) with modifications. Briefly; whole rumen contents were filtered through 4 layers of cheesecloth and solids were rinsed once with saline, and the filtrate (A) was treated with formalin (0.1% v/v in final solution) and stored at 4°C. The solids retained on the cheesecloth were incubated for 1 h a 39°C in a 0.1% methylcellulose solution, mixed for 1 min at low speed (Omni Mixer, Omni International, Kennesaw, GA) to detach solids associated bacteria, and held at 4°C for 24h. The contents were then squeezed through 4 layers of cheesecloth and the filtrate (B) was treated with formalin (0.1% v/v in final solution). Filtrates A and B were then combined and centrifuged at 1000 x *g* for 5 min at 4°C to remove small feed particles. The supernatant was centrifuged at 15,000 x *g* for 20 min at 4°C and the bacterial pellet, representing both solid and liquid associated bacteria, was collected and stored at -20°C until lyophilization and later analysis.

Protozoa were isolated from whole omasal contents using the procedure described by Denton et al. (2015) with modifications (Figure 3.1). Strained omasal fluid (250 mL) was combined 1:1 with pre-warmed, anaerobically prepared Simplex type buffer and added to a pre-warmed

separatory funnel. Plant particles were removed by aspiration after 1 h of incubation at 39°C, allowing for removal of 50 mL of fluid to a pre-calibrated 450 mL line on the funnel. Funnel contents were then preserved with formalin (0.1% v/v in final solution) and stored for < 4 d at 4°C. At the end of each sampling period, preserved contents were centrifuged at 1000 x *g* for 5 min, the pellet was re-suspended in saline, and protozoa were isolated on a nylon cloth with a 20 μm pore size (14% open area, Sefar, Buffalo, NY). The protozoa isolate was washed several times with saline (500 mL) to reduce bacterial contamination. Microscopic inspection of the retained protozoa and filtrate indicated low feed contamination and good recovery of small protozoa. After isolation, protozoa were stored at -20°C, followed by lyophilization and measurement of DM amount to calculate yield of protozoal DM per L of omasal fluid (Ahvenjärvi et al., 2002).

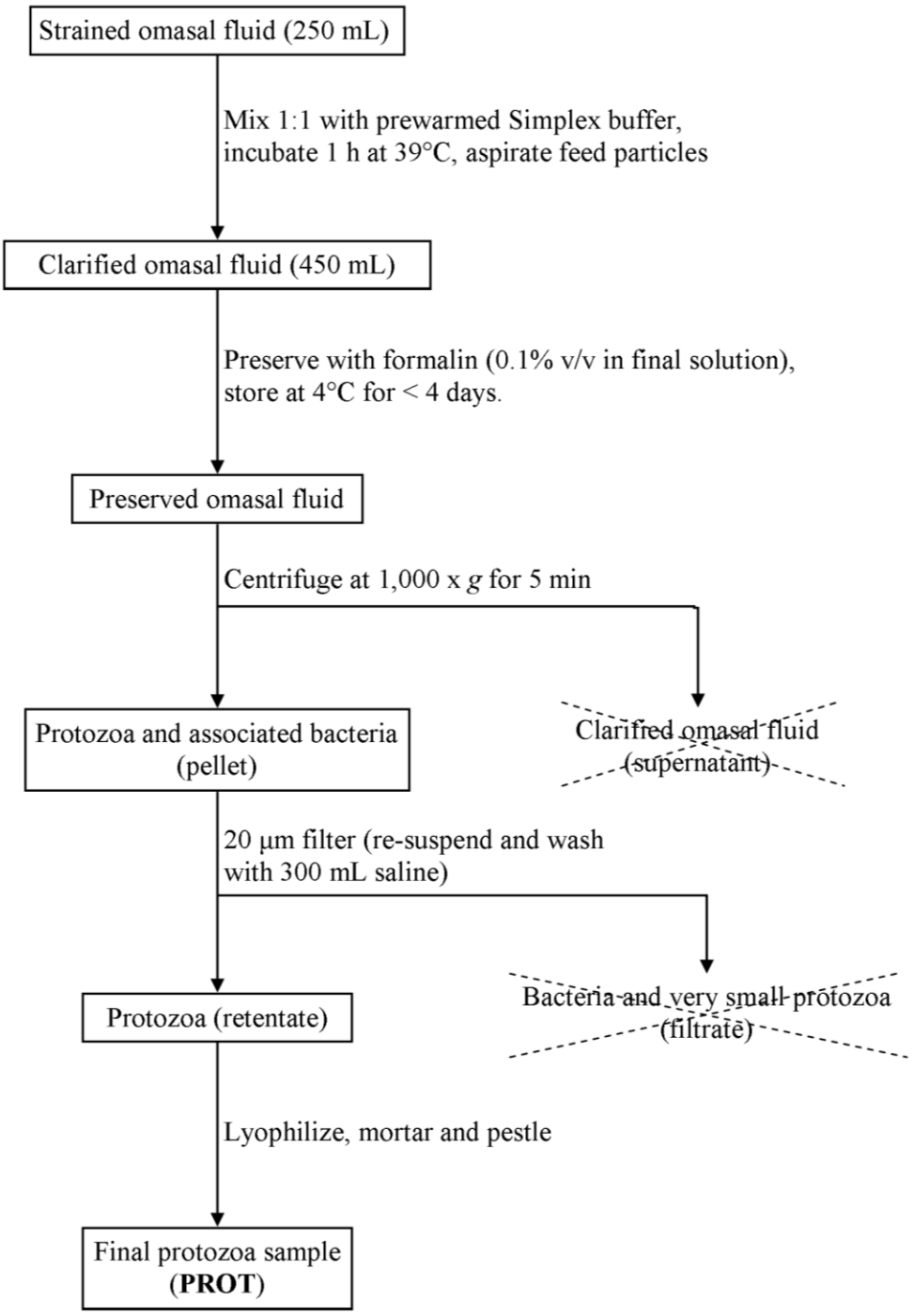

Figure 3.1. Flowchart for preparation of protozoa isolates. Fractions discarded are crossed out.

Sample Analysis

Samples of bacteria, protozoa, omasal fractions and rumen contents were analyzed for DM after 6 h at 105°C and ash according to AOAC (2005). Total N was determined using a combustion assay (Leco FP-528 N Analyzer, Leco Corp., St. Joseph, MI). Samples were analyzed for non-ammonia nitrogen (**NAN**) and ^{15}N as follows: 20 μg of N from each sample was weighed into tin capsules and 10 μL of 72 m*M* K_2CO_3 were added and incubated at 60°C overnight to volatilize ammonia. Samples were then analyzed for NAN and ^{15}N using a Carlo Erba NC2500 elemental analyzer interfaced with an isotope ratio mass spectrometer (Cornell University Stable Isotope Laboratory, Ithaca, NY).

Amino acid content of bacteria, protozoa, and omasal fractions was determined by HPLC. For all AA excluding Met, Cys, and Trp, sample containing 2 mg N was weighed into hydrolysis tubes with 25 μL of 250 mM Norleucine as an internal standard. Samples were then hydrolyzed at 110ºC for 21 h in a block heater (Gehrke et al., 1985) with high-purity 6 M HCl (5 mL) after flushing with N_2 gas. For Met and Cys, aliquots containing 2 mg N and the internal standard were preoxidized with 1 mL performic acid (0.9 mL of 88 % formic acid, 0.1 mL of 30% H_2O_2 and 5mg phenol) for 16 h at 4°C prior to acid hydrolysis as described above (Mason et al., 1980, Elkin and Griffith, 1984). After hydrolysis, tube contents were filtered through Whatman 541 filter paper and filtrate was diluted to 50 mL in a volumetric flask with HPLC grade H_2O. Aliquots (0.5 mL) were evaporated at 60°C under constant N_2 flushing, with 3 rinses and re-evaporations with HPLC grade H_2O to remove acid residues. After final evaporation, hydrolysate was dissolved in 1 mL of Na diluent (Na220, Pickering Laboratories, Mountain View, CA).

Individual AA hydrolysates were separated using an Agilent 1100 series HPLC (Agilent Technologies, Santa Clara, CA) fitted with a sodium cation exchange column (Cat. no

1154110T, Pikering Laboratories, Mountain View, CA) using a 4 buffer step gradient and column temperature gradient. Detection of separated AA was performed at 560 nm following post-column ninhydrin derivation. Standards (250 nM/mL) for the individual amino acids were prepared by diluting a pure standard in sample buffer. The volume of sample and standards loaded onto the column was 10 μL. For Trp determination, a separate aliquot of sample containing 2 mg N was hydrolyzed with 1.2 g of $Ba(OH)_2$ at 110°C for 16 h on a block heater according to the method of Landry and Delhaye (1992). Included in the hydrolysis was 125μL of 5-Methyl-Trp (5m*M*) as an internal standard. After cooling to precipitate barium ions, an aliquot (3 μL) of the hydrolysate was added to 1 mL of acetate buffer (0.07 *M* sodium acetate) and analyzed using fluorescence detection (excitation = 285 nm, emission – 345 nm) after HPLC separation.

Calculations

Calculation of ^{15}N atom percent excess (**APE**) in rumen contents, omasal fractions and microbial samples; omasal nutrient flow, and partitioning of NAN has been described previously (Chapter 2). Briefly, total N entering the omasal canal was partitioned into three fractions: ammonia N, microbial N, and non-ammonia non-microbial N (**NANMN**). Total NAN flow was calculated as the difference between total N and ammonia N. Flows of bacteria, protozoa, and total microbial NAN flow was partitioned as described in Chapter 2.

The N content of bacteria and protozoa was used to calculate flow of OM and DM in each microbial fraction. Partitioning of the microbial pool into bacterial and protozoal pool takes into account the differences in ^{15}N APE between bacteria and protozoa samples, therefore reducing the underestimation bias introduced by assuming bacterial ^{15}N enrichment as representative of all

microbial biomass (Brito et al., 2007). Protozoal predation was estimated using the ^{15}N enrichment of the microbial fractions in the following manner

$$\text{Engulfed bacterial N} = \frac{\text{protozoa N flow (g/d)} \times \text{protozoa } ^{15}\text{N APE (g/g)} \times 0.9 \,/\, 0.5}{\text{bacteria } ^{15}\text{N APE (g/g)}}$$

In the preceding calculation, it is assumed that 90 % of the enriched ^{15}N in the protozoa is of bacterial origin; recognizing the capability of protozoa for de-novo synthesis of AA from ammonia (Williams and Harfoot, 1976; Williams and Coleman, 1997; Newbold et al., 2005). The calculation also assumes that 50 % of the engulfed N is incorporated into cell N (Hristov and Jouany, 2005), an assumption also incorporated into a dynamic version of the CNCPS (Higgs, 2014). Protozoa consumption of bacterial DM and OM was determined using the N and OM content of the omasal bacteria.

Rumen OM, fermentable carbohydrate (**CHO**), NAN, and microbial NAN pool sizes was determined from nutrient analysis of the samples taken during the rumen evacuations. Measured ^{15}N APE of the total rumen NAN pool was used to partition microbial and non-microbial N in the same manner as described for omasal NAN flows. Pool size calculations for digestible OM and CHO are as follows:

Digestible OM (kg) = Rumen OM (kg) – Microbial OM pool (kg) – rumen uNDF pool (kg)

Digestible CHO (kg) = Rumen digestible OM pool (kg) - (Rumen CP pool – Microbial CP pool) – (rumen DM pool * diet fat content (g/g of DM))

To estimate the partition of the rumen microbial N pool into bacteria and protozoal pools, relative flows of bacteria and protozoa were multiplied by a factor representing selective retention of protozoa in the rumen. Reported rumen protozoa retention in rumen vs. post-ruminal

measurements vary widely, and range from < 5 % (Sylvester et al., 2005) to over 70% (Punia et al., 1992). Therefore, rumen protozoa ^{15}N proportion of the total rumen ^{15}N pool (**PP**) was calculated at 4 different levels representing 0 to 75 % retention:

Protozoa ^{15}N proportion of the total ^{15}N, (PP; g/g) = [Protozoa NAN flow (g/d) x protozoa ^{15}N APE (g/g NAN)] / [OTD ^{15}N flow (g/d) x (1, 0.25, 0.5, 0.75,)]

The protozoa proportion of the rumen ^{15}N at each of the 4 levels of selective retention, along with the APE of rumen contents and the microbial fractions was used to calculate the rumen pool sizes for bacteria, protozoa, and total microbial NAN:

Protozoa NAN pool size (g) = [Rumen contents ^{15}N APE (g/g) x Rumen total N (g) x PP, (g/g)] / protozoa ^{15}N APE (g/g)

Bacteria NAN pool size (g) = [Rumen contents ^{15}N APE (g/g) x Rumen total N (g) x (1-PP, g/g)] / bacteria ^{15}N APE (g/g)

Microbial NAN pool size (g) = Protozoa NAN pool size (g) + Bacteria NAN pool size (g)

The value obtained when using a selective retention rate of 25 % was used in calculations requiring a total rumen microbial pool size. Justification for this approach is discussed later in this chapter. Rumen pool size and omasal flow was then used to calculate the fractional growth rate of total microbial, bacteria and protozoa fractions:

$$\text{Fractional growth rate } (h^{-1}) = \frac{\text{flow of microbial, bacterial, or protozoal N, g/h}}{\text{Rumen pool size of microbial, bacterial, or protozoal N g}}$$

Since flows and pool sizes were measured values, the fractional growth rate accounts for lysis and turnover in the rumen. The same pool size and flow approach was used to calculate the

absolute and fractional degradation rates of OM and CHO in the rumen, where the numerator was the hourly rate of disappearance of OM or CHO, and the denominator is the rumen pool size of digestible OM or CHO. Fractional rate of CHO degradation and fractional rate of microbial growth was then used to calculate yield of microbial cells per gram of CHO degraded ($\mathbf{Y_g}$)

$$Y_g \text{ (g cell DM / g CHO degraded)} = \frac{\text{fractional rate of microbial growth}}{\text{fractional rate of CHO degradation}}$$

Flows of individual AA in OTD was calculated using the concentration of AA in each omasal fraction and the triple marker system described in Chapter 2. Bacteria, protozoa, total microbial, and non-microbial AA flow were then calculated as follows:

Bacterial AA flow (g/d) = bacteria N flow (g/d) × bacteria AA (g/g N)

Protozoa AA flow (g/d) = protozoa N flow (g/d) × protozoa AA (g/g N)

Microbial AA flow (g/d) = protozoa AA flow (g/d) + bacteria AA flow (g/d)

Non-microbial AA flow (g/d) = OTD AA flow (g/d) – microbial AA flow (g/d)

All relevant farm, cattle, and diet information was entered into CNCPS v. 7 (Higgs, 2014) to provide comparisons with reported values. The model mechanistically describes substrate degradation using rates of passage and degradation (Waldo et al., 1972), and relates microbial growth to substrate availability (Russell et al., 1992) with modifications (Higgs, 2014). Protozoa, endogenous N transactions, N recycling, and large intestine degradation of substrate are all represented in a mechanistic manner. Comparisons were made of model predicted vs. measured values for substrate degradation, microbial growth, and post-ruminal AA flows.

Statistical Analyses

All data were analyzed using SAS version 9.3 (SAS Institute Inc. Cary, NC). The same model as described in chapter 2 is reproduced here:

$$Y_{ijkl} = \mu + S_i + C_{j:i} + P_k + T_l + ST_{il} + \varepsilon_{ijkl}$$

where Y_{ijkl} = dependent variable, μ = overall mean, S_i = fixed effect of sequence i, $C_{j:i}$ = random effect of cow within sequence, P_k = fixed effect of period k, T_l = fixed effect of treatment l, ST_{il} = fixed interaction effect of sequence i and treatment l, and ε_{ijkl} = residual error. Degrees of freedom were calculated using the Kenward-Roger option. Means were determined using the least squares means statement, and treatment means were compared using the PDIFF option. Statistical significance was considered at $P \leq 0.05$ and trends were considered at $0.05 < P \leq 0.10$.

RESULTS AND DISCUSSION

Microbial Nutrient Composition

The OM content of omasal bacteria and protozoa did not differ between diets, and averaged 84.1 and 87.6 %, respectively (Table 3.2). Organic matter content was similar to values obtained previously from rumen and omasal isolates (Brito et al., 2006; 2007), although OM content is strongly influenced by the isolation procedures used (Martin et al., 1994). Nitrogen content of bacteria and protozoa were both affected by diet, with increased N content in microbial isolates from cows fed EXP (7.9 vs. 8.3 and 7.9 vs. 8.6 % of DM for CON and EXP bacteria and protozoa, respectively). This increased N content can arise from several possible mechanisms. With decreased protein degradation in cows fed EXP (Chapter 2), more AA and peptides could be available for microbial incorporation, leading to increased N content. Brito et al. (2007) reported a similar 5% increase in NAN content of fluid associated bacteria when urea *vs*. true protein supplements were fed to lactating dairy cows. An alternative possibility is a change in microbial reserve carbohydrate synthesis, resulting in more glycogen to dilute the measured NAN value. In competition studies for substrate Denton et al. (2015) demonstrated that protozoa sequester up to 60 % of available glucose and store it as glycogen, while less than 2 % was recovered in bacteria. In this study, cows fed CON demonstrated lower microbial N content, which could indicate more reserve CHO synthesis. This also provides a tempting explanation for the relative change in bacteria vs. protozoa NAN content. Bacteria NAN increased by 5% (7.9 vs. 8.3 % of DM for cows fed CON vs. EXP, respectively), while protozoa NAN content increased by approximately 8 % (7.9 vs. 8.6 % of DM for cows fed CON vs. EXP, respectively). Omasal protozoa NAN content has been reported as low as 2.3 % of DM (Brito et al., 2006), although sucrose was used in the isolation procedure, likely influencing N content. The protozoa

isolation method employed in the current study deliberately omitted any addition of glycogenic compounds to avoid biasing the NAN measurement. Enrichment of ^{15}N in bacteria and protozoa was similar for cows fed CON vs. EXP. The mean protozoal:bacterial ^{15}N enrichment ratio of 0.62 was within the range of values reported in the literature: 0.40 (Ahvenjärvi et al., 2002), 0.63 (Hristov and Broderick, 1996), 0.75 (Cecava et al., 1991). Some authors attributed low enrichment to feed contamination in the isolation method, although ^{15}N enrichment is likely more related to the sources of N used for growth (Atasoglu et al., 2001; Brito et al., 2006) and possibly the amount of time the bacteria had to take up the label. The approach used in this experiment followed the concept of a plateau in enrichment and cows were infused for 72 h before any measurements were made (Recktenwald et al. 2014), thus different enrichment levels was likely not due to non-plateau of ^{15}N.

Table 3.2. Chemical composition and isotopic enrichment of omasal bacteria and protozoa in lactating dairy cattle fed two different sources of rumen available nitrogen

	Diet[1]			
Item	CON	EXP	SEM	*P*
Bacteria				
OM, % of DM	83.6	84.7	0.5	0.18
N, % of DM	7.90	8.29	0.13	0.02
^{15}N atom% excess	0.035	0.034	0.002	0.74
Total AA, % of DM	28.8	30.8	0.4	0.01
AA, % of N	50.1	50.6	0.8	0.67
Protozoa				
OM, % of DM	86.1	89.1	1.5	0.17
N, % of DM	7.93	8.60	0.24	0.05
^{15}N atom% excess	0.023	0.020	0.002	0.11
Total AA, % of DM	31.4	34.4	0.9	0.02
AA, % of N	53.4	54.6	0.9	0.32

[1]CON = 3% of diet DM as urea control mix; EXP = 3% of diet DM as fermentation byproduct.

Total AA content in bacteria and protozoa was increased in cows fed EXP vs. CON. Amino acid content as a % of N was not different between diets, and averaged 50.3 and 54.0 % for omasal bacteria and protozoa, respectively. These values are lower than reported previously in the literature (Storm and Ørskov, 1983). Hvelplund (1986) reported a mean AA N as a % of N of 67.4 % in mixed rumen bacteria, and demonstrated a curvilinear relationship of diet starch and sugar content vs. AA N as a % of N, with AA N decreasing rapidly as starch+sugar content exceeded 30 % of diet DM. Reporting of AA N in this experiment did not include DAPA, which can represent greater that 10% of the amino acid content in rumen micro-organisms. Volden and Harstad (1998) reported a decrease in total AA N as a % of N when formalin treatment was used in the isolation procedure. Formalin has been shown previously to affect the AA composition of isolated cells (Stern et al., 1983), likely through cross-linkage of protein chains. This process can create products resistant to acid hydrolysis, rendering incomplete extraction of AA from the sample matrix (Barry, 1976).Therefore, bacterial and protozoal AA profiles and contributions to AA flow might be underestimated in this study; however total flow of AA in OTD is unaffected, as no formalin was used in the separation of LQ, SP, or LP fractions.

Omasal bacteria and protozoa AA composition was unaffected by diet (Table 3.3). Profiles of bacterial amino acids generally agree well with literature reports (Volden et al., 1999) with the exception of Lys and Tyr, which were all decreased in the current study. Formalin is known to specifically affect these AA (Volden and Harstad, 1998). Protozoal AA profile was similar when compared with bacterial AA profile, with the exception of Met and Lys showing numerically increased levels in protozoa. Volden et al. (1999) also reported increased Lys concentration in protozoa vs. bacteria; however Met was very similar between isolates. Cockburn and Williams (1984) reported mean Met concentrations of 2.4 g / 100 g AA. Inconsistent use and poor

reporting of pre-oxidation procedures used among studies makes comparisons difficult, as recoveries from pre-oxidation are rarely reported (Spindler et al., 1984). It is possible that formalin treatment increased the relative proportion of Met in our microbial isolates; however this effect should also be distributed across other AA not affected by formalin treatment.

Table 3.3. Omasal bacteria and protozoa amino acid composition in lactating dairy cattle fed two different sources of rumen available nitrogen

	Bacteria AA, g/100 g AA				Protozoa AA, g/100 g AA			
	Diet[1]				Diet[1]			
Item	CON	EXP	SEM	*P*	CON	EXP	SEM	*P*
Essential AA								
ARG	5.60	5.38	0.23	0.49	5.16	5.84	0.36	0.20
HIS	1.96	2.02	0.04	0.30	2.67	2.67	0.12	0.96
ILE	4.70	4.66	0.06	0.60	5.15	4.91	0.25	0.51
LEU	4.42	3.60	0.30	0.07	5.59	5.61	0.40	0.96
LYS	4.13	4.04	0.05	0.24	6.15	6.16	0.26	0.98
MET	2.34	2.56	0.16	0.34	3.99	4.00	0.10	0.93
PHE	6.50	6.44	0.15	0.79	7.08	6.85	0.14	0.29
TRP	4.94	4.98	0.13	0.85	3.74	3.63	0.09	0.26
THR	6.08	5.93	0.21	0.62	4.79	4.90	0.25	0.75
VAL	6.55	6.71	0.09	0.16	5.83	6.05	0.22	0.49
Total EAA	47.2	46.3	0.6	0.26	50.1	50.8	0.4	0.23
Nonessential AA								
ALA	7.07	7.18	0.08	0.36	5.66	5.62	0.17	0.86
ASP	10.65	10.67	0.32	0.95	11.45	11.87	0.67	0.62
CYS	1.20	1.14	0.06	0.49	2.84	2.80	0.05	0.62
GLU	14.36	14.59	0.15	0.30	13.87	14.11	0.28	0.49
GLY	5.77	5.89	0.05	0.10	4.84	4.84	0.13	0.99
PRO	7.22	8.57	0.46	0.04	3.96	2.73	0.95	0.37
SER	5.31	4.81	0.20	0.10	4.49	4.74	0.20	0.40
TYR	1.15	0.82	0.16	0.02	2.72	2.60	0.20	0.67
Total NEAA	52.8	53.7	0.6	0.26	49.9	49.2	0.4	0.23

[1]CON = 3% of diet DM as urea control mix; EXP = 3% of diet DM as fermentation byproduct.

Omasal Flows of AA in OTD, Microbial, and Non-microbial Fractions

Microbial nutrient flow of DM, OM and NAN flow was not different between diets (Table 3.4). Effects of rumen degradable protein source on omasal nutrient flows and have been previously reported in Chapter 2. Cows fed CON vs. EXP tended to have increased ($0.10 > P > 0.05$) bacterial DM and OM flow (4808 vs, 4056 g DM/d and 4023 vs, 3433 g OM/d for CON vs. EXP, respectively). Since bacterial OM and DM flow are calculated using microbial N as a marker, it is likely that the observed difference in microbial N composition contributed to lower calculated DM and OM flows. Protozoa nutrient flows followed similar numeric trends, but flows did not differ significantly between diets. Protozoa accounted for 15.8 and 17.9 % of the total microbial NAN flow in cows fed CON vs. EXP, respectively. This estimate is slightly higher than has previously been reported in the literature with animals at similar levels of intake. Sylvester et al. (2005) reported protozoa N accounting for 5.9 – 11.9 % of the microbial N flow using 18S rDNA techniques, while Ahvenjärvi et al. (2002) reported 7 % of microbial N flow as protozoa N using a very similar technique as our study. Using a linear programming approach, Shabi et al. (2000) estimated protozoal N to account for 7 to 19 % of microbial N flow. This was a result similar to that estimated by Steinhour et al. (1982) using a differential ^{15}N enrichment approach, although many assumptions were made pertaining to pool size and turnover in that study. Alternatively, computer simulations by Dijkstra et al. (1998) indicated that protozoa N could account for 10.7 to 26.1 % of microbial N in cattle at 17.1 kg of DMI. Simulations using CNCPS v. 7 indicated that overall microbial flow in cows fed CON was well predicted; however the model was insensitive to the numerical difference in microbial flow in CON vs. EXP fed cows. Protozoa flow was slightly under predicted, as was predation of protozoa on bacteria. Possible updates to the microbial sub-model to address this will be discussed in later chapters.

Table 3.4. Omasal microbial nutrient flows and protozoa predation in lactating dairy cattle fed two different sources of rumen available nitrogen

	Diet[1]			
Item	CON	EXP	SEM	*P*
Dry matter intake, kg/d[2]	23.8	23.9	0.7	0.91
Total microbial nutrient flow, g/d				
DM flow	5718	4930	358	0.14
OM flow	4815	4210	310	0.19
NAN flow[2]	450	409	28	0.31
Bacterial nutrient flow, g/d				
DM flow	4808	4056	286	0.08
OM flow	4023	3433	240	0.10
NAN flow	378	337	23.0	0.22
% of microbial N flow	84.2	82.1	1.0	0.12
Protozoa nutrient flow, g/d				
DM flow	909	850	82	0.61
OM flow	790	764	81	0.82
NAN flow	72.1	73.9	7.3	0.84
% of microbial N flow	15.8	17.9	1.0	0.12
Protozoa predation of bacteria, g/d				
DM consumed	1159	929	166	0.33
OM consumed	967	783	138	0.35
N consumed	90.6	76.3	12.9	0.45
% of bacterial N flow	23.4	22.2	2.4	0.70
CNCPS v. 7 output				
Predicted microbial N flow, g/d	412	417	-	-
Bacteria N flow	371	375	-	-
Protozoa N flow	41	42	-	-
% of microbial N flow	9.9	10.1	-	-
Predation estimate, bacterial N consumed, g/d	75	76	-	-

[1]CON = 3% of diet DM as urea control mix; EXP = 3% of diet DM as fermentation byproduct.
[2]Previously reported in Chapter 2

Omasal Flows of AA in Bacteria and Protozoa

Flow of AA in OTD is displayed in Table 3.5. Most AA demonstrated increased flow in cows fed EXP vs. CON. Total AA flow was increased by 211 g/d in cows fed EXP compared to CON (2456 v. 2245 g/d for CON vs. EXP, respectively; $P < 0.01$). Omasal flow of Lys, Met, and Phe, were similar between diets, while all other EAA were increased in cows fed EXP compared with CON. Total non-essential AA flow was increased by 116 g/d in cows fed EXP, while Cys flow was the only individual NEAA that was similar between diets. Reynal and Broderick (2005) reported similar results in omasal AA flows when diets with varying RDP from soybean meal vs. treated soybean meal were fed to lactating dairy cows. When soybean meal, cottonseed meal, and canola meal were compared with a urea control, flows of all AA increased greatly in a study by Brito et al. (2007). The increase in AA flows in the current study was directly related to the lower dietary CP degradation in cows fed EXP (Chapter 2). This is demonstrated by the data found in table Table 3.6, where OTD AA flow is divided into microbial AA flow and non-microbial AA flow. Inclusion of the fermentation byproduct had no effect on microbial AA flow, while non-microbial AA flow was increased for most individual AA. This further supports the protein sparing effect of the fermentation byproduct on RDP, as microbial AA flow was not significantly lower, while non-microbial AA flow increased by 316 g/d in cows fed EXP compared to CON ($P = 0.03$). Microbial protein synthesis was apparently not negatively affected by decreased CP degradation, indicating that sufficient AA and N compounds were present to support high rates of microbial growth.

Table 3.5. Effect of rumen available nitrogen source on omasal true digesta flow of AA in lactating dairy cattle

	Diet[1]			
AA flow, g/d	CON	EXP	SEM	*P*
Essential AA				
ARG	108.2	120.1	3.4	0.01
HIS	62.2	68.0	1.5	< 0.01
ILE	87.3	100.2	2.7	< 0.01
LEU	131.0	149.2	7.0	0.03
LYS	139.0	127.4	14.0	0.56
MET	57.3	57.3	5.5	1.00
PHE	147.7	165.8	8.3	0.11
TRP	78.9	86.3	2.4	< 0.01
THR	119.9	131.6	3.3	< 0.01
VAL	116.8	131.1	3.6	< 0.01
Total EAA	1045.8	1141.8	28.4	0.03
Nonessential AA				
ALA	140.9	153.0	4.0	< 0.01
ASP	212.3	236.2	5.7	< 0.01
CYS	39.3	38.0	3.4	0.78
GLU	280.1	314.0	6.3	< 0.01
GLY	105.9	116.5	2.7	< 0.01
PRO	172.1	189.4	6.2	0.03
SER	120.0	131.4	3.3	< 0.01
TYR	130.4	138.7	4.4	0.05
Total NEAA	1200.9	1316.8	31.4	< 0.01
Total AA	2245.2	2456.5	57.1	< 0.01

[1]CON = 3% of diet DM as urea control mix; EXP = 3% of diet DM as fermentation byproduct.

Table 3.6. Effect of rumen available nitrogen source on omasal flow of microbial and non-microbial AA in lactating dairy cattle

	Microbial AA flow, g/d				Non-microbial AA flow, g/d			
	Diet[1]				Diet[1]			
Item	CON	EXP	SEM	*P*	CON	EXP	SEM	*P*
Essential AA								
ARG	96.9	88.4	6.7	0.38	10.7	31.8	7.9	0.08
HIS	36.4	34.7	2.3	0.57	25.8	33.2	2.7	0.04
ILE	83.1	75.4	5.7	0.34	4.2	23.4	6.4	0.01
LEU	79.5	61.7	6.3	0.06	52.9	87.9	9.7	0.02
LYS	78.4	71.9	5.8	0.42	60.5	56.3	14.7	0.84
MET	43.1	44.8	3.4	0.67	14.0	12.8	6.1	0.88
PHE	114.0	103.0	7.8	0.35	33.4	61.9	10.4	0.06
TRP	82.7	74.5	5.2	0.28	-4.1	11.4	6.7	0.09
THR	103.3	92.9	7.8	0.35	16.4	37.0	8.9	0.05
VAL	112.4	106.3	8.0	0.58	4.3	23.1	10.0	0.09
Total EAA	830.2	749.0	51.4	0.28	213.6	371.7	64.6	0.04
Nonessential AA								
ALA	119.8	111.2	8.0	0.43	20.5	40.5	9.7	0.07
ASP	188.7	177.8	13.8	0.51	23.1	56.7	15.5	0.04
CYS	24.8	23.0	1.5	0.34	14.4	15.3	3.7	0.86
GLU	249.8	234.9	17.7	0.51	30.0	76.7	19.9	0.04
GLY	98.1	91.7	6.8	0.48	7.6	23.7	7.8	0.08
PRO	117.0	122.1	9.6	0.66	55.7	65.8	12.7	0.42
SER	90.5	79.5	6.7	0.17	29.5	50.7	8.3	< 0.01
TYR	24.2	19.4	2.5	0.09	106.2	119.1	4.4	< 0.01
Total NEAA	913.1	861.0	61.7	0.49	287.1	445.6	73.7	0.03
Total AA	1744.2	1611.4	113.9	0.39	500.0	815.9	135.4	0.03

[1]CON = 3% of diet DM as urea control mix; EXP = 3% of diet DM as fermentation byproduct.

Flows of individual amino acids in bacterial and protozoa fractions were generally not affected by diet (Table 3.7). Bacterial Leu flow was increased in cows fed CON vs. EXP (63.4 vs. 45.1 g/d for CON vs. EXP, respectively; $P = 0.03$). Bacterial Ser and Tyr flows tended to be increased in cows fed CON vs. EXP, while all other bacterial AA flows were not different between diets. Protozoa AA flows were unaffected by treatment. The contribution of protozoa to total microbial AA flow is in Table 3.8. Inclusion of the fermentation byproduct in the EXP diets increased protozoa contribution to Leu and Lys flow. Protozoa flow of Lys accounted for 21.5 vs. 29.5 % of the total microbial flow of Lys in cows fed EXP vs. CON, respectively, ($P < 0.01$). Contribution of protozoa to total EAA flow tended to be greater in cows fed EXP vs. CON (19.0 vs. 22.8 % of microbial flow, respectively; $P = 0.07$). These results demonstrate the importance of protozoa to post ruminal AA flows. Protozoa NAN contribution to microbial NAN flows averaged 16.9 % of microbial N, while contribution of protozoa AA to microbial AA flows ranged from 8 to 46 % for individual AA. Models that do not take into account the difference in AA profile, composition, and contribution of protozoa to microbial AA flow might have poor predictions of post-ruminal AA flow. For models seeking to describe the diet adequacy to support milk protein production, accurate predictions of post ruminal supply are needed if the models are to be applied in practical feeding situations (Pacheco et al., 2012). Higgs (2014) evaluated CNCPS v.7 against a large literature dataset, and found the model adequately predicts post ruminal total NAN and microbial NAN supply; however individual AA were generally over-predicted. The same tendency to over-predict AA flow was observed in the current evaluation and this might be related to methodology associated with the use of the triple-marker system, as all studies in the dataset utilized this approach.

Table 3.7. Effect of rumen available nitrogen source on omasal flow of bacteria and protozoa AA flow in lactating dairy cattle

	Bacteria AA flow, g/d				Protozoa AA flow, g/d			
	Diet[1]				Diet[1]			
Item	CON	EXP	SEM	*P*	CON	EXP	SEM	*P*
Essential AA								
ARG	81.8	70.5	5.9	0.20	15.3	18.4	2.5	0.35
HIS	28.6	26.5	2.0	0.45	7.8	8.0	0.6	0.77
ILE	68.0	60.2	4.7	0.27	15.0	15.1	1.6	0.95
LEU	63.4	45.1	5.4	0.03	16.1	16.6	1.4	0.79
LYS	60.2	53.4	4.3	0.26	18.1	18.6	1.9	0.85
MET	31.5	32.5	2.8	0.77	11.7	12.1	1.2	0.84
PHE	93.3	82.5	6.4	0.25	20.6	20.7	1.9	0.96
TRP	71.8	63.5	4.7	0.23	10.9	11.0	1.0	0.91
THR	89.0	77.4	6.4	0.21	14.2	15.1	1.9	0.71
VAL	95.1	87.2	6.5	0.41	17.1	18.6	2.2	0.60
Total EAA	682.9	597.1	42.2	0.17	146.9	154.1	14.8	0.71
Nonessential AA								
ALA	103.1	93.7	6.9	0.34	16.6	17.1	1.9	0.86
ASP	154.8	141.7	10.6	0.31	34.0	36.2	4.6	0.69
CYS	16.6	14.4	1.1	0.17	8.3	8.4	0.7	0.93
GLU	208.8	191.5	14.9	0.38	40.9	42.9	4.3	0.70
GLY	83.8	76.4	5.7	0.38	14.3	14.9	1.7	0.77
PRO	105.5	114.0	10.3	0.50	11.4	8.2	2.4	0.37
SER	77.2	64.5	5.7	0.10	13.3	14.3	1.7	0.66
TYR	16.1	11.2	2.5	0.06	8.1	8.0	1.0	0.94
Total NEAA	766.1	708.6	51.5	0.39	147.1	149.5	15.0	0.90
Total AA	1449.7	1304.3	93.6	0.27	293.9	303.7	29.8	0.80

[1]CON = 3% of diet DM as urea control mix; EXP = 3% of diet DM as fermentation byproduct.

Table 3.8. Effect of rumen available nitrogen source on protozoa proportion of omasal microbial AA flow in lactating dairy cattle

	Diet[1]			
Protozoa AA contribution to microbial AA flow, %	CON	EXP	SEM	*P*
Essential AA				
ARG	17.0	22.0	2.0	0.07
HIS	22.5	25.9	1.7	0.18
ILE	18.8	21.5	1.6	0.15
LEU	21.5	29.5	1.6	< 0.01
LYS	23.9	28.2	1.6	0.05
MET	28.6	31.3	2.5	0.46
PHE	19.0	22.0	1.7	0.22
TRP	17.8	24.4	4.1	0.27
THR	14.7	17.8	1.5	0.15
VAL	16.0	19.0	1.4	0.17
Total EAA	19.0	22.8	1.5	0.07
Nonessential AA				
ALA	14.7	17.1	1.4	0.25
ASP	18.9	22.6	1.8	0.09
CYS	34.4	40.0	2.4	0.11
GLU	17.2	20.1	1.5	0.15
GLY	15.2	17.8	1.5	0.23
PRO	10.6	7.7	2.7	0.46
SER	15.6	21.0	1.7	0.04
TYR	35.4	45.8	5.5	0.21
Total NEAA	16.8	19.4	1.3	0.20
Total AA	17.8	20.9	1.4	0.13

[1]CON = 3% of diet DM as urea control mix; EXP = 3% of diet DM as fermentation byproduct.

Rumen Pool Size and Kinetics of OM, CHO, Bacteria and Protozoa

Rumen pool sizes of digestible OM, CHO, NAN, and microbial NAN were not affected by diet (Table 3.9). Generally, microbial N pool size of 3.5 g of microbial N / L was similar to that observed by Sylvester et al. (2005), who reported a microbial pool of approximately 3 g of microbial N / L, although the authors point out that the estimation of bacterial N contains the error of both the 18S RNA procedure and the purine determination. Purines have been shown to be inconsistent microbial markers relative to ^{15}N (Klopfenstein et al., 2001; Firkins and Reynolds, 2005; Ipharraguerre et al., 2007). Cows fed CON had greater microbial DM contribution to the total DM pool than cows fed EXP (27.7 vs. 23.6 % for CON vs. EXP, respectively; $P = 0.05$). Values were within the range of 17 – 27 % of the rumen DM pool as microbial DM reported by Craig et al. (1987).

Selective retention of protozoa in the rumen is not well understood, and estimates range from < 5 % (Sylvester et al., 2005) to over 70% (Punia et al., 1992). Since rumen protozoa mass was not quantified directly in this study, the effect of 4 different levels of retention is described in Table 9. Since total rumen microbial pool size was estimated from ^{15}N enrichment of the rumen NAN pool, it is possible to evaluate the effect of selective retention on pool sizes, assuming total microbial ^{15}N pool size remains constant. Therefore, at 0 % selective retention, we expect the protozoa to account for the same proportion of total microbial N as measured in OTD. At greater levels of retention, protozoa account for larger portions of the microbial pool. Bacteria pool size was decreased in cows fed EXP vs. CON, and bacteria vs. protozoa pool sizes diverged as selective retention increased. At the highest estimation of selective retention, protozoa were calculated to represent 55 to 58 % of the total microbial pool (CON vs. EXP, respectively, $P = 0.40$).

To assess which level of selective retention of protozoa is likely most correct, it is possible to use pool size and flow to estimate fractional rates of turnover (Table 3.10). In this case, since actual flows and microbial pool size were measured, the rate of turnover of the pool can be used as an index of microbial growth rate (Wells and Russell, 1996). Recognizing that the main energy substrate for rumen bacteria is CHO (Russell et al., 1992), and assuming the maximum yield of cell DM / g of CHO degraded is 0.5 (Isaacson et al., 1975), one can quickly determine which retention values allows for realistic growth rates. In this instance, selective retention at 50 % indicate that bacteria would have to grow at a fractional rate of 0.07 h^{-1}, corresponding to a CHO degradation rate of 0.14 h^{-1} (0.07 / 0.5). Given the estimated pool size (g) and digestion (g/h), the fractional rate of CHO availability in this study averaged 0.138 h^{-1} of the available pool; therefore theoretical maximal fractional growth rate was estimated at 0.138 x 0.5, or ~ 0.069 h^{-1}. Using the measured total microbial pool at 25 % selective retention, it was calculated that the fractional growth rate of all microbes in the rumen was 0.061 h^{-1}. This corresponds to an estimated Y_g of 0.44 g / g of CHO degraded. This is close to the theoretical maximums for individual species reported in pure cultures (Russell and Baldwin, 1979; Theodorou and France, 2005). In vitro measurements of mixed rumen microbes often give yields on the high range of those observed in pure culture (Russell and Wallace, 1997). The range of yields (29 to 100 g / mol of hexose) reported by Russell and Wallace (1997), correspond to Y_g of 0.16 to 0.55 g / g of glucose degraded. Stouthamer (1973) estimated a maximal Y_g of approximately 0.8 g / g of glucose using biochemical pathways, indicating the possibility for much higher yields in some bacterial species; however values this high are rarely reported in vitro with mixed rumen microbial fermentations.

The CNCPS relates cell growth directly to CHO availability in the manner described above, so accurate estimates of CHO degradation are key to accurately predicting microbial yield. To provide the model with rates of CHO degradation, feedstuffs were analyzed according to the methods of Fessenden (2011) and Raffrenato (2011) for starch and fiber degradation, respectively. Simulations indicated that the model characterized CHO digestion fairly well (Table 3.10) with predicted absolute rates of degradation approximately 37 grams lower than observed. This corresponds to a fractional rate of degradation of CHO at 0.124 h^{-1}. Given the predicted microbial yield (Table 3.4), the apparent Y_g used by the model was 0.45 g / g of CHO degraded in the rumen; very similar to the value observed in vivo. This agreement of predicted vs. independent measured values indicates the structure of the model is likely adequate to provide accurate estimates of microbial yield from substrate degradation. This provides a strong basis from which to improve AA supply predictions, as microbial N represents a large portion of MP flowing from the rumen.

Table 3.9. Effect of rumen available nitrogen source on rumen pool sizes of organic matter, carbohydrate, and non-ammonia nitrogen in lactating dairy cattle

	Diet[1]			
Item	CON	EXP	SEM	*P*
Rumen pool sizes				
Digestible OM, kg[2]	6.61	7.06	0.50	0.50
Total fermentable CHO, kg[3]	3.98	4.10	0.32	0.79
Total NAN, g	586	614	38	0.53
Microbial NAN pool at 25% selective retention, g	340	300	21	0.21
Microbial DM proportion of rumen DM pool, %	27.7	23.6	1.4	0.05
Bacteria NAN rumen pool sizes, g[4]				
0% selective retention	281	240	18	0.13
25% selective retention	271	229	17	0.11
50% selective retention	250	209	17	0.07
75% selective retention	181	148	17	0.07
Protozoa NAN rumen pool sizes, g[5]				
0% selective retention	53	53	5	0.98
25% selective retention	70	70	7	0.98
50% selective retention	105	105	10	0.98
75% selective retention	210	211	21	0.98
Protozoa NAN pool, % of total microbial NAN pool				
0% selective retention	15.8	17.9	1.0	0.12
25% selective retention	20.7	23.2	1.3	0.13
50% selective retention	30.1	33.1	1.8	0.17
75% selective retention	55.1	58.0	2.9	0.40

[1]CON = 3% of diet DM as urea control mix; EXP = 3% of diet DM as fermentation byproduct.
[2]Measured OM from rumen evacuation, corrected for microbial OM and uNDF240
[3]Rumen OM pool – (Rumen CP pool – Microbial CP pool) – (rumen DM pool * diet fat content)
[4]Microbial NAN pool – Protozoa NAN pool at 4 levels of selective retention of protozoa
[5]Microbial NAN x Protozoa % of omasal flow x level of selective retention

Table 3.10. Fractional rates of microbial growth, nutrient digestion, and rumen fermentation parameters in lactating dairy cattle fed two different sources of rumen available nitrogen

	Diet[1]			
Item	CON	EXP	SEM	*P*
Fractional growth rate of bacteria[2], h^{-1}				
0% selective retention	0.061	0.061	0.004	0.99
25% selective retention	0.064	0.064	0.005	0.99
50% selective retention	0.070	0.070	0.006	1.00
75% selective retention	0.108	0.103	0.012	0.74
Fractional growth rate of protozoa[2], h^{-1}				
0% selective retention	0.061	0.061	0.004	0.99
25% selective retention	0.046	0.046	0.003	0.99
50% selective retention	0.030	0.030	0.002	0.99
75% selective retention	0.015	0.015	0.001	0.99
Omasal flows and ruminal digestion parameters				
True OM flow, kg/d[3]	7.08	7.19	0.47	0.87
Microbial NAN flow, g/d[3]	450	409	28	0.31
Ruminal true OM digestion rate, g/h	626	619	17	0.77
Ruminal true CHO digestion rate, g/h	518	526	15	0.72
Fractional rate of OM digestion[4], h^{-1}	0.101	0.094	0.008	0.54
Fractional rate of CHO digestion[4], h^{-1}	0.139	0.138	0.011	0.91
Microbial growth parameters				
Fractional growth rate, h^{-1}	0.060	0.060	0.004	0.94
Theoretical maximum CHO allowable growth[5], h^{-1}	0.070	0.069	0.005	0.91
Observed Yg, g of cells / g of CHO degraded[6]	0.44	0.44	0.03	0.99
% of theoretical maximum Yg	88.4	88.3	6.6	0.99
CNCPS v. 7 output				
Predicted CHO degradation, g/h	484	487	-	-
Predicted fractional rate of CHO digestion, h^{-1}	0.124	0.124	-	-
Predicted Yg, g of cells / g of CHO degraded	0.45	0.45	-	-

[1]CON = 3% of diet DM as urea control mix; EXP = 3% of diet DM as fermentation byproduct.
[2]bacteria or protozoa daily flow (g/h) / bacteria or protozoa pool size (g) at 4 levels of protozoa selective retention
[3]Previously reported in chapter 2
[4]Measured microbial NAN flow (g/h) / measured rumen microbial NAN pool (g)
[5]Fractional rate of CHO digestion x 0.5
[6]Fractional microbial growth rate / fractional rate of CHO digestion

CONCLUSIONS

The inclusion of the fermentation byproduct in EXP diets increased microbial N and AA N flows compared with CON cows fed a urea control. Microbial amino acid composition did not differ between diets; however estimates of total AA N and specific AA were likely lower than literature values due to formalin treatment. Daily flows of AA in were increased in OTD as a result of decreased degradation of feed N (Chapter 2). This was reflected in the current study with increased non-microbial AA flow in cows fed EXP vs. CON. Average protozoa contribution to microbial NAN flow was 16.8 %, yet protozoa AA accounted for 21 % of the microbial EAA flow, with a range of 8 to 46 % for individual AA. Cows in this study maintained an average pool size of 320 g of microbial N in the rumen, while bacterial and protozoal pools were estimated at 4 different theoretical levels of retention. Fractional growth rate of all microbes in the rumen was measured at 0.069 h^{-1}, with a Yg of 0.44 g / g of CHO degraded. A dynamic versions of the CNCPS (v.7) was able to accurately predict CHO degradation and total microbial yield, however improvements are needed for bacteria vs. protozoa partitioning and individual AA flow predictions. Overall, the current structure of the CNCPS v.7 provides a strong base for predicting supply of microbial NAN. Future model improvements in microbial AA profiles, intestinal digestibility, protozoa partitioning, and dietary protein degradation might be needed to improve estimates of individual AA flow.

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CHAPTER 4: Effect of rumen degradable protein source on intake, milk production, and total tract nutrient digestion in lactating dairy cattle

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ABSTRACT: The objective of this study was to evaluate effects of three different dietary sources of protein on intake, milk performance, total tract nutrient digestion, and environmental and economic outcomes. Primiparous (n=48) and multiparous (n=144) lactating dairy cattle were stratified by milk production and randomly allocated into 12 pens containing 4 primiparous and 12 multiparous animals each. Cattle averaged 118 days in milk and 712 kg of body weight at trial start. Treatment diets on a dry matter (**DM**) basis consisted of 42% corn silage, 13% alfalfa forage, 20% grain corn, and 25% protein premix containing either soybean meal (Diet A), Fermenten (Diet B), or an rumen protected soybean meal (Diet C), at a 3.5% inclusion rate. All 3 diets provided a similar level (dry matter basis) of aNDFom (31 %), CP (14.9 %), starch (26 %), metabolizable energy (2.7 Mcal/kg), and differed in rumen degradable protein. The trial consisted of a 2 week adaptation and covariate period where all cows were fed diet C and covariate measurements were taken. Pens were then randomly allocated to treatments and weekly measurements of milk production, intake, body weight and condition score were made for 10 weeks. All data were analyzed using the Proc Mixed procedure in SAS. Increased dry matter intake was observed for cows fed diet B compared with cows fed diet A and C (28.3 kg/d vs. 26.9 and 26.7, respectively; $P = 0.03$). Cows fed diet B produced more energy corrected milk (45.3 kg/d) compared with cows fed diets A and C (43.6 and 43.7 kg/d, respectively; $P = 0.05$). Milk protein yield and percent was also increased in cows fed diet B. No differences were

observed with body weight or condition score gain throughout the trial. Apparent total tract digestibility of fiber was decreased in cows fed diet B, likely as a result of increased intake. The results from this experiment demonstrate beneficial milk performance responses to Fermenten when fed with a source of rumen degradable protein. Responses are consistent with a potential decrease in ruminal CP degradation as demonstrated by previous research in our lab. Results also demonstrate the economic and environmental value of rumen degradable protein vs. rumen undegradable protein when fed in nitrogen efficient diets in high producing dairy cattle.

Keywords: milk performance, nitrogen, rumen degradable protein, Fermenten, dairy cow

INTRODUCTION

The ability of rumen microbial populations to utilize substrate that would otherwise be unavailable to mammalian enzyme systems allows lactating dairy cattle cow to produce copious amounts of milk on relatively poor quality feed ingredients. Microbial populations in the rumen can convert non-protein N compounds into high quality, metabolizable protein (**MP**) when provided sufficient degradable carbohydrate (Firkins, 1996, Hackmann and Firkins, 2015). However, fermentation and conversion of rumen degradable protein to ammonia can cause excessive N losses to the environment (Castillo et al., 2000). The most effective way to reduce N losses is through reduced CP feeding while maintaining MP balance and high levels of milk protein output (Huhtanen and Hristov, 2009).

Byproducts from human production provide an opportunity to utilize feedstuffs that would otherwise be discarded (VandeHaar and St-Pierre, 2006). Fermenten (Church & Dwight, Inc., Princeton, NJ) is a commercially available fermentation byproduct derived from glutamic acid production and contains high amounts of rumen available nitrogen compounds in the form of soluble amino acids (**AA**) and small peptides. Lean et al. (2005) indicated that fermentation

byproducts can increase the flow of microbial protein from continuous flow fermenters with rumen microbes through stimulation of microbial protein synthesis (Cotta and Russell, 1982). However, production responses in vivo have been inconsistent (Broderick et al., 2000; Penner et al., 2009). Previous research in our lab (Chapters 1 and 2) demonstrated that fermentation byproduct inclusion did not stimulate microbial protein synthesis, but did increase daily feed non-ammonia nitrogen flow from the rumen beyond increased dietary protein supply, resulting in a 15 % reduction in CP degradation in the rumen. This sparing effect on RDP could provide some benefit when included in diets containing higher levels of rumen available AA.

Predicting such responses when balancing rations in the field can be difficult without a mechanistic understanding of microbial substrate metabolism, animal requirements, and subsequent utilization of supplied nutrients. Integrated mathematical models such as the Cornell Net Carbohydrate and Protein System (**CNCPS**) (Van Amburgh et al., 2015) can be successfully used in production systems across the world to improve economic and environmental efficiency of milk production (Tylutki et al., 2008, Russomanno et al., 2013). Successful model development requires proper characterization of farm, animal and feed characteristics; a requirement for practical model use in the field as well.

The hypothesis of this study was that inclusion of fermentation byproduct in a diet with adequate RDP from soybean meal would increase milk performance. Therefore, the objectives of this study were to 1) evaluate the effects of soybean meal with wheat middlings and urea, soybean meal with Fermenten or soybean meal with rumen protected soybean meal on intake, milk production and total tract nutrient digestion in lactating dairy cattle; 2) provide comparisons of economic and environmental impacts of the three diets; and 3) provide well characterized information to aid in future model development and evaluation.

MATERIALS AND METHODS

The experiment was conducted from February – April 2016 at the Cornell University Ruminant Center in Harford, NY. All animals involved in this experiment were cared for according to the guidelines of the Cornell University Animal Care and Use committee. The committee reviewed and approved the experiment and all procedures carried out in the study.

Animals and Experimental Design

Lactating dairy cattle (n=192) were enrolled in a 12 week longitudinal experiment with 2 weeks of acclimation and covariate sampling followed by 10 weeks of experimental data collection. Cattle averaged (mean ± SD) 118 ± 37 days in milk and 712 ± 88 kg of body weight (**BW**) at trial start. Primiparous (n=48) and multiparous (n=144) cattle were stratified by pre-trial milk production and randomly allocated into 12 pens containing 4 primiparous and 12 multiparous cows each. All cows were fed the same diet during the acclimation and covariate periods. After 1 week of acclimation to pens, covariate measurements were taken in the same manner described for the experimental period. Pens were then randomly allocated to one of three dietary treatments, and were fed those treatment diets for the remainder of the study. All cows were administered bovine somatotropin (500 mg of Posilac^{TM}, Elanco Animal Health, Greenfield, IN) on the same 14 d interval according to label. Cattle were housed in a modern 4-row freestall barn in pens with at least 16 stalls and 16 headlocks per pen and had free access to feed, water, and bedding at all times. Cows were milked 3 x daily at 08:00, 16:00, and 00:00 h in a double-16 parlor. Feed refusals were collected and measured daily at 06:00 hr and cattle were fed 1x daily at 08:00 h to target feed refusals of 5 % of daily intake.

Diets and Sample Collection

Treatment diets consisted of (DM basis) 42% corn silage, 5% alfalfa silage, 8% alfalfa hay, 12.4% steam flaked corn, 7.6% corn meal, and 25% protein premix containing either soybean meal (**Diet A**), soybean meal and Fermenten (**Diet B**), or soybean meal and rumen protected soybean meal (**Diet C**). All diets were balanced (assuming 45 kg/d energy corrected milk) to be sufficient in metabolizable energy (**ME**) and rumen NH_3-N using CNCPS v. 6.55. The protein premix for each diet was mixed at a commercial feed mill (CNY Feeds, Inc., Jordan, NY) and delivered every 3 weeks for the duration of the trial. All cows were fed Diet C during the acclimation and covariate period. Weekly samples of forages were collected and a portion was sent to a commercial lab (Cumberland Valley Analytical Services, Maugansville, MD) for chemical analysis (Table 4.1), while another portion was analyzed for DM by drying at 60°C for 96 h followed by grinding through a 1-mm screen (Wiley Mill no. 4, Arthur H. Thomas, Philadelphia, PA). Ingredient inclusion was adjusted for DM to maintain intended formulation (Table 4.2). Samples of corn meal, steam flaked corn, and refusals were sampled weekly, dried and ground as described above, and composited by ingredient into 3 separate samples representing 3 or 4 consecutive weeks and analyzed for major nutrients (data not shown). Individual ingredients in the premixes were obtained from the mill 3 times during the experiment and analyzed for major nutrients (data not shown). Total mixed ration (**TMR**) was prepared fresh each day. Samples of TMR were collected twice weekly, analyzed for DM, ground and composited as described above and analyzed for nutrient composition (Table 4.3). Daily dry matter intake per pen was calculated from daily feed amount offered and refused corrected for DM determined from individual ingredient values and verified by the DM determinations on TMR, feeds, and refusals. Deviation from intended formulation was tracked with a commercially available feed management program (FeedWatch, Valley Agricultural Software, Tulare, CA).

Table 4.1. Chemical composition (mean ± SD)[1] of forages used in the experiment

Item	Corn silage	Alfalfa silage	Alfalfa hay
DM, %	32.3 ± 2.4	41.9 ± 7.6	86.3 ± 1.6
CP, % of DM	7.8 ± 0.4	17.1 ± 1.6	18.8 ± 1.6
NH_3-N, % of CP	11.3 ± 0.7	9.9 ± 3.3	10.4 ± 1.5
Soluble protein, % of CP	54.8 ± 2.8	61.2 ± 6.1	48.7 ± 2.8
ADICP, % of CP	9.2 ± 0.4	10.5 ± 1.3	7.7 ± 0.8
NDICP, % of CP	11.6 ± 0.8	15.7 ± 3.1	11.9 ± 1.0
aNDFom, % of DM	41.3 ± 2.7	44.2 ± 2.2	39.1 ± 3.8
30h uNDFom, % of aNDFom	40.8 ± 1.0	50.3 ± 2.5	54.6 ± 2.1
120h uNDFom, % of aNDFom	35.9 ± 1.0	44.1 ± 3.1	47.7 ± 2.4
240h uNDFom, % of aNDFom	23.9 ± 1.2	40.1 ± 3.5	44.9 ± 2.6
ADF, % of DM	24.9 ± 1.8	35.4 ± 1.8	35.5 ± 3.2
Lignin, % of DM	2.8 ± 0.2	7.0 ± 0.6	7.5 ± 0.7
Sugars, % of DM	1.5 ± 0.2	4.3 ± 1.7	8.2 ± 0.7
Starch, % of DM	29.4 ± 3.2	2.4 ± 0.8	2.4 ± 0.5
Ether extract, % of DM	3.1 ± 0.1	3.3 ± 0.2	2.2 ± 0.2
Ash, % of DM	2.7 ± 0.2	8.8 ± 1.0	10.5 ± 1.7

[1]Analyzed values from 10 weekly samples per feed ingredient.

Table 4.2. Ingredient composition (mean ± SD)[1] of experimental diets

	Diet		
Ingredient, % DM	A	B	C
Corn silage	41.9 ± 0.1	41.9 ± 0.1	42.0 ± 0.1
Alfalfa silage	4.9 ± 0.1	4.9 ± 0.1	4.9 ± 0.1
Alfalfa hay	8.1 ± 0.1	8.0 ± 0.1	8.1 ± 0.1
Steam flaked corn	12.5 ± 0.8	12.6 ± 0.9	12.6 ± 0.8
Corn meal	7.7 ± 0.7	7.6 ± 0.8	7.7 ± 1.0
Protein premix	24.9 ± 0.1	25.0 ± 0.1	25.0 ± 0.2
Soybean meal	6.98 ± 0.02	7.2 ± 0.03	3.57 ± 0.09
Wheat middlings	3.49 ± 0.01	-	-
Urea	0.51 ± 0.01	-	0.09 ± 0.01
Rumen protected soybean meal[2]	-	-	7.14 ± 0.18
Fermentation byproduct meal[3]	-	3.59 ± 0.01	-
Dried molasses	3.68 ± 0.02	3.61 ± 0.01	3.56 ± 0.09
Soybean hulls	3.81 ± 0.18	3.80 ± 0.15	3.64 ± 0.06
Saturated fatty acid[4]	1.97 ± 0.09	1.98 ± 0.07	1.85 ± 0.10
Blood meal with added methionine[5]	1.05 ± 0.01	1.08 ± 0.01	1.07 ± 0.03
Calcium sulfate, dihydrate	1.05 ± 0.01	-	1.07 ± 0.03
Ground limestone	0.35 ± 0.01	1.08 ± 0.01	0.36 ± 0.01
Sodium sesquicarbonate	0.70 ± 0.01	0.72 ± 0.01	0.71 ± 0.02
White salt	0.52 ± 0.01	0.54 ± 0.01	0.53 ± 0.01
Vitamin and mineral mix[6]	0.79 ± 0.02	0.79 ± 0.03	0.80 ± 0.04

[1]Composition obtained from feed management software and mill mixing information.
[2]SOYPLUS (West Central Cooperative, Ralston, IA).
[3]FERMENTEN (Church & Dwight, Inc., Princeton, NJ).
[4]ENERGY BOOSTER 100 (MSC Company, Dundee, IL).
[5]LYSAAMET (Perdue AgriBuisness, Salisbury, MD)
[6]Contained (DM basis) 8.1 % Ca; 0.21 % P; 21.6 % Mg; 0.7 % K; 1.9 % S; 0.4 % Na; 79 mg/kg of Fe; 3523 mg/kg of Zn; 1050 mg/kg of Cu; 1355 mg/kg Mn; 33 mg/kg of Se; 68 mg/kg of Co; 32 mg/kg of I; 740 KIU/kg of vitamin A; 207 KIU/kg of vitamin D; 4589 IU/kg of vitamin E; and 2550 mg/kg RUMENSIN (Elanco Animal Health, Greenfield, IN).

Table 4.3. Chemical and nutrient composition (mean ± SD)[1] of experimental diets

	Diet		
Item	A	B	C
DM, %	48.3 ± 0.9	48.4 ± 0.9	48.3 ± 0.9
OM, % of DM	92.8 ± 0.4	92.3 ± 0.8	92.8 ± 0.4
CP, % of DM	14.9 ± 0.2	14.8 ± 0.2	14.9 ± 0.5
SP, % of CP	40.9 ± 7.3	40.4 ± 4.5	31.8 ± 4.9
RDP, % of DM[2a]	9.4 ± 0.7	8.8 ± 0.4	8.1 ± 0.3
ADICP, % of CP	5.9 ± 0.5	5.6 ± 0.2	5.7 ± 0.4
NDICP, % of CP[a]	7.1 ± 0.7	7.4 ± 0.8	10.9 ± 0.5
aNDFom, % of DM	31.3 ± 0.5	31.3 ± 0.4	31.0 ± 0.1
240h uNDFom, % of aNDFom	27.8 ± 2.3	27.9 ± 0.6	26.3 ± 1.1
ADF, % of DM	19.3 ± 0.8	20.2 ± 0.4	19.8 ± 0.2
ADL, % of DM	2.9 ± 0.2	2.7 ± 0.1	2.9 ± 0.1
Sugars, % of DM	5.9 ± 1.0	5.7 ± 1.3	5.8 ± 1.5
Starch, % of DM	26.2 ± 0.9	26.0 ± 1.0	26.3 ± 1.1
Ether extract, % of DM	4.6 ± 0.2	4.7 ± 0.1	4.7 ± 0.3
Ash, % of DM	7.2 ± 0.4	7.7 ± 0.8	7.2 ± 0.4
Metabolizable energy, Mcal/kg[2]	2.7 ± 0.1	2.7 ± 0.1	2.7 ± 0.2

[1]Analyzed values from 3 composite samples per diet from twice weekly TMR samples.
[2]Calculated by the Cornell Net Carbohydrate and Protein System v. 6.55.
[a]Diet C differs significantly from diets A and B ($P < 0.05$).

Body weights on every animal were measured weekly after the 16:00 h milking, and condition score (**BCS**; 1-5 scale) was recorded weekly as the average of two trained scorers. Milk yield was recorded at every milking by farm software systems (ALPRO; DeLaval Inc., Kansas City, MO). Weekly milk samples were taken at 3 consecutive milkings and analyzed for fat, true protein, lactose, somatic cell count, total solids, and milk urea nitrogen (**MUN**) (Dairy One, Ithaca, NY) using Fourier transform infrared spectroscopy (Milkoscan 6000; Foss Electric, Hillerød, Denmark). Daily milk composition was calculated using a weighted average of the three milkings. Weekly energy corrected milk (**ECM**) yield was then calculated using the equations of Tyrrell and Reid (1965).

Fecal samples were collected from a randomly selected subset of cows (2 primiparous and 6 multiparous) in each pen during the covariate, and weeks 5 and 10 of the experimental period. On the day following milk and feed sampling for each respective week, three spot fecal samples (~500 g/cow) were taken at 06:00, 12:00, and 22:00 hours, composited by pen, and frozen at −20°C. Samples were later thawed, mixed, and a subsample was dried at 60°C for 96 h and ground to pass a 1mm screen. Dried and ground fecal samples were analyzed for starch, aNDFom (Mertens, 2002), and for undigested NDF (**uNDFom**) after 240 h of in vitro incubation with rumen fluid, according to Raffrenato (2011). Apparent total tract digestion of DM, starch and aNDFom was calculated using uNDFom as a marker, as described by Huhtanen et al. (1994).

Blood samples were collected during the covariate period, and wk 5 and 9 from the same subset of cows as the fecal sampling. Blood (8 mL) was collected 8 h after feeding via the coccygeal vein into tubes containing sodium heparin and immediately placed on ice. Within 30 minutes of collection, samples were then centrifuged (3,000 × *g* for 20 min at 4°C) and plasma was harvested and frozen at -20°C for later analysis. Plasma urea nitrogen (**PUN**) concentration

was determined using an enzymatic colorimetric assay based on a commercial kit (No. 640, Sigma-Aldrich, St. Louis, MO).

A comparison of milk income and feed costs was performed using the milk component pay price received and an estimation of feed costs incurred by the Cornell University Ruminant Center during the duration of the trial. Regional feed prices were used to estimate ingredient costs when insufficient information was available. For N utilization comparisons among diets, all relevant farm, cattle, and diet information was entered into the CNCPS v. 6.55. Body condition score change was inputted as measured, while weight change was computed by the target growth system (Fox et al., 1999) within the CNCPS. Body weight gain in the CNCPS was assumed to be associated with frame growth, thus entering observed BW gain for cattle gaining body condition through the lactation cycle will result in overestimation of growth requirements for energy and protein.

Statistical Analyses

All data were analyzed using SAS version 9.3 (SAS Institute Inc. Cary, NC). Diet chemical composition was analyzed using PROC GLM and means were compared using the LSMEAN statement. Pen was considered as the experimental unit, with covariate adjustment applied at the individual cow level when suitable. (St-Pierre, 2007). For variables containing individual cow information with covariate and repeated weekly measurements (milk, PUN, BW and BCS), the following model was used:

$$Y_{ijkl} = \mu + T_i + W_j + TW_{ij} + P_{k:i} + C_{l:ik} + BX_{lik} + L_m + \varepsilon_{ijkl}$$

where Y_{ijklm} = dependent variable, μ = overall mean, T_i = fixed effect of treatment i, W_j = fixed effect of week j, TW_{ij} fixed interaction of treatment i and week j, $P_{k:i}$ = random effect of pen k

within treatment i, $C_{l:ik}$ = random effect of cow within pen k and treatment I, BX_{ilk} = the covariate adjustment for each cow, L_m = fixed effect of parity, and ε_{ijklm} = residual error. For variables containing pen-level repeated measurements (intake and digestibility data), terms corresponding to individual cow information were removed, and covariate adjustment was applied at the pen level if available. Statistical analysis of non-repeated measures (BW and BCS change) did not contain repeated or covariate adjustments. Nine cows left the experimental pens throughout the trial for health or management reasons (2, 4, and 3 cows for diet A, B and C, respectively). These cows were excluded from the analysis for the duration of the absence and, if returned to pens, were evaluated for re-inclusion after 2 weeks if milk production indicated adequate recovery. In all statistical models, the degrees of freedom were calculated using the Kenward-Roger option to account for any imbalance from missing observations. Treatment means were determined using the least squares means statement, and means were compared using the PDIFF option. Statistical significance was considered at $P \leq 0.05$ and trends were considered at $0.05 < P \leq 0.10$.

RESULTS AND DISCUSSION

Diets and Milk Performance

Corn silage fed during the trial averaged 41.3 % aNDFom, 24.9 % ADF and 29.4 % starch, and did not vary appreciably for the duration of the trial (Table 4.1). Alfalfa silage averaged 44.2% aNDFom, 35.4% ADF, and 17.1% CP, although CP decreased in the middle portion of the trial, reaching a nadir of 15 % CP in week 7 (Figure 4.1). Alternatively, alfalfa hay maintained a slightly greater than average CP concentration in weeks 4-8, reaching a peak CP concentration of 21.1% CP in week 8, so no diet adjustments were deemed necessary. Experimental diets were formulated to be iso-nitrogenous and iso-energetic (Table 4.3). Using the measured chemical composition and intakes, calculated RDP using the CNCPS v. 6.55 was decreased in diet C compared with diets A and B (7.5 vs. 9.0 and 8.5 % of DM, respectively; $P < 0.05$). Consequently, the inclusion of rumen protected soybean meal in diet C resulted in increased NDICP (10.9 vs. 7.1 and 7.4 % of CP for diets C, A and B, respectively, $P = 0.03$). These differences in ruminal protein degradability were intended given the diet formulation and expected mode of action of the fermentation byproduct. All other analyzed nutrients were not different between diets ($P > 0.05$; Table 4.2).

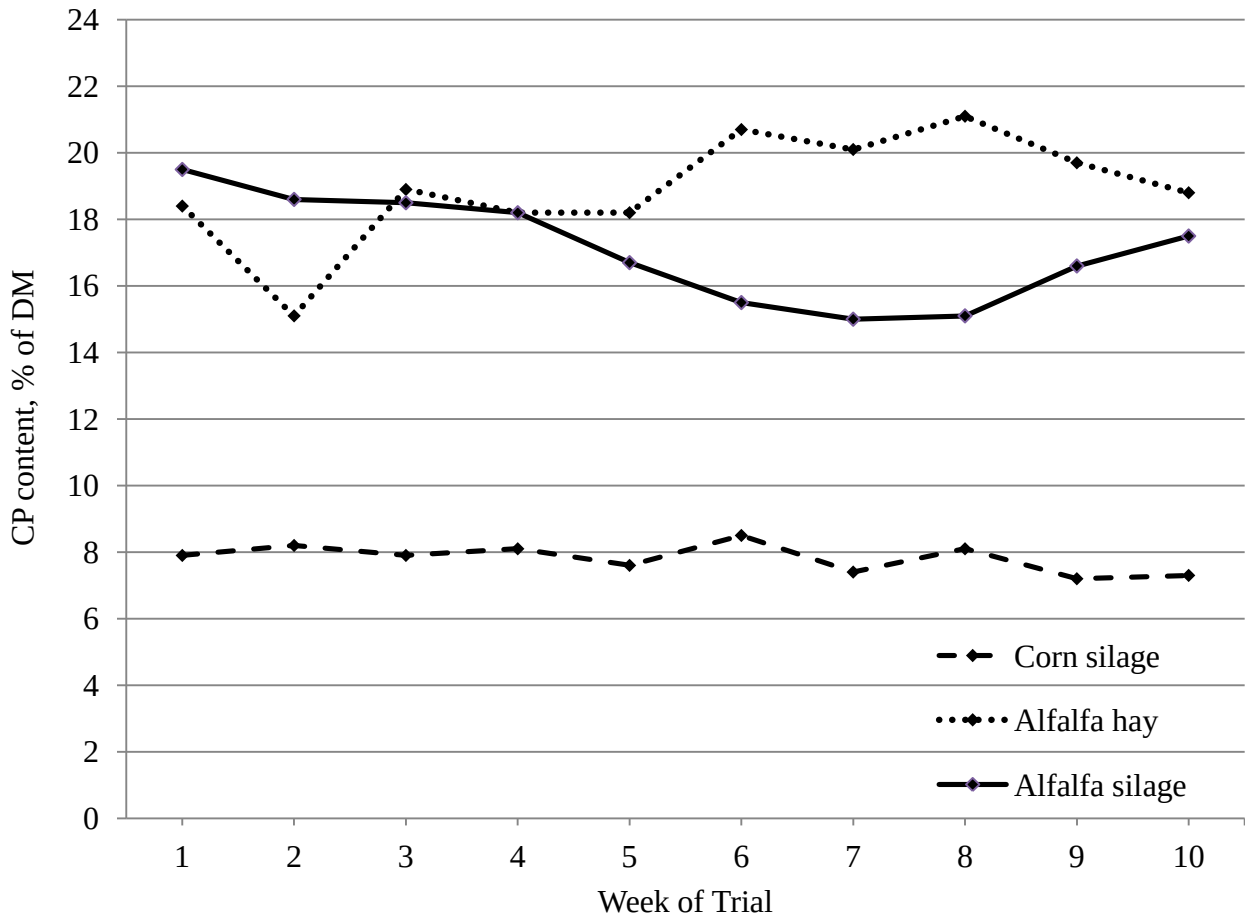

Figure 4.1. Analyzed weekly crude protein content of the forages for the duration of the trial.

Cows fed diet B had increased intake and energy corrected milk compared with cows fed diets A and C ($P < 0.05$; Table 4.4 and Figure 4.2). Total milk yield was significantly higher in cows fed diet B compared with cows fed diet A (40.7 vs. 42.5 kg/d for diets A and B, respectively, $P < 0.05$), while milk yield for cows fed diets B and C were not significantly different (P = 0.21). Milk protein percent and yield were also both increased in cows fed diet B compared with diets A and C. Milk fat percent and yield were not significantly different amoung any of the treatments. The magnitude and direction of the observed difference in intake and energy corrected milk was similar to that reported the omasal flow study with the same fermentation byproduct meal (Chapter 2) when compared to cows fed control diet with similar wheat middlings and urea inclusion rates. It was hypothesized that the addition of fermentation byproduct meal in diet B would reduce dietary CP degradation in the rumen, thus allowing cows fed diet B to have increased flow of MP to the small intestine relative to cows fed diet A. Given the similarity of the diets, cows, and management, a similar protein sparing mechanism as described in Chapter 2 would allow for the observed milk production results reported here.

Table 4.4. Effect of rumen degradable protein source on dry matter intake and milk performance in lactating dairy cattle.

	Diet[1]				*P*		
Item	A	B	C	SEM	Diet	Week	D*W
Dry matter intake, kg/d	26.9[a]	28.3[b]	26.7[a]	0.4	0.03	< 0.01	< 0.01
Milk yield, kg/d	40.7[a]	42.5[b]	41.6[ab]	0.5	0.05	< 0.01	< 0.01
ECM, kg/d	43.6[a]	45.3[b]	43.7[a]	0.4	0.05	< 0.01	< 0.01
Milk fat, %	3.82	3.73	3.72	0.05	0.30	< 0.01	0.01
Milk fat, kg/d	1.62	1.64	1.61	0.02	0.39	< 0.01	< 0.01
Milk true protein, %	3.11[ab]	3.17[b]	3.08[a]	0.03	0.11	< 0.01	< 0.01
Milk true protein, kg/d	1.31[a]	1.38[b]	1.33[a]	0.01	0.02	< 0.01	< 0.01
Milk urea N, mg/dL	7.1[a]	6.7[b]	6.2[c]	0.1	< 0.01	< 0.01	< 0.01
Plasma urea N, mg/dL	9.5[a]	8.5[b]	8.3[b]	0.2	0.01	< 0.01	0.11
Feed efficiency[3]	1.60	1.59	1.61	0.02	0.77	< 0.01	< 0.01

[1]A= Diet containing soybean meal premix, B= Diet containing fermentation byproduct premix, C= Diet containing rumen protected soybean meal premix.

[2]ECM/DMI.

[a,b,c]Within a row, means without a common superscript differ ($P < 0.05$).

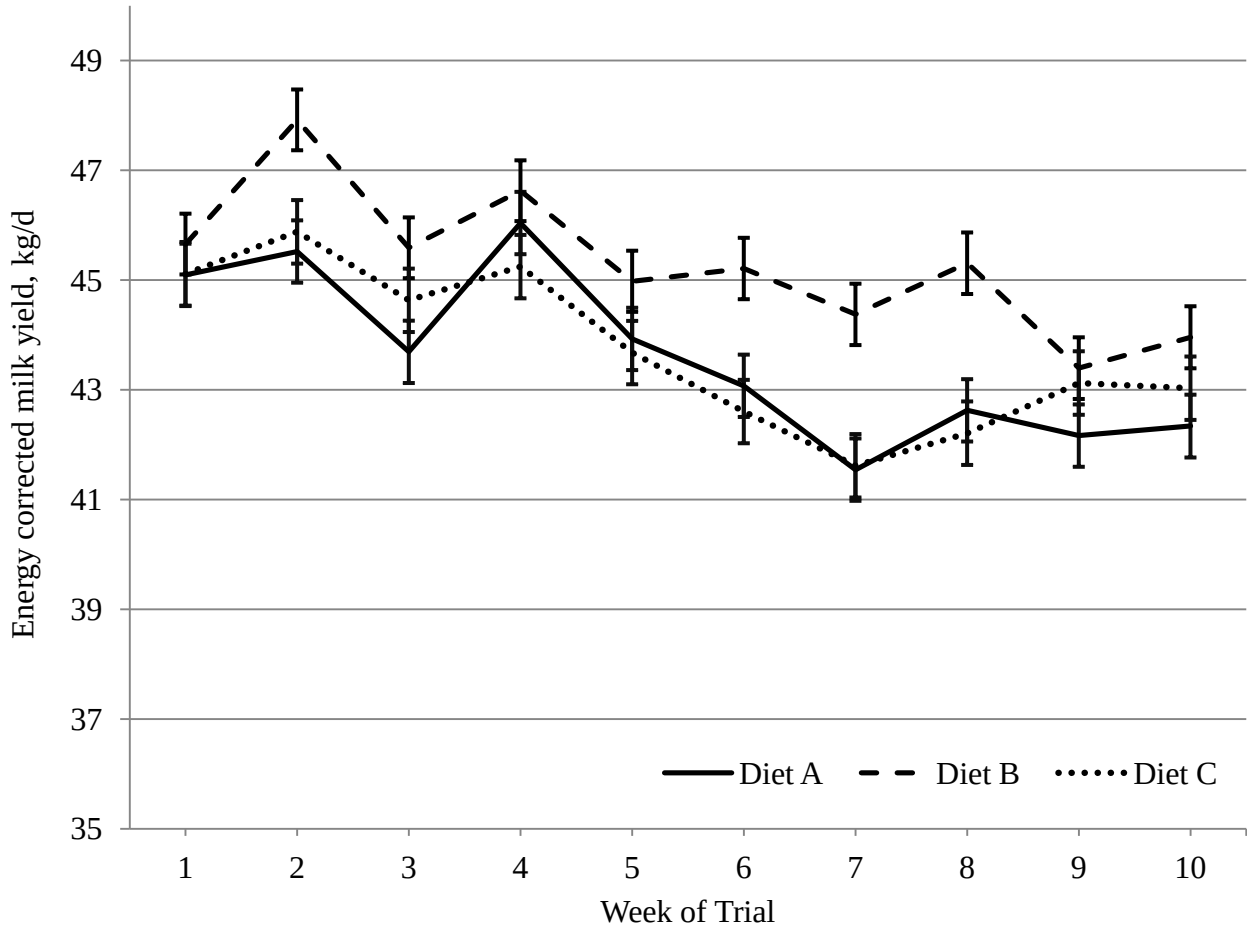

Figure 4.2. Energy corrected milk yield by week for the duration of the trial. Error bars represent the standard error. Diet A= Diet containing soybean meal premix, Diet B= Diet containing fermentation byproduct premix, Diet C= Diet containing rumen protected soybean meal premix.

Previous research with similar fermentation byproducts demonstrated little to no positive benefits when compared to a urea control (Broderick et al., 2000). The diets in that study had very little non-forage protein supplementation other than the fermentation byproduct, possibly resulting in low true soluble and degradable protein balance in the urea control and fermentation byproduct treatments. As such, soybean meal supplementation produced an expected positive response in milk production compared with a control diet containing wheat middlings and urea (Broderick et al., 2000). While recycling of urea N and endogenous secretions might provide additional rumen-available N, the ability of microbes to utilize these sources for rapid, efficient growth is likely lower than preformed AA of dietary origin (Atasoglu et al., 2001; Wallace et al., 2001). In diets averaging approximately 20 % CP, Penner et al. (2009) observed a numerical increase in milk yield and milk energy when Fermenten was fed in low sugar diets compared with a canola meal control. Direction and magnitude of the effect in the Penner et al. (2009) study was similar to observed response in the current study. These results indicate that the ability for a Fermenten to improve MP flow and therefore production is likely dependent on positive rumen peptide and degradable protein balance.

As the trial progressed, ECM in cows fed diets A and C decreased (Figure 4.2) to a nadir at week 7, the same week that alfalfa silage CP content reached its nadir (Figure 4.1). It is possible that the protein sparing effect of the fermentation byproduct described in Chapter 2 might have allowed cows fed diet B to maintain MP supply despite a decrease in soluble N from alfalfa silage. Use of ECM to evaluate production differences takes into account the animal's ability to utilize substrate and metabolites in a variety of milk synthesis pathways, depending on which nutrient is most limiting (Lobley, 2007; Lemosquet et al., 2010). This is demonstrated in cows fed diet C, where milk yield was not significantly different from cows fed diet A or B, however

milk protein yield was significantly lower (1.38 vs. 1.31 kg/d for cows fed diets B and C, respectively). Diet C was formulated to be sufficient in rumen degradable N, with inclusion of rumen protected soybean meal to allow for increased passage of intestinally available feed protein; therefore cows fed B and C were expected to perform in a similar manner. Given the relatively lower milk yield in cows fed diet C, it is possible that rumen degradable protein balance was insufficient to promote adequate microbial protein synthesis and thus MP supply. In separate reviews of soybean meal replacement with RUP supplements, Santos et al. (1998) and Ipharraguerre and Clark (2005) found inconsistent increases in milk performance, and concluded that rumen degradable protein balance can be limiting in many diets with high RUP supplementation, although neither analysis sought to control for the possibility of ME limitations in the datasets. Lower than expected intestinal digestibility of the rumen protected meal may also contribute to insufficient MP compared with diets A and C.

Dietary protein source strongly influenced MUN and PUN concentrations in this study (Table 4.4). Milk urea nitrogen was greatest in diet A, followed by diets B and C (7.1, 6.7, and 6.2 mg/dL for A, B and C, respectively). Plasma urea nitrogen followed the same trend. Responses of this sort have been well documented before in low CP diets (Colmenero and Broderick, 2006; Brito and Broderick, 2007). In a study investigating the effects of rumen available N in the form of urea, Broderick and Reynal (2009) demonstrated a clear, linear response of MUN and PUN to increased RDP. In the present study, steadily decreasing RDP in the form of true protein similarly resulted in lower MUN and PUN. In a meta-analysis of omasal sampling studies, Broderick et al., (2010) suggested that in diets of 14.7 % CP and RDP of 10.6 % resulted in ruminal N balance of 0. Compared with the results in chapter 2, the inclusion of fermentation byproduct had an opposite effect on urea N concentrations. This is consistent with

the hypothesis of a protein sparing effect of fermentation byproduct on degradable protein. In the previous trial, ruminal CP degradation was decreased, yet greater ammonia accumulation occurred, indicating possible shifts in the fate of soluble AA and peptides between diets. Since peptide uptake and transport a rate limiting step in protein degradation (Wallace et al., 1990), accumulation of AA and peptides in the rumen fluid might simultaneously allow for increased rumen NH_3-N accumulation and increased RUP flow from soluble amino-N. This is likely to occur when relatively greater numbers of hyper-ammonia producing bacteria exist (Russell et al., 1988). A key difference between the omasal study (Chapter 2) and the current study is the use of monensin in this study. Hyper-ammonia producing bacteria have been shown to be sensitive to monensin (Yang and Russell, 1993), and suppression of these microbes would allow for concomitant decreased MUN and PUN levels. Further investigation of possible fermentation byproduct and monensin interactions might be warranted.

Body Weight, Condition Score, and Total Tract Nutrient Digestibility

Dietary protein source did not have any appreciable effects on body weight, condition score, or rate of BW and BCS change (Table 4.5; Figure 4.3). Recorded BW and BCS change was consistent with normal temporal changes in condition score in mid-lactation dairy cattle. (Waltner et al., 1993; Roche et al., 2009). Total tract apparent nutrient digestibility was affected by protein source (Table 4.6). Daily dry matter intakes reported are from sampling weeks only. Intake of uNDFom was significantly different among diets, with 2.34, 2.48, and 2.15 kg/d uNDFom consumed for diets A, B, and C, respectively ($P < 0.01$). These differences are likely due to the combination of small numerical differences in intake and uNDFom concentration between diets. Rumen degradable protein intake was significantly lower in diet C relative to diets A and B (2.16 vs. 2.57 and 2.51 g of RDP intake / d, respectively; $P < 0.01$). Since microbial

contribution to fecal nutrients was not quantified, all total-tract digestibilities are treated as apparent. Dry matter digestibility tended to be lower in diet B relative to diets A and C (P = 0.09), while OM digestibility was increased in Diet C vs. Diet B (73.8 and 76.7 %, respectively; $P < 0.05$) although this is of limited biologic significance due to microbial and metabolic fecal contributions (Van Soest, 1994). Total tract digestion of aNDFom was significantly lower in diet B compared with diet C ($P < 0.01$), while no significant difference was observed between cows fed diets A vs. B or A vs. C. Similar directionality and magnitude of differences was observed with total tract digestibility of the potentially digestible NDF fraction, however no significant differences were detected. These results indicate that degradation of NDF in the total tract was not positively affected by increased dietary supply of RDP. Results observed in this study is similar to those reported by Brito and Broderick (2007) in cattle fed canola compared with a urea control, where increased RDP did not increase total tract NDF digestion. In an omasal study with processed vs. unprocessed canola meal, increased RDP had no effect on ruminal or total tract NDF digestion, although increased CP level in the diet had negative effects on NDF digestion (Mutsvangwa et al., 2016). This was likely a result of positive rumen N balance in most of these studies. When cattle were fed diets formulated to be deficient in rumen N, total tract NDF digestion was decreased (Higgs, 2014). Digestion of NDF is influenced not only by RDP but also many other factors, a principal one being intake. In the current study, it is likely that the increased intake observed in cows fed diet B resulted in increased passage rate from the rumen, thus reducing the extent of digestion (Waldo et al., 1972; Van Soest, 1994). Interestingly, NDF digested per gram of RDP intake was increased in cows fed diet C compared with cows fed diets A and B. (2.03 vs. 1.70 and 1.68 g of NDF digested / gram of RDP intake, respectively). This increase in apparent NDF digestion efficiency is likely due to increased N recycling to the GI

tract, allowing the rumen to maintain high levels of microbial fiber degradation even when dietary RDP supply is low (Reynolds and Kristensen, 2008; Mutsvangwa et al., 2016).

Table 4.5. Effect of rumen degradable protein source on body weight and condition score in lactating dairy cattle

	Diet[1]				*P*		
Item	A	B	C	SEM	Diet	Week	D*W
Body weight, kg	721	719	723	2.8	0.68	<0.01	0.97
Body condition score	2.97	3.00	2.98	0.02	0.32	<0.01	0.11
Body weight change, kg/week	3.4	3.8	4.0	0.90	0.74	-	-
Condition score change, score/month	0.03	0.05	0.06	0.01	0.32	-	-

[1]A= Diet containing soybean meal premix, B= Diet containing fermentation byproduct premix, C= Diet containing rumen protected soybean meal premix.

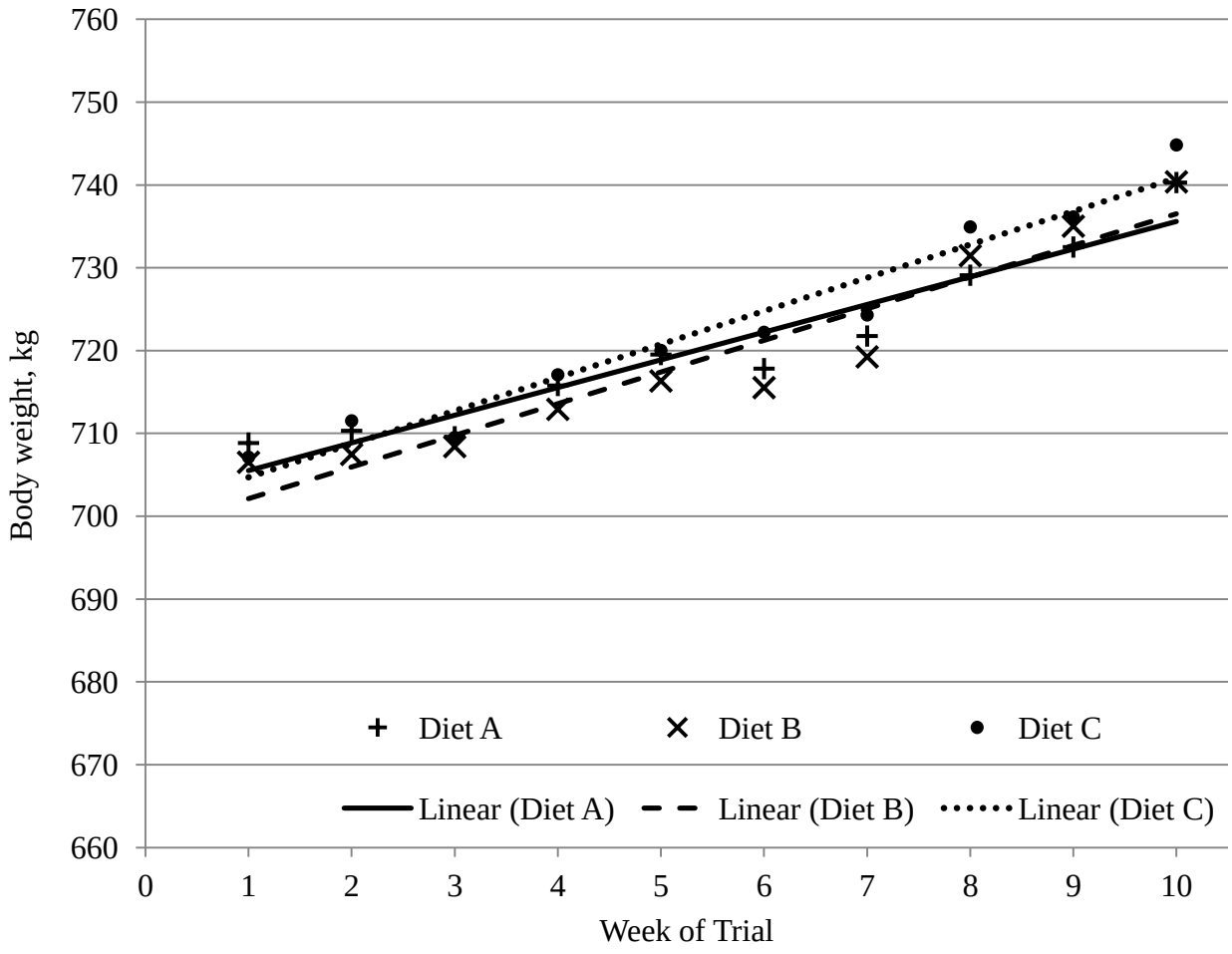

Figure 4.3. Body weight by week of trial for lactating dairy cows fed three different rumen degradable protein sources, where Diet A= diet containing soybean meal premix, Diet B= diet containing fermentation byproduct premix, Diet C= diet containing rumen protected soybean meal premix. The equation describing the linear fit line in cattle fed Diet A is y = 3.35x + 702; for Diet B is y = 3.82x + 698; for Diet C is y = 4.02x + 701.

Table 4.6. Effect of rumen degradable protein source on apparent total tract nutrient digestion

	Diet[1]				
Item	A	B	C	SEM	*P*
Intake, kg/d					
DM	27.8	28.1	27.0	0.5	0.28
OM	25.7	25.8	25.1	0.4	0.44
aNDFom	8.67	8.78	8.35	0.14	0.15
pdNDF	6.34	6.30	6.21	0.10	0.68
uNDFom	2.34[b]	2.48[c]	2.15[a]	0.04	< 0.01
RDP[2]	2.57[a]	2.51[a]	2.16[b]	0.04	< 0.01
Starch	6.86[a]	6.85[a]	6.39[b]	0.11	0.03
Apparent total tract digestibility, %					
DM	71.2	70.3	72.7	0.7	0.09
OM	74.6[ab]	73.8[a]	76.7[b]	0.6	0.02
aNDFom	50.3[ab]	47.9[a]	52.4[b]	0.9	0.02
pdNDF	68.8	66.8	70.6	1.2	0.14
Starch	99.2	99.2	99.3	0.2	0.96
NDFD/RDP[3]	1.70[a]	1.68[a]	2.03[b]	0.03	< 0.01

[1]A= Diet containing soybean meal premix, B= Diet containing fermentation byproduct premix, C= Diet containing rumen protected soybean meal premix.

[2]Calculated by the Cornell Net Carbohydrate and Protein System v. 6.55.

[3]kg of aNDFom digested per kg of rumen degradable protein intake.

[a,b,c]Within a row, means without a common superscript differ ($P < 0.05$).

Economic and Environmental Comparisons

Results of the economic comparisons are reported in Table 4.7. The pricing of the fermentation byproduct and rumen protected soybean meal numerically increased the calculated price of the protein mix relative to the soybean meal only mix (543, 550, 556 $/ton of DM for mix A, B, and C, respectively). Combined with slightly higher intakes in cows fed diet B, the average diet cost per head/d was numerically increased for cows fed diet B (7.38, 8.38, and 7.92 $/h/d for cows fed diets A, B, and C, respectively). Milk component pay price averaged 2.65 $ / lb for butterfat, 1.75 $/lb for milk true protein, and 0.05 $ / lb for other solids. The observed production resulted in a daily milk component income of 14.22, 14.72, and 14.24 $/h/d for cows fed diets A, B, and C, respectively. Therefore, the calculated income over feed cost (IOFC) based on milk component pricing and estimated feed costs was 6.33, 6.37, and 6.32 $/h/d for cows fed diets A, B, and C, respectively. The approximately $ 0.05 numerically greater IOFC in cows fed diet B relative to those fed A and C results in an additional $18,250 of IOFC for a 1000 cow dairy over a 1 year period. This benefit does not take into account the dilution of fixed costs associated with higher productivity per cow (Schmidt and Pritchard, 1987; Eicker et al., 2006). Results of this comparison demonstrate that the use of fermentation byproducts can be economically beneficial when priced competitively with soybean meal.

Table 4.7. Effect of rumen degradable protein source on economics associated with feed costs and milk income.

		Diet cost or income, $/h/d[1]		
Item	Prices	A	B	C
Feed cost	$ / ton of DM[2]			
Corn silage	145	1.80	1.90	1.79
Alfalfa silage	130	0.19	0.20	0.19
Alfalfa hay	300	0.71	0.75	0.71
Steam flaked corn	185	0.75	0.79	0.74
Corn meal	200	0.42	0.44	0.41
Protein premix A	543	4.02	-	-
Protein premix B	550	-	4.29	-
Protein premix C	556	-	-	4.09
Total ration cost, $/h/d		7.89	8.35	7.92
Production income	$ / lb pay price[3]			
Milk fat	2.65	9.08	9.26	9.04
Milk protein	1.75	4.88	5.20	4.94
Other milk solids	0.05	0.26	0.27	0.26
Total component income, $/h/d		14.22	14.72	14.24
Daily IOFC, $/h		6.33	6.37	6.32

[1]Diet cost and income amounts calculated using previously reported diet composition, intake, and milk performance (Tables 4.2 and 4.3).
[2]Cost per ton of dry matter determined as price paid by the Cornell University Ruminant Center when available or by regional feed markets during the duration of the trial.
[3]Milk component pay prices obtained from milk checks received by the Cornell University Ruminant Center during the duration of the trial.

Comparisons of the nitrogen intake, excretion, and nitrogen utilization for each diet are provided in Table 4.8. The values reported are the raw values from simulation with the CNCPS v. 6.55. Cows fed diet B had numerically greater intake of N compared with diets A and C. Fecal N was predicted to be greater in diet B, likely due to increased OM intake (Higgs et al., 2012). Overall milk nitrogen efficiency (**MNE**) averaged approximately 32%, which is higher than the mean, yet well within the range reported by Huhtanen and Hristov (2009). The authors concluded that reduction of CP intake was the most effective way at increasing MNE in lactating dairy cattle. The mean CP content of diets fed in this trial was less than 15%, a value on the lower end of the Huhtanen and Hristov (2009) meta-analysis. When the MNE number is calculated without the contribution of intake from forage N, efficiently improves. This recognizes the fact that non-ruminant species are not typically fed forage-based diets, and might provide a more meaningful comparison to the 50-60 % N retention seen with non-ruminant species (Campbell et al., 1984, Noblet et al., 1987, Sakomura et al., 2007). When MNE is calculated excluding forage and non-protein N from byproducts, MNE can be as high as 60-70%. Due to the inclusion of rumen protected meal and soybean meal as the main digestible protein source, diet C exhibits the lowest MNE when excluding forage and byproduct non-protein N. This comparison demonstrates the value of formulating diets using byproduct feeds that do not compete directly with human-edible products (Clark et al., 1987). Russomanno et al. (2012) performed a comparison of several byproduct inclusion levels on greenhouse gas emissions when fed to a cow or incineration, demonstrating the positive aspects of byproduct inclusion. Similar large scale comparisons have previously been used to demonstrate the progress made by the dairy industry toward a more environmentally sustainable food production system (VandeHaar and St-Pierre, 2006; Bradford and Mullins, 2012).

Table 4.8. Comparisons of efficiency of nitrogen use in dairy cattle fed three different protein sources

	Diet[1]		
Item	A	B	C
N intake g/d	641	667	637
N output from milk true protein g/d	205	216	208
N excretion, g/d[2]			
Fecal N	250	262	246
Urine N	180	179	177
Productive N:Urinary N	1.13:1	1.20:1	1.17:1
Milk nitrogen efficiency (MNE), %			
MNE, all intake N	32.0	32.4	32.8
MNE, excluding intake of forage N	51.7	53.8	52.6
MNE, excluding intake of forage and byproduct N	64.0	72.1	55.6

[1]A= Diet containing soybean meal premix, B= Diet containing fermentation byproduct premix, C= Diet containing rumen protected soybean meal premix.

[2]Predicted values from the Cornell Net Carbohydrate and Protein System, v. 6.55

CONCLUSIONS

Inclusion of fermentation byproduct increased milk yield, protein yield, and subsequently energy corrected milk yield when compared with soybean meal and rumen protected soybean meal in mid-lactation dairy cows. Milk and plasma urea nitrogen concentrations were decreased as protein degradability decreased in the diet. Body weight and condition score change was unaffected by diet. Total tract digestion of NDF was slightly reduced with fermentation byproduct inclusion; likely due to an increase in DM intake. Fermentation byproduct meal can be successfully used to improve milk performance and economic returns while reducing negative environmental impacts compared with processed and unprocessed soybean meal based diets.

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CHAPTER 5: Rumen and omasal bacteria and protozoa composition, digestibility and amino acid profile determined by multiple hydrolysis times

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ABSTRACT: Microbial samples from several independent experiments in lactating dairy cattle were obtained and analyzed for nutrient composition, AA digestibility, and AA profile after multiple hydrolysis times ranging from 2 to 168 h. Similar bacterial and protozoal isolation techniques were used for all isolations. Omasal bacteria and protozoa samples from two trials were analyzed for AA digestibility using a new in vitro technique. Multiple time point hydrolysis and least-squares non-linear regression were used to evaluate the true amino acid content of omasal bacteria and protozoa, and comparisons were made against single time point hydrolysis. Formalin was used in one experiment, which strongly affected AA digestibility and likely limited the complete release of AA during acid hydrolysis. The mean AA digestibility was 87.8 and 81.6 % for non-formalin treated bacteria and protozoa, respectively. Formalin treatment decreased recovery of several individual AA. Results from the multiple time point hydrolysis indicated that Ile, Val, and Met hydrolyze at a slower rate compared with other essential AA. Serine, Ile, and Val were under-predicted using AA determination after a single 24 h hydrolysis. Results indicate that models for predicting post-ruminal supply of AA might need to consider potential bias present in post-ruminal AA flow literature when AA determinations are performed after single time point hydrolysis and when using formalin as a preservative for microbial samples.

Keywords: rumen bacteria, rumen protozoa, digestibility, amino acids, hydrolysis

INTRODUCTION

Nutrient supply and requirement models such as the Cornel Net Carbohydrate and Protein System (**CNCPS**) (Van Amburgh et al., 2015) and the NRC (NRC, 2001) along with their derivative models predict post ruminal flows of bacterial biomass. Bacterial protein flow is assigned an AA content, AA profile, and a digestibility of individual AA to calculate supply of metabolizable AA. These field applicable models typically use the AA profile of bacteria obtained from the literature (Storm et al., 1983; Clark et al., 1992; Volden and Harstad, 1998), and few account for protozoal AA flows, which can contribute a substantial amount to total microbial AA flow (Chapter 3). A new, dynamic version of the CNCPS (v. 7; Higgs, 2014) utilizes a similar approach in a nitrogen based model, and mechanistically accounts for protozoa and endogenous AA contributions to total AA flow. To characterize this model, accurate representations of the AA content and digestibility of bacterial and protozoal AA were needed.

The methods used for isolation of microbial fractions and analysis of AA vary widely across the literature, and much of the data used for nutrition models still rely on older methods where more robust alternatives now exist. An example can be found in the isolation of protozoa, where differential centrifugation has historically been used to isolate microbial cells with significant contamination from bacteria and feed particles. A procedure developed for isolation of cultivatable mixed rumen protozoa for qPCR and competition studies (Sylvester et al., 2004; 2005; Denton et al., 2015) can be used to isolate protozoa for more accurate nutrient analysis.

Intestinal digestibility of individual microbial AA has been estimated by several different methods, including regression approaches (Tas et al., 1981; Hvelplund and Hesselholt, 1987) and in vitro assays such as the modified three-step assay (Gargallo et al., 2006) and the mobile bag technique. Procedures relying on retention in bags are largely inadequate, as indigestible protein

can pass through the large pores in the bags resulting in inflated digestibility values. The precision-fed cecectomized rooster bioassay has recently been applied to rumen bacteria (Fonseca et al., 2014); however data are still lacking on protozoa AA digestibility. Ross et al. (2013) recently developed an assay to determine intestinally unavailable N (**uN**) in ruminant feeds that addressed the issues of non-physiologic or species specific enzyme activities and poor retention of small particles. This procedure might provide an adequate assessment of intestinal digestibility of rumen bacteria and protozoa isolates.

Amino acid content of feeds and microbes has historically been determined by single time point hydrolysis, as this represents a compromise between maximal release of AA from the matrix while minimizing the loss of acid labile AA (Rutherfurd, 2009). Determination at multiple time points followed by least-squares non-linear regression provides more accurate estimates of the true amino acid profile (Darragh and Moughan, 2005). This approach has been utilized in purified protein (Darragh et al., 1996), cat hair, (Hendriks et al., 1998), milk protein (Rutherfurd et al., 2008) and common animal feedstuffs (Rutherfurd, 2009). To our knowledge, AA determination after multiple hydrolyses times has not been performed on rumen microbial biomass.

The hypothesis for this study was that updated AA characterization methods would result in different values for microbial AA content and availability than what is currently used in nutrition models. The specific objectives of this study were to: 1) characterize the chemical composition and AA profile of rumen bacteria and protozoa from high producing lactating dairy cows; 2) evaluate the intestinal digestibility of microbial AA using a new in-vitro assay; and 3) Determine the amino acid content of omasal bacteria and protozoa using multiple hydrolysis times and least-squares non-linear regression.

MATERIALS AND METHODS

All cannulated cows used as rumen or omasal fluid donors for the microbial isolations in this experiment were cared for according to the guidelines of the Institutional Animal Care and Use committee appropriate for the university responsible for their care. The committees reviewed and approved the experiment and all procedures carried out in the study.

Microbial Isolation Procedures

Microbial samples from several independent experiments in lactating dairy cattle were obtained and analyzed for nutrient composition, AA content, and intestinal nutrient and AA digestibility. Bacteria and protozoa included in the analysis were from the following experiments: **Trial A**: An omasal sampling trial with 8 cows in a 2 treatment switchback design investigating effects of a fementation byproduct on omasal nutrient flow (Chapters 2 and 3); **Trial B**: An omasal sampling trial with 12 cows in a 3 treatment Latin Square design investigating effect of rapidly degradable starch on omasal nutrient flow (Foskolos et al., unpublished data); **Trial C**: A rumen nitrogen balance and recycling trial with 12 cows in a 3 treatment randomized complete block design investigating rumen N and/or MP deficient diets (Recktenwald, 2010). One additional protozoal sample was obtained from T. Hackmann at the University of Florida from repeated isolations from the rumen of a lactating dairy cow at the Ohio State University Columbus campus **(Trial D)**. For trials A-C, equal parts DM were combined within microbial type, resulting in a composited sample of bacteria and protozoa from each experiment. Therefore, the possible effects of treatments from trials A – C are not represented in this dataset. Information regarding the chemical composition of the average diet fed to cows in each experiment, along with the number of isolations represented by each composited sample, if available, is in Table 5.1.

For Trials A and B, microbial samples were obtained using the omasal sampling technique developed by Huhtanen et al. (1997) and adapted by Reynal and Broderick (2005). Samples of whole omasal contents were collected from the omasal canal every 2 h during three 8 h intervals. Details of sampling for trial A are described in Chapter 2 and 3. Trial B sampling occurred according to a very similar sampling schedule as Trial A by the same researchers (A. Foskolos and S. Fessenden). Trial C collection methods are described in Chapter 2 of Recktenwald (2010). Trial D protozoa were collected 4 separate days with two separate aliquots filtered per day for 8 aliquots total (T. Hackmann, personal communication).

Bacterial isolations were performed in a similar manner for trials A-C. Bacteria isolation from trials A and B were performed in the same manner by the same researchers for both trials, as described in Chapter 2, with the exception that formalin was omitted from the isolation of bacteria in trial B. This omission was based on modeling work by Higgs (2014) that suggested data from omasal flow studies with formalin treated microbes did not fit the predictions for amino acid flows, despite a robust prediction for total N flow. Trial C bacterial isolation was performed by S. Fessenden, as described by Recktenwald (2010). All bacterial isolations followed, with some exceptions (mentioned in their respective papers), the procedures of Whitehouse et al. (1994) and Martin et al. (1996).

Protozoa from trials A and B were isolated from whole contents using the same procedure as described by Denton et al. (2015) and modified as reported in Chapter 3. The only difference between trials was the omission of formalin in Trial B. The isolations were performed by the same researcher for both trials (S. Fessenden). A diagram of the isolation procedure is in Figure 5.1. Protozoa from trial C were isolated from strained rumen fluid by flocculation followed by preservation with formalin and centrifugation at 500 x *g* for 5 min at 10°C. The pellet obtained

after centrifugation was assumed to be representative of the rumen protozoa (Recktenwald, 2010). Protozoa isolation from Trial D was performed by T. Hackmann in the laboratory of J. Firkins at the Ohio State University according to Denton et al., (2015) except 25 mL instead of 30 mL or clarified fluid was filtered per isolation.

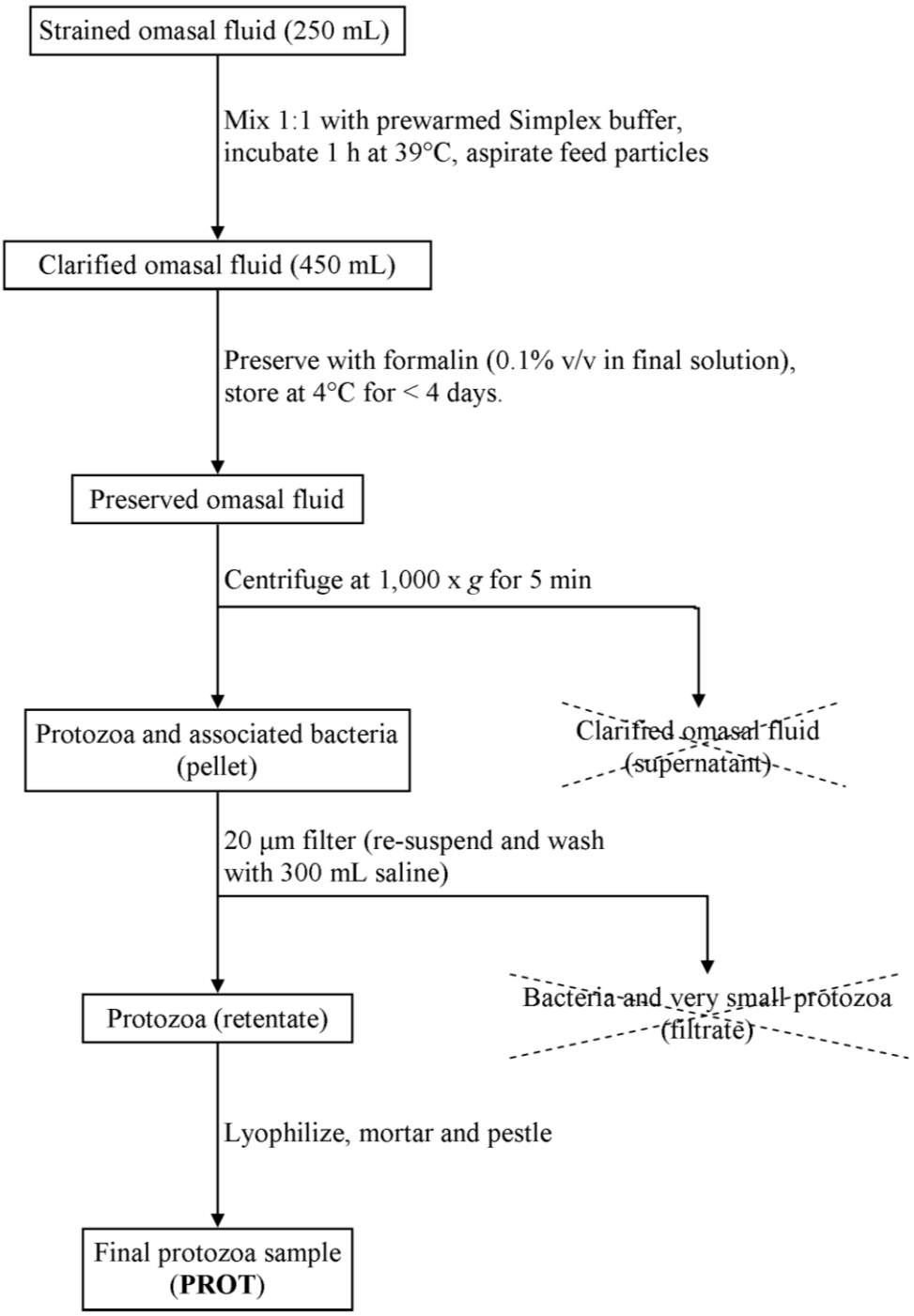

Figure 5.1. Flowchart for preparation of protozoa isolates in trials A and B. Fractions discarded are crossed out. Trial B isolation differed only in the omission of the steps in the box outlined by dashed lines.

Chemical Analysis and Hydrolysis Times

All samples were analyzed for DM after 6 h at 105°C and ash according to AOAC (2005). Total N was determined using a combustion assay (Leco FP-528 N Analyzer, Leco Corp., St. Joseph, MI). Amino acid content of all samples was determined by HPLC following hydrolysis at 110°C in a block heater (Gehrke et al., 1985) for 2, 4, 6, 12, 18, 21, 24, 30, 48, 72, 120 and 168 h. The time points chosen were based on a similar analysis performed on milk proteins (Rutherfurd et al., 2008) and practical limitations of the laboratory apparatus. The entire time course was performed twice for each sample, and the reported values are the mean of the two determinations.

For all AA excluding Met, Cys, and Trp, sample containing 2 mg N was weighed into hydrolysis tubes with 25 μL of 250 mM norleucine as an internal standard. Samples were then hydrolyzed as described above with high-purity 6 M HCl (5 mL) after flushing with N_2 gas. For Met and Cys, additional aliquots containing 2 mg N and the internal standard were pre-oxidized with 1 mL performic acid (0.9 mL of 88 % formic acid, 0.1 mL of 30% H_2O_2 and 5 mg phenol) for 16 h at 4°C prior to acid hydrolysis as described above (Mason et al., 1980, Elkin and Griffith, 1984). After hydrolysis, tube contents were filtered through Whatman 541 filter paper and filtrate was diluted to 50 mL in a volumetric flask with HPLC grade H_2O. Aliquots (0.5 mL) were evaporated at 60°C under constant N_2 flushing, with 3 rinses and re-evaporations with HPLC grade H_2O to remove acid residues. After final evaporation, the hydrolysate was dissolved in 1 mL of Na diluent (Na220, Pickering Laboratories, Mountain View, CA).

Individual AA hydrolysates were separated using an Agilent 1100 series HPLC (Agilent Technologies, Santa Clara, CA) fitted with a sodium cation exchange column (Cat. no 1154110T, Pikering Laboratories, Mountain View, CA) using a 4 buffer step gradient and

column temperature gradient. Detection of separated AA was performed at 560 nm following post-column ninhydrin derivation. Standards (250 n*M*/mL) for the individual AA were prepared by diluting a pure standard in sample buffer. The volume of sample and standards loaded onto the column was 10 µL.

For Trp determination, a separate aliquot of sample containing 2 mg N was hydrolyzed with 1.2 g of $Ba(OH)_2$ at 110°C for the same time course as other AA on a block heater according to the method of Landry and Delhaye (1992). Included in the hydrolysis was 125µL of 5-Methyl-Trp (5m*M*) as an internal standard. After cooling to precipitate barium ions, an aliquot (3 µL) of the hydrolysate was added to 1 mL of acetate buffer (0.07 *M* sodium acetate) an analyzed using fluorescence detection (excitation = 285 nm, emission = 345 nm) after HPLC separation.

In vitro Digestibility of N and Amino Acids

Microbial samples from Trials A and B were analyzed for intestinal digestibility of N and AA according to the assay described by Ross et al. (2013) with minor modifications. Since microbial samples were isolated from the omasum, no in vitro fermentation step was needed. For each sample, 150 mg of DM was weighed in duplicate into 125 mL Erlenmeyer Flasks and 40 mL of pre-warmed rumen buffer was added (Van Soest, 2015). Samples were then acidified to a pH of 2 with 3*M* HCl followed by addition of 2 mL of pepsin solution (282 U/mL). After 1 h of incubation at 39°C in a shaking water bath, contents of the flask were neutralized with ~2 ml of 2 *M* NaOH. Ten milliliters of enzyme mixture containing trypsin (24 mg/mL) chymotrypsin (20 mg/mL) amylase (50 mg/mL) and lipase (4 mg/mL) was then added to the flasks, followed by 24 h of incubation at 39°C in a shaking water bath. After incubation, flask contents were filtered on previously tared Whatman 934AH filters under vacuum. Samples were allowed to air dry, followed by drying and storage in a desiccator. Filter + residue weight was then recorded, and

DM remaining on the filter was corrected for a blank carried through the whole process. Each filter was quantitatively cut in half; with one half used for determination of residual N, while the other half used for AA analysis of the residual material. Determination of residual AA except Trp was performed after 21 h of hydrolysis with pre-oxidation of Met and Cys, as described previously. Insufficient sample N on the filters precluded the determination of Trp on the residues.

Calculations and Statistical Analysis

Digestibility of DM, N and individual AA was calculated as the disappearance of DM, OM, N or AA after enzymatic hydrolysis, corrected for the procedure blank. Determination of the true amino acid concentration of microbes was performed using a method similar to Rutherfurd et al. (2008) and Rutherfurd (2009). Amino acid concentration (mg/g of DM) was plotted against hydrolysis time and a non-linear equation was used to fit the curves to each plot:

$$B(t)=\frac{A_o h\left(e^{-lh}-e^{-ht}\right)}{h-l}$$

where $B(t)$ is the amino acid concentration at time t, h is the hydrolysis rate (proportion of bound amino acid hydrolyzed per hour), l is the loss rate (proportion of bound amino acid destroyed per hour) and A_o is the actual amino acid content of the protein within the sample. A_o,h and l for each sample were derived from each amino acid using least-squares non-linear regression with the constraints that $A_o > 0$, and $h > 0$. Rutherfurd (2009) utilized an additional term to account for free AA content (analyzed as AA determined before hydrolysis). This was not included in the current model, as free AA in bacterial and protozoal samples was considered to be negligible.

Amino acid digestibility and composition determined after multiple hydrolysis times were compared using the TTEST procedure of SAS version 9.3 (SAS Institute, Cary, NC). Only means from trials A and B (n=2 for each comparison) were analyzed, as limited amount of sample precluded digestibility and multiple hydrolysis time analysis for trials C and D.

RESULTS AND DISCUSSION

Microbial Chemical Composition and Digestibility

All donor cows were fed diets with fairly similar chemical and nutrient composition (Table 5.1). Diets were typical of the Northeastern and Midwestern US with corn silage and alfalfa silage as the principal forages. Organic matter content in bacteria and protozoa was similar to values obtained previously from rumen and omasal isolates (Brito et al., 2006;2007) although OM content is strongly influenced by the isolation procedures used (Martin et al., 1994). Trial A bacteria and protozoa had numerically decreased DM, OM, and N digestibility compared with trial B. This is likely due to the use of formalin in trial A vs. trial B. Formaldehyde readily reacts with proteins to form products resistant to digestion (Barry, 1976). While diet and animal differences between trials might also have contributed to observed differences, the direction and magnitude of the difference between trial A and B for OM, DM, and N digestibility; formalin treatment is a likely cause of the differences observed. Bacteria isolates had similar AA as a percent of DM, while the lower N content of bacteria from trial C (7.5 %) resulted in AA N contributing more to total N compared with bacteria from trials A and B. Protozoa from trial D had the highest AA N as a % of N. Values for trials A and B were on the low end of the range (54.9 – 86.7 AA-N, % of total N) reported by Clark et al. (1992). This could be related to the fact that microbes in trials A and B were isolated from omasal contents, while trials C and D were isolated from rumen contents, which could contribute to different amounts of non-amino

nitrogen. Volden et al. (1999b) reported DAPA and purine bases were affected by diet and differed between protozoa and bacterial fractions in the rumen, and Illg and Stern (1994) noted wide ranges in non-amino N concentration between duodenal and rumen samples.

Table 5.1. Donor cow diet chemical composition, intake and milk production

	Trial[1]			
Item	A	B	C	D[2]
Number of isolations in each sample:	72	108	NA	8
Diet chemical composition				
OM, % of DM	93.9	92.6	92.5	91.8
CP, % of DM	16.0	16.9	14.8	16.3
Soluble protein, % of CP	35.7	43.7	31.9	34.1
RDP, % of CP[3]	51.9	59.8	54.3	62.0
aNDFom, % of DM	31.1	30.6	33.6	32.1
ADF, % of DM	19.8	20.5	-	20.2
ADL, % of DM	3.0	3.4	1.9	3.1
Sugars, % of DM	5.4	3.5	5.4	2.8
Starch, % of DM	27.6	24.6	25.4	26.7
Ether extract, % of DM	4.9	3.2	4.5	5.6
ME, Mcal/kg[3]	2.5	2.6	2.7	2.6
Cattle intake and production				
Dry matter intake, kg/d	27.3	26.1	23.8	NA
Milk production, kg/d	41.7	41.6	30.9	NA

[1]Trial A: Chapters 1 and 2; Trial B: Foskolos et al., (unpublished); Trial C: Recktenwald (2010); Trial D: T. Hackmann, personal communication

[2]Chemical composition estimated using CNCPS v. 6.55 using diet ingredient composition

[3]Metabolizable energy predicted using CNCPS v. 6.55

Table 5.2. Chemical composition and intestinal digestibility of bacteria and protozoa isolates

	Trial[1]			
Item	A	B	C	D[2]
Bacteria				-
OM, % of DM	83.9	92.1	90.1	-
N, % of DM	8.1	8.9	7.5	-
uN, % of total N[2]	36.4	15.1	-	-
AA, % of DM	28.2	34.3	32.5	
AA N, % of N	47.3	51.8	57.8	-
DM digestibility, %	53.6	69.0	-	-
OM digestibility, %	68.2	81.9	-	-
N digestibility, %	63.6	84.9	-	-
Protozoa				
OM, % of DM	87.0	85.0	90.8	93.8
N, % of DM	8.3	8.2	8.1	8.4
uN, % total N[2]	46.7	24.7	-	-
AA, % of DM	28.4	31.7	37.4	45.3
AA N, % of N	53.2	53.7	65.9	71.3
DM digestibility, %	48.7	61.9	-	-
OM digestibility, %	65.9	88.4	-	-
N digestibility, %	53.3	75.3	-	-

[1]Trial A: Chapters 1 and 2; Trial B: Foskolos et al., (unpublished); Trial C: Recktenwald (2010); Trial D: T. Hackmann, personal communication

[2]intestinally unavailable N as determined by the procedure of Ross et al. (2013)

Microbial isolates averaged 50.6 % and 49% EAA as a % of total AA for bacteria and protozoa, respectively (Table 5.3). Bacterial isolates from trial B had numerically increased concentrations of Lys and Met, while trials B and D protozoa also demonstrated increased Lys and Met concentrations. For NEAA, bacteria and protozoa isolations were similar amoung trials with the exception of tyrosine, which was reduced in trials A and C. The differences in microbial isolates from trial A and C for individual AA are likely due to formalin treatment. Volden et al. (1999b) reported decreased recoveries of Lys, Met, and Tyr with vs. without formaldehyde treatment in solid and liquid associated bacteria. Whitehouse et al. (1994) reported approximately 20% less Tyr in microbial samples after treatment with formaldehyde. Beyond these differences likely due to formaldehyde treatment, the AA profile of bacteria and protozoa agreed fairly well with literature reports with some exceptions. Met averaged 3.2 % of total AA among all samples, and was at the high end of the range reported by Clark et al. (1992). The variability of reported AA composition is likely related more to the isolation techniques rather than true differences among microbial populations. Protozoa AA composition has been shown to remain fairly constant among sampling times (Martin et al., 1996; Volden et al., 1999a). Differences amoung microbial fractions (solid associated bacteria, liquid associated bacteria, and protozoa) have been well documented (Chiquette and Benchaar, 1998; Korhonen et al., 2002); however reasons for the differences are not clear. Procedures used to detach microbes report recoveries ranging from 20 % (Martín-Orúe et al., 1998) to 80 % (Whitehouse et al., 1994). This might call into question the true ability of recovered bacteria to represent the particle associated bacteria (Korhonen et al., 2002). Ultimately it is likely that differences in isolation methods are responsible for much of the reported ranges of AA composition, while a smaller portion of the variation can be considered a true difference in AA composition (Fonseca et al., 2014)

Table 5.3. Amino acid profile (% of total AA) of bacteria and protozoa isolates

	Bacteria AA				Protozoa AA			
	Trial[1]				Trial[1]			
Item	A	B	C	D	A	B	C	D
Essential AA								
ARG	5.4	4.8	5.0	-	5.5	5.4	5.4	4.7
HIS	2.1	2.2	2.0	-	2.9	2.5	2.3	2.0
ILE	5.0	4.2	4.9	-	4.7	4.0	5.7	5.5
LEU	4.8	5.4	6.8	-	5.5	6.3	4.6	4.2
LYS	4.7	7.5	4.8	-	5.7	8.8	5.3	10.2
MET	3.3	4.6	2.6	-	2.7	3.3	2.1	3.8
PHE	6.6	6.0	6.8	-	7.4	6.7	7.3	7.6
TRP	5.7	5.5	5.3	-	4.6	4.3	3.1	1.4
THR	6.3	5.6	5.4	-	5.4	4.9	6.1	4.7
VAL	6.7	6.0	5.6	-	5.7	4.7	4.7	4.7
Total EAA	50.8	51.8	49.3	-	50.1	50.9	46.5	48.8
Non-essential AA								
ALA	7.9	6.9	6.7	-	5.9	5.3	4.7	4.0
ASP	12.2	10.9	8.8	-	11.8	10.2	12.1	11.6
CYS	1.4	1.5	1.1	-	2.0	2.2	1.7	2.2
GLU	12.4	11.4	13.0	-	14.0	13.3	15.2	13.6
GLY	5.7	5.0	5.2	-	4.7	4.6	4.3	4.0
PRO	2.1	2.0	6.9	-	2.7	3.0	7.9	5.6
SER	5.5	4.7	5.4	-	5.3	5.2	5.5	4.2
TYR	2.0	5.8	3.5	-	3.5	5.4	2.0	6.1
Total NEAA	49.2	48.2	50.7	-	49.9	49.1	53.5	51.2

[1]Trial A: Chapters 1 and 2; Trial B: Foskolos et al., (unpublished); Trial C: Recktenwald (2010); Trial D: T. Hackmann, personal communication

Trial A bacteria and protozoa demonstrated decreased digestibility (Table 5.4) for most AA, which again is likely related directly to formalin treatment. Total bacterial EAA digestibility averaged 74.9 and 88.0 % for trials A and B, respectively ($P = 0.01$). Protozoa AA digestibility was also affected by formalin treatment, however not all AA were significantly different between diets. Arginine, Leu, Val and Glu all demonstrated decreased digestibility with formalin treatment. The ability of the uN assay to detect differences in digestibility due to formalin treatment indicates the assay might be a useful evaluation tool for other protein containing feedstuffs, especially rumen protected protein supplements. Other in vitro techniques such as the mobile bag technique (Hvelplund et al., 1992) and the modified three step assay (Gargallo et al., 2006) rely on retention of undigested proteins in bags, and as such estimates of microbial digestibility of AA from those assays are of limited value due to potential for loss from the bag. The use of the precision-fed cecectomized rooster bioassay has been used in ruminant feeds (Titgemeyer et al., 1990, Boucher et al., 2009), and Fonseca et al. (2014) recently applied the technique to rumen bacteria isolations. Total AA digestibility reported by Fonseca et al. (2014) averaged 76%, with a range of 62 % (Cys) to 82 % (Met). Average AA digestibility in non-formalin treated bacteria in the current trial was 88%, with a range of 84 % (Tyr) to 95 % (Cys). Differences between the studies are likely related to the different enzyme activities and digestive processes between these two methods (Ross et al., 2013). In sheep maintained on VFA, minerals and isolated rumen microorganisms, Storm et al., (1983) calculated a mean intestinal digestibility of 85%, with a range of 80 to 88 %. Tas et al. (1981) utilized a regression approach to estimate true digestibility of 87% for rumen bacteria in sheep, and a similar approach was utilized by Hvelplund and Hesselholt (1987) who reported true AA digestibilities between 80 and 91% for most AA. General agreement between the previously utilized techniques and the current

application of the assay developed by Ross et al. (2013) imply that in-vitro uN determination might be useful for future studies of AA digestibility in diverse feedstuffs.

Table 5.4. Intestinal digestibility (% of AA) of amino acids of omasal bacteria and protozoa

	Bacteria				Protozoa			
	Trial[1]				Trial[1]			
Item	A	B	*SEM*	*P*	A	B	SEM	*P*
Essential AA								
ARG	74.0	88.2	0.9	0.03	69.0	89.0	0.4	0.01
HIS	78.4	90.7	1.9	0.03	69.7	70.9	11.9	0.93
ILE	76.8	88.9	0.6	0.00	75.3	85.7	3.0	0.08
LEU	78.5	92.2	1.5	0.01	74.3	92.5	1.1	0.01
LYS	75.5	91.0	1.8	0.02	68.2	77.5	12.6	0.54
MET	80.7	88.8	1.0	0.07	78.4	90.1	0.9	0.17
PHE	69.0	83.2	1.2	0.01	67.5	79.7	2.7	0.06
TRP	-	-	-	-	-	-	-	-
THR	76.3	89.8	1.1	0.01	73.5	79.2	9.3	0.61
VAL	69.9	88.0	4.0	0.05	63.2	83.3	3.8	0.04
Total EAA	74.9	88.0	1.4	0.01	70.6	82.8	4.4	0.11
Non-essential AA								
ALA	72.5	87.2	2.2	0.02	67.5	80.1	6.6	0.20
ASP	77.5	90.8	1.2	0.01	75.8	86.3	5.0	0.18
CYS	82.1	94.8	0.7	0.05	89.3	93.6	1.7	0.15
GLU	68.5	85.2	3.6	0.04	67.5	87.7	0.8	0.01
GLY	69.4	85.8	3.7	0.05	63.5	72.8	13.4	0.56
PRO	77.9	88.4	1.4	0.10	78.1	80.7	4.7	0.66
SER	75.0	89.3	1.3	0.01	71.9	56.7	33.6	0.70
TYR	34.0	84.3	0.4	0.00	55.9	73.8	4.5	0.06
Total NEAA	71.6	87.5	1.9	0.02	70.2	80.2	7.6	0.32
Total AA	73.3	87.8	1.7	0.01	70.4	81.6	6.0	0.21

[1]Trial A: Chapters 1 and 2; Trial B: Foskolos et al., (unpublished); Trial C: Recktenwald (2010); Trial D: T. Hackmann, personal communication. n=2 for each comparison

Amino Acid Determination from Multiple Hydrolysis Times

Due to the effect of formalin on Trial A samples, AA composition after multiple hydrolysis times for trial A and B are presented as separate data points and least-squares non-linear regression lines for bacteria (Figures 2-4) and protozoa (Figures 5-7) . Comparisons of bacteria and protozoa AA composition between trials A and B are in Table 5.5. Of the EAA in bacterial isolates (Figure 5.2), extraction of His, Leu, Lys, and Met was numerically increased in trial B samples. Total AA composition was decreased in trial A bacteria (283 vs.339 mg/g DM for trial A vs.trial B, respectively; $P = 0.03$). Extraction of Ile, Met, and Val demonstrated greater release over time and thus positive slopes at time points greater than 24 h. Hydrolysis rate (*h*; Table 5.5) were lowest for these AA. Of the NEAA, Cys and Pro also demonstrated increasing concentrations of AA as hydrolysis time increased. Ser concentrations decreased markedly after 24 h of hydrolysis, as indicated by a relatively high loss rate (*l*; Table 5.5). Hydrolysis of protozoa isolates demonstrated very similar results (Figure 5.5 - 5.7) compared to bacteria, with the lowest *h* reported for Ile, Met and Val. Overall, total AA were hydrolyzed from the sample matrix at a rate of 0.415 and 0.357 mg/h for bacteria and protozoa, respectively. The same least-squares non-linear regression approach has been previously employed in the analysis of other AA containing compounds, including lysozyme (Darragh et al., 1996), cat hair (Hendriks et al., 1998), human milk (Darragh and Moughan, 1998) and some common feedstuffs (Rutherfurd, 2009). To our knowledge, no previous work has reported rumen microbial AA content after multiple hydrolysis times. For this reason, comparisons will principally be made to Rutherfurd (2009), as the feedstuffs reported by those authors are likely the most relevant comparison to microbial AA. Rutherfurd (2009) reported similarly low *h* for Ile and Val, while Ser was reported to have the highest *l* of any amino acid.

Overall, the use of multiple hydrolysis times provides some insight into the appropriateness of single time point hydrolysis for AA in rumen microbial samples. The true AA content (determined from the regression) compared with the value determined at 24 h was used to determine the under or over-estimation of AA content (Table 5.6). Of EAA, the 24 h time point underestimated Ile content by 8.4 and 11.2 % for bacteria and protozoa, respectively. This is similar to the results of Rutherfurd (2009), where soybean meal Ile content was underestimated by 8.4 %, followed by Val (7.0%), Ser (4.6%), and Thr (4.3%). In bacteria, 7 out of 10 EAA were accurately determined (≤ 3% error) with a single 24 h hydrolysis. Protozoa AA determinations were less accurate, with 5 out 10 EAA exhibiting an error less than 3%. Total EAA were over-predicted by 2.5% and 1.2 % for bacteria and protozoa respectively. The ability of a single time point to accurately determine NEAA content varied more than EAA. In bacteria, 4 out of 8 NEAA were determined with less than 3% error, while only 3 of 8 protozoa NEAA met the same criterion. Finally, total NEAA content was under-predicted by 4.8% and 1.8 % for bacteria and protozoa, respectively. Overall, a single time point at 24 h over-predicted total AA content by 2.2 and 1.4 % for bacteria and protozoa, respectively.

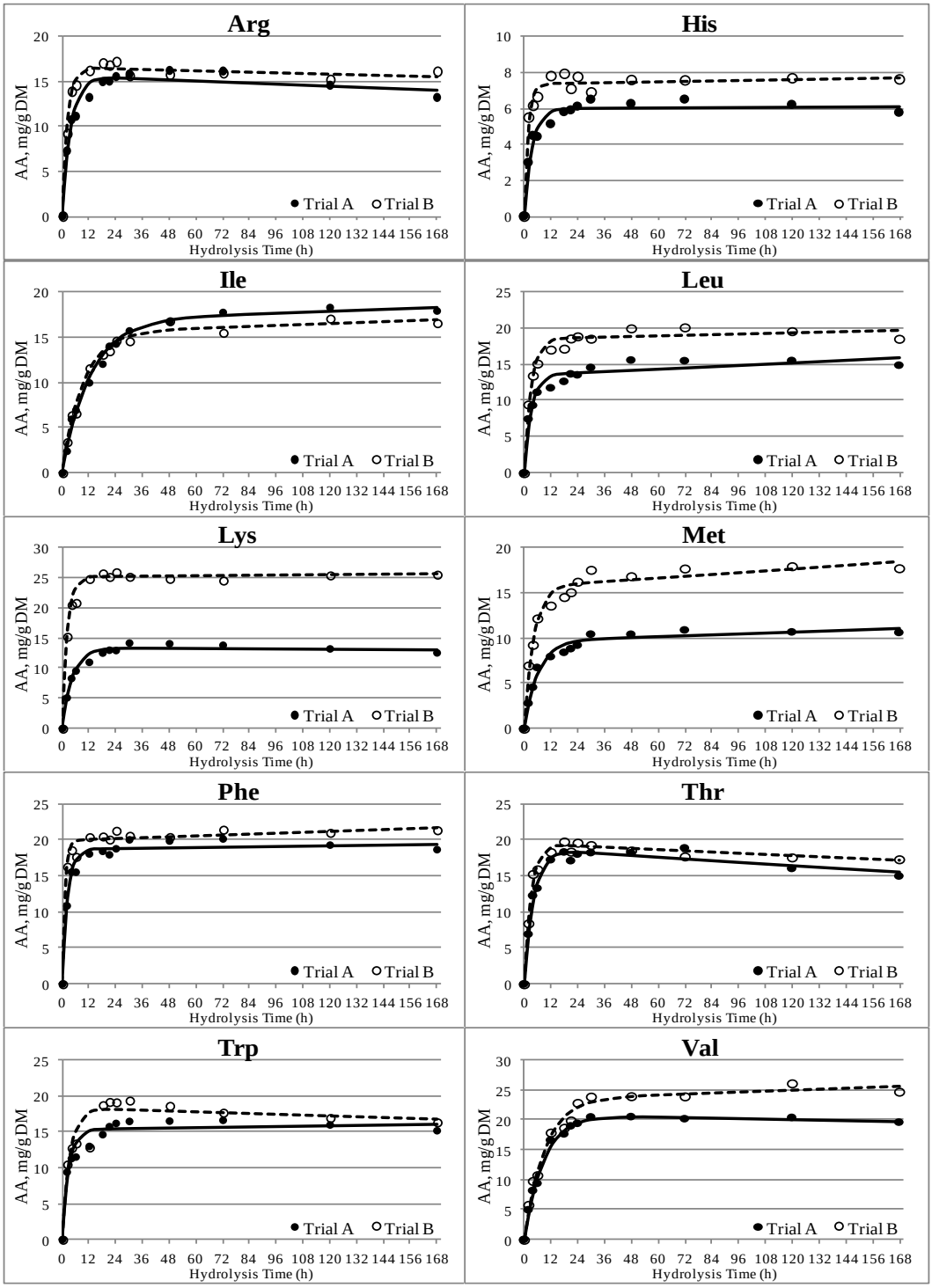

Figure 5.2. Effect of hydrolysis time (h) on yield of essential AA (mg/g DM) from freeze dried isolations of omasal bacteria from Trial A (●) and Trial B (○). The mean of each time point (2 replicates each) is plotted against the least squared regression line for each respective dataset.

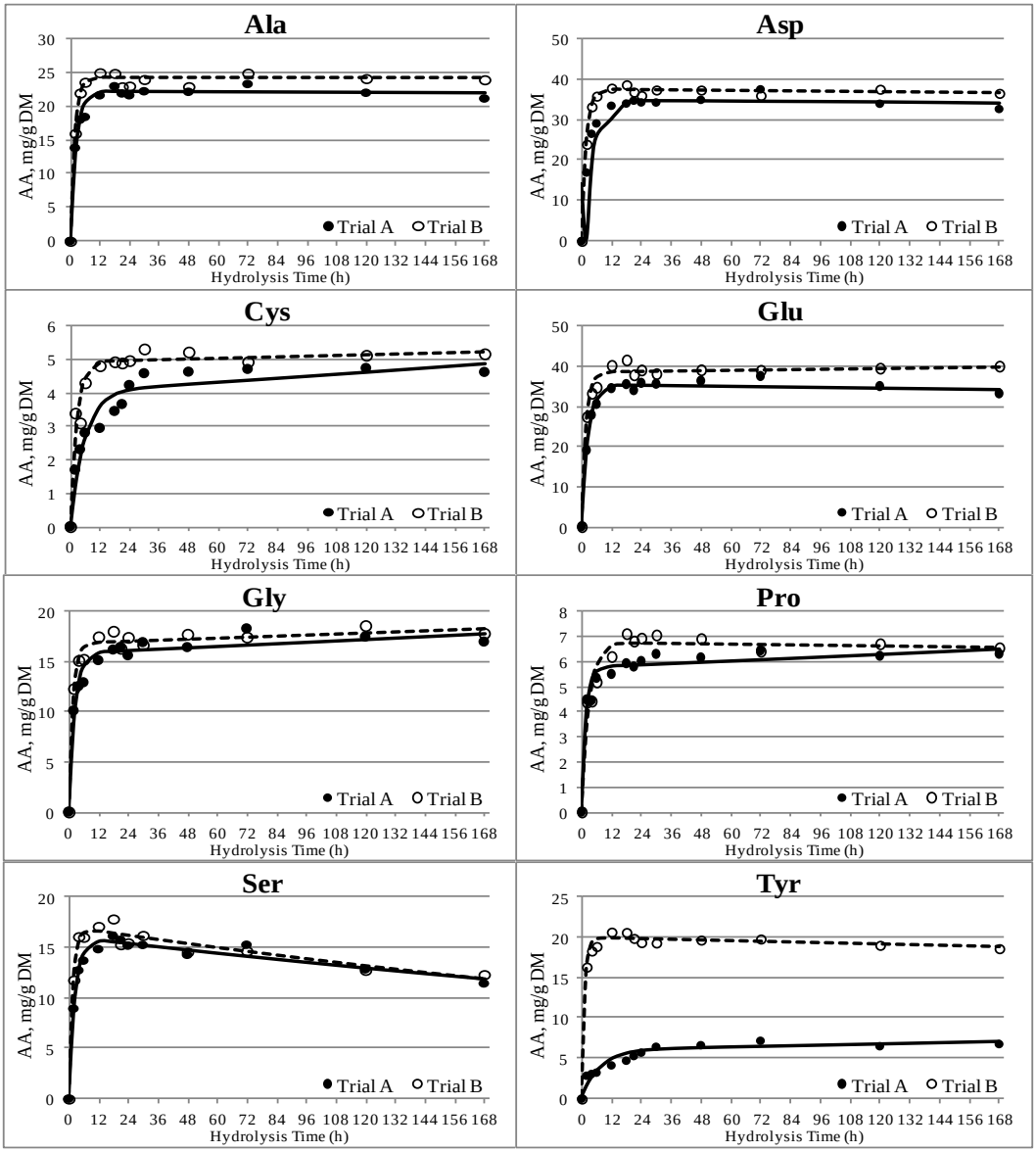

Figure 5.3. Effect of hydrolysis time (h) on yield of non-essential AA (mg/g DM) from freeze dried isolations of omasal bacteria from Trial A (●) and Trial B (○). The mean of each time point (2 replicates each) is plotted against the least squared regression line for each respective dataset.

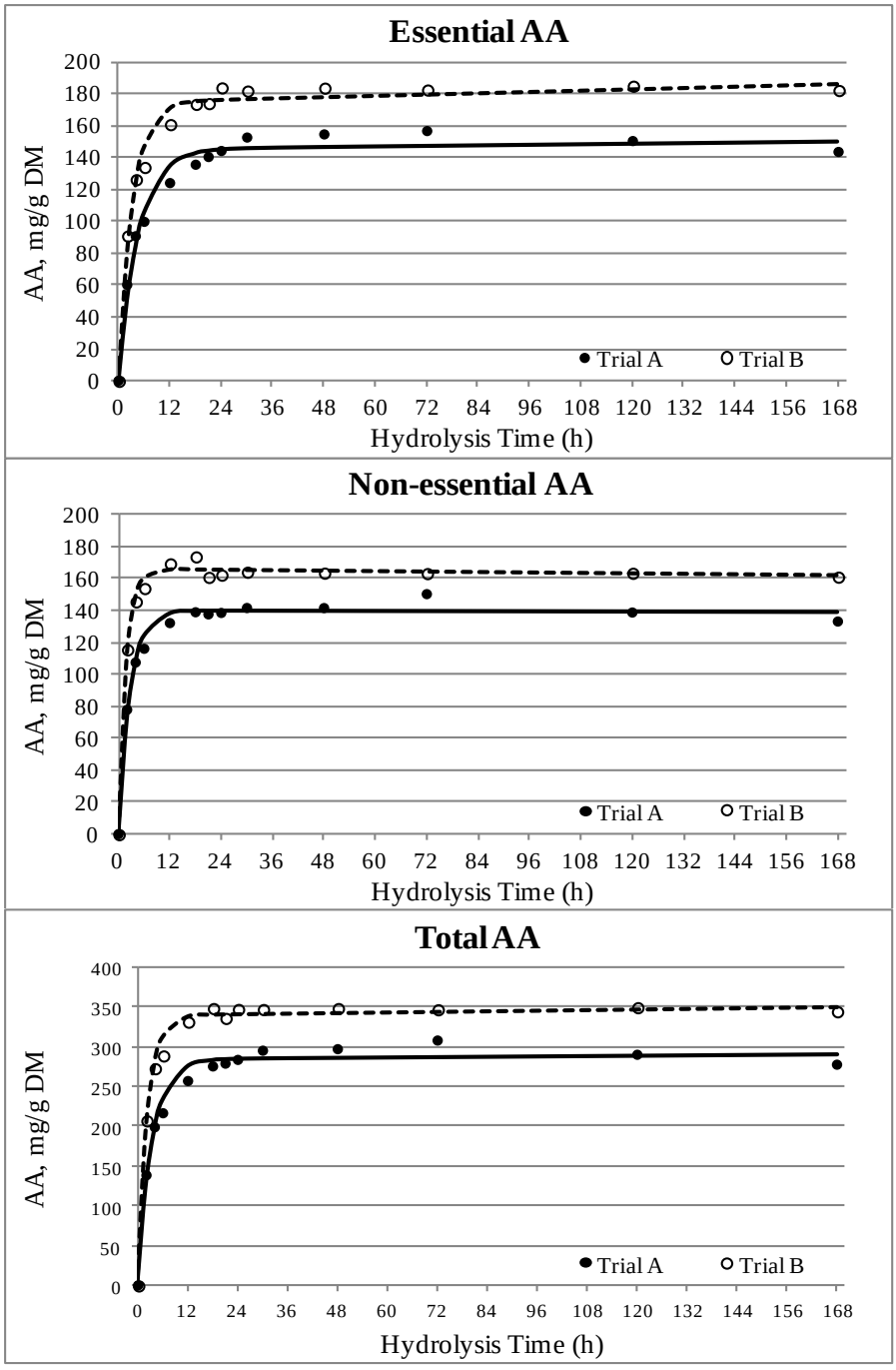

Figure 5.4. Effect of hydrolysis time (h) on yield of essential, non-essential, and total AA (mg/g DM) from freeze dried isolations of omasal bacteria from Trial A (●) and Trial B (○). The mean of each time point (2 replicates each) is plotted against the least squared regression line for each respective dataset.

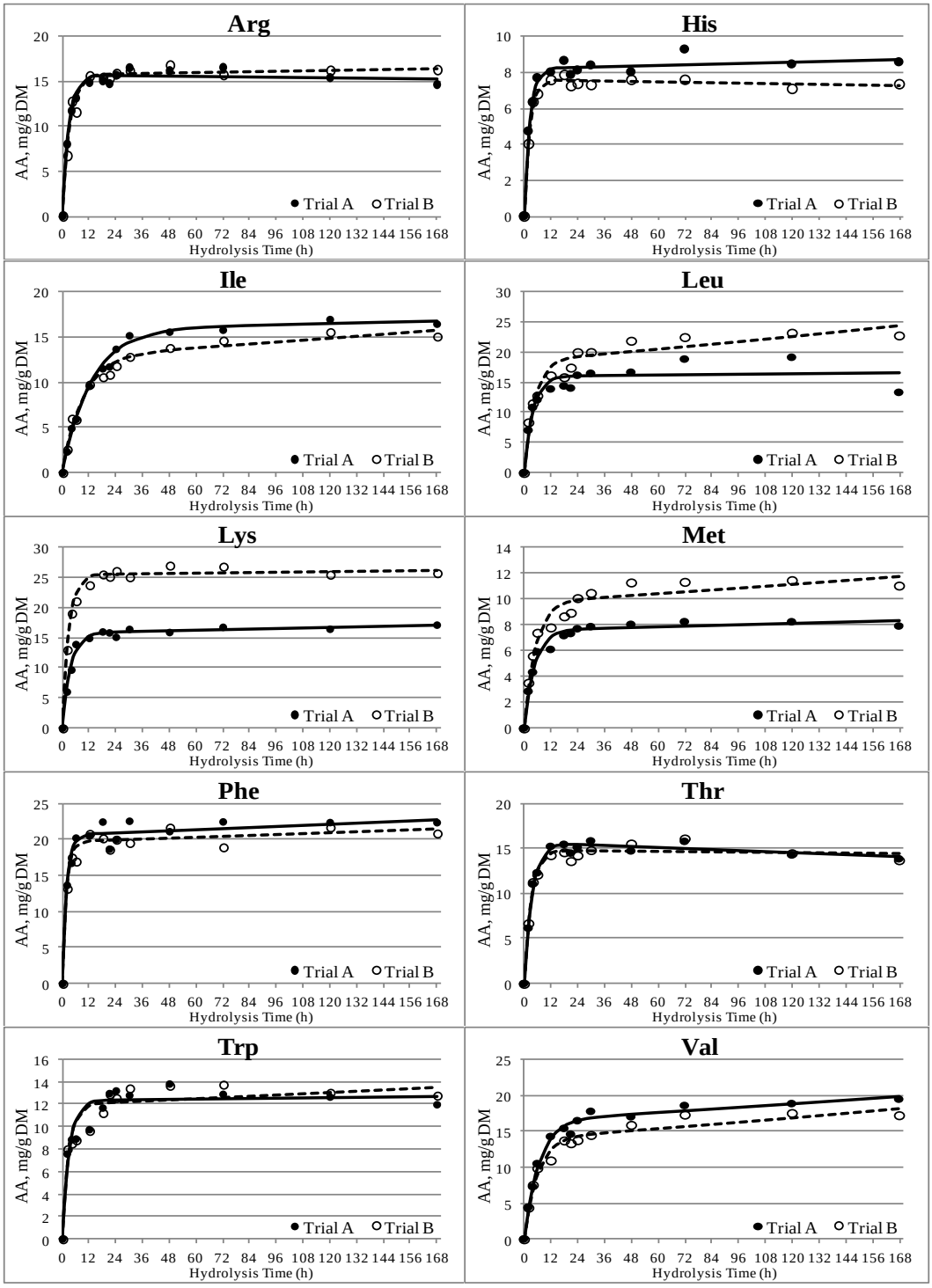

Figure 5.5. Effect of hydrolysis time (h) on yield of essential AA (mg/g DM) from freeze dried isolations of omasal protozoa from Trial A (●) and Trial B (○). The mean of each time point (2 replicates each) is plotted against the least squared regression line for each respective dataset.

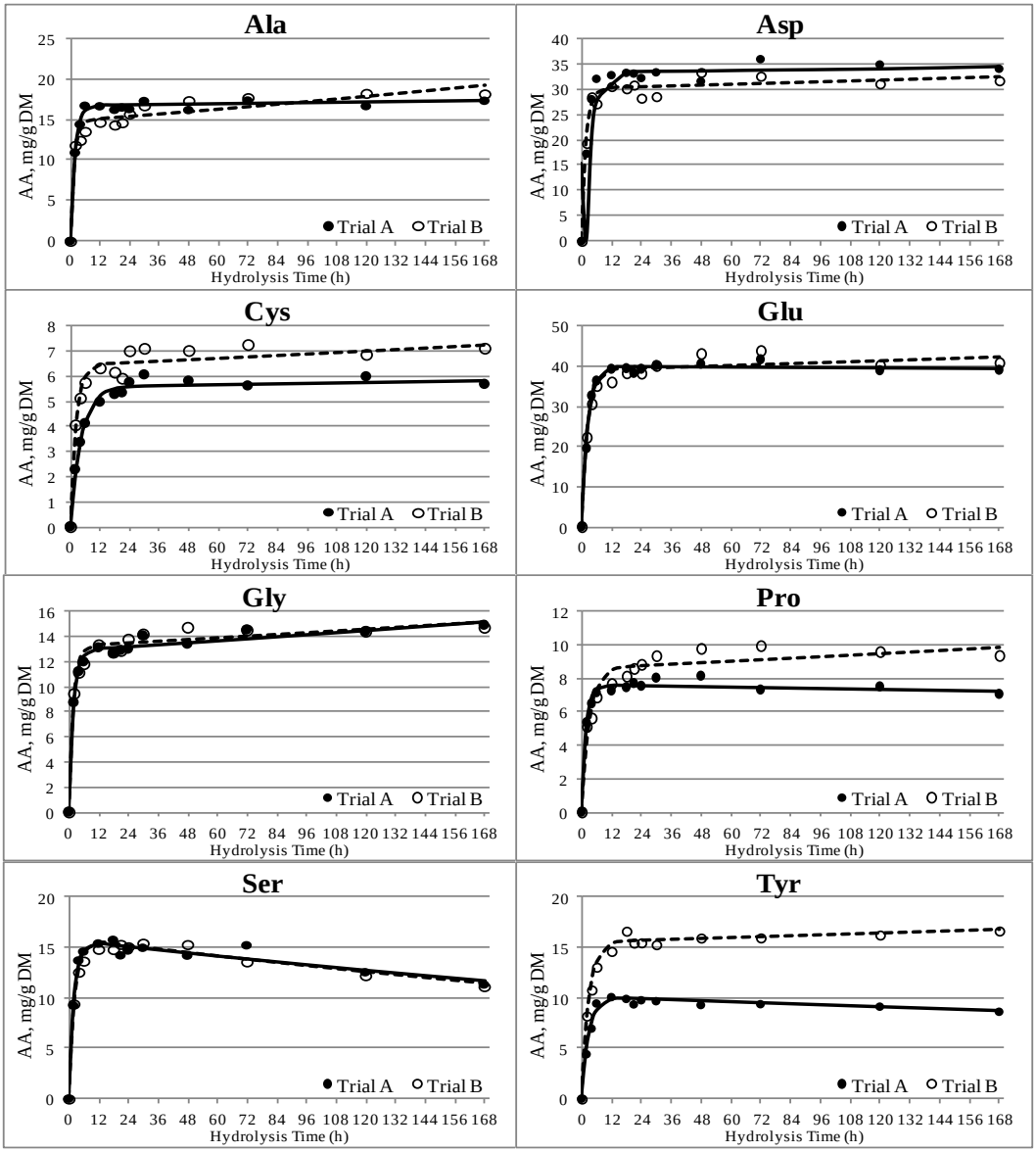

Figure 5.6. Effect of hydrolysis time (h) on yield of non-essential AA (mg/g DM) from freeze dried isolations of omasal protozoa from Trial A (●) and Trial B (○). The mean of each time point (2 replicates each) is plotted against the least squared regression line for each respective dataset.

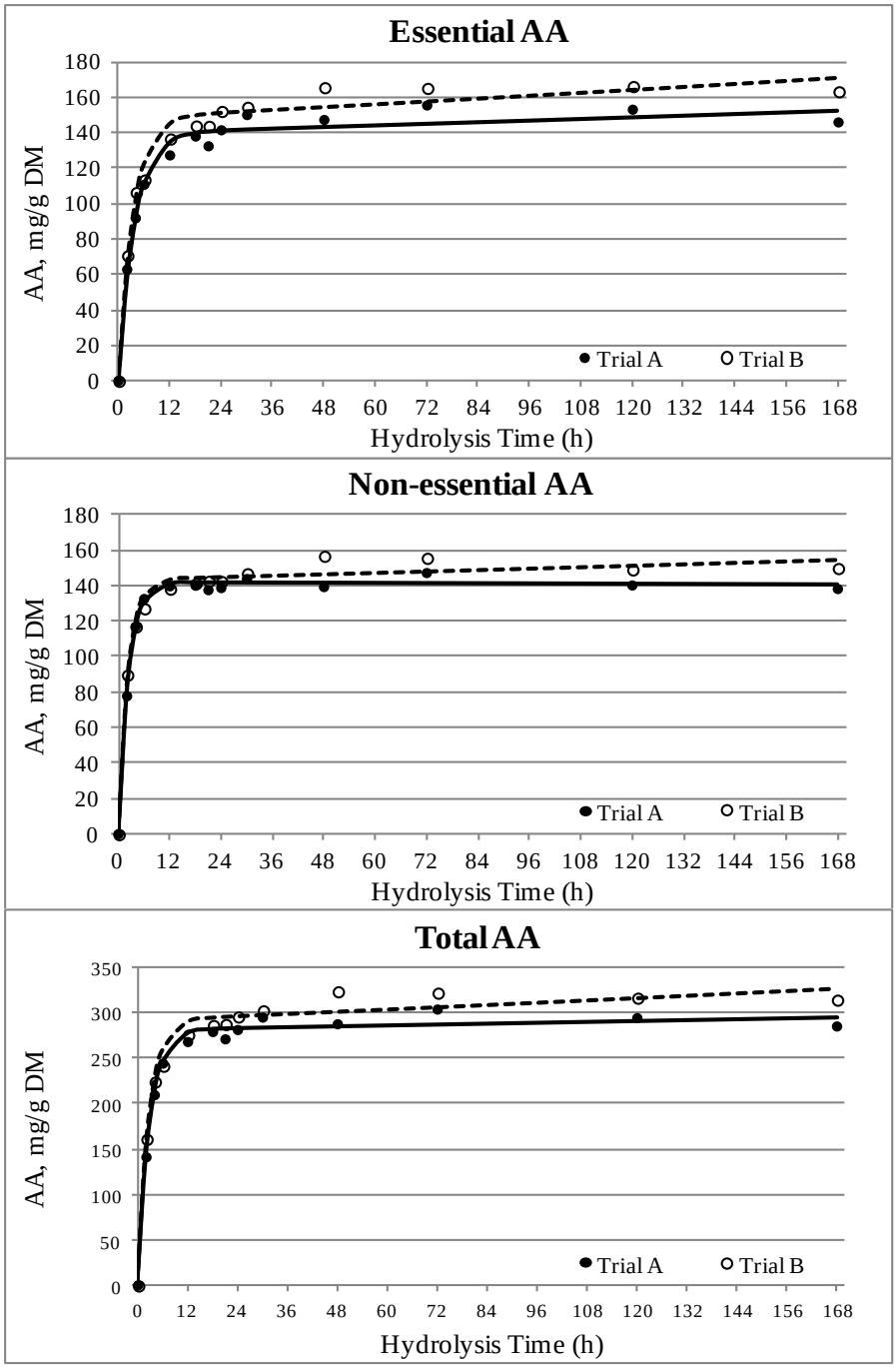

Figure 5.7. Effect of hydrolysis time (h) on yield of essential, non-essential, and total AA (mg/g DM) from freeze dried isolations of omasal protozoa from Trial A (●) and Trial B (○). The mean of each time point (2 replicates each) is plotted against the least squared regression line for each respective dataset.

Table 5.5. Comparison of the AA composition (mg/g of DM) of rumen bacteria and protozoa from trials A and B determined after multiple hydrolysis times.

	Bacteria				Protozoa			
	Trial[1]				Trial[1]			
AA, mg/g DM	A	B	SEM	*P*	A	B	SEM	*P*
Essential AA								
ARG	15.6	16.5	0.5	0.22	15.7	15.7	0.9	0.98
HIS	6.1	7.4	0.2	0.02	8.2	7.5	0.3	0.42
ILE	17.3	16.2	1.2	0.48	16.3	13.1	1.5	0.19
LEU	13.8	18.5	2.3	0.17	18.0	18.7	3.4	0.89
LYS	13.5	25.1	1.1	0.03	15.9	25.5	1.2	0.01
MET	10.2	16.3	3.9	0.36	8.0	11.3	1.4	0.38
PHE	18.8	20.1	1.2	0.59	20.8	19.6	0.2	0.65
TRP	6.6	20.1	1.2	0.05	10.1	16.0	0.5	0.05
THR	18.7	19.3	0.4	0.59	15.6	14.8	0.3	0.45
VAL	20.8	24.2	6.4	0.66	16.6	14.2	0.4	0.12
Total EAA	145.3	175.4	7.0	0.06	140.9	148.3	2.3	0.08
Non-essential AA								
ALA	22.2	24.2	0.2	0.02	16.7	15.0	0.2	0.04
ASP	35.0	37.7	1.3	0.17	33.3	30.3	2.6	0.37
CYS	4.6	4.9	1.2	0.85	5.8	6.4	1.7	0.80
GLU	35.6	38.6	0.6	0.33	39.9	38.9	1.4	0.82
GLY	15.9	16.9	0.6	0.22	12.9	13.2	0.0	0.54
PRO	5.9	6.7	0.1	0.06	7.6	8.6	1.1	0.59
SER	16.1	17.1	0.9	0.44	15.7	15.8	0.9	0.98
TYR	16.1	19.7	0.8	0.05	13.4	14.0	1.4	0.73
Total NEAA	139.5	165.8	1.1	0.00	141.4	143.1	4.6	0.85
Total AA	283.1	339.0	8.8	0.03	280.6	290.6	2.6	0.31

[1]Trial A: Chapters 1 and 2; Trial B: Foskolos et al., (unpublished); Trial C: Recktenwald (2010); Trial D: T. Hackmann, personal communication. n=2 for all comparisons

Table 5.6. Rate of hydrolysis (h)[1] and loss (l)[2] for individual and total amino acids from omasal bacteria and protozoa isolates from Trial B.

	Bacteria		Protozoa	
Item	h (h^{-1})	l (h^{-1})	h (h^{-1})	l (h^{-1})
Essential AA				
ARG	0.414	0.00039	0.303	-0.00025
HIS	0.577	-0.00027	0.577	-0.00027
ILE	0.107	-0.00054	0.107	-0.00054
LEU	0.323	-0.00037	0.323	-0.00037
LYS	0.421	-0.00012	0.421	-0.00012
MET	0.234	-0.00097	0.234	-0.00097
PHE	0.782	-0.00050	0.782	-0.00050
TRP	0.283	0.00054	0.283	0.00054
THR	0.323	0.00077	0.323	0.00077
VAL	0.112	-0.00054	0.112	-0.00054
Total EAA	0.303	-0.00038	0.303	-0.00038
Non-essential AA				
ALA	0.571	0.00001	0.658	-0.00156
ASP	0.523	0.00014	0.548	-0.00044
CYS	0.377	-0.00035	0.429	-0.00070
GLU	0.550	-0.00019	0.397	-0.00048
GLY	0.599	-0.00049	0.541	-0.00081
PRO	0.324	0.00018	0.304	-0.00081
SER	0.622	0.00218	0.418	0.00198
TYR	0.804	0.00036	0.328	-0.00046
Total NEAA	0.568	0.00014	0.447	-0.00045
Total AA	0.415	-0.00018	0.357	-0.00067

[1]proportion of bound AA hydrolyzed per hour.
[2]proportion of bound AA destroyed per hour.

Table 5.7. Comparison of measured AA composition after single hydrolysis time point vs. estimated AA composition determined using least-squares non-linear regression after multiple hydrolysis times for omasal bacteria and protozoa isolates from trial B.

	Bacteria			Protozoa		
Item	24 h[1]	Mult[2]	% Δ	24 h[1]	Mult[2]	% Δ
Essential AA, % of AA						
ARG	4.96	4.88	1.6	5.37	5.41	-0.7
HIS	2.24	2.17	3.0	2.50	2.59	-3.6
ILE	4.25	4.77	-12.4	4.03	4.51	-12.0
LEU	5.48	5.47	0.3	6.83	6.43	5.8
LYS	7.52	7.40	1.6	8.90	8.79	1.2
MET	4.71	4.81	-2.0	3.44	3.87	-12.6
PHE	6.15	5.94	3.4	6.79	6.76	0.4
TRP	5.51	5.93	-7.7	4.26	5.49	-29.1
THR	5.67	5.70	-0.5	4.84	5.09	-5.1
VAL	6.58	7.14	-8.4	4.67	4.88	-4.6
Total EAA	53.07	51.73	2.5	51.61	51.01	1.2
Non-essential AA, % of AA						
ALA	6.68	7.15	-7.0	5.36	5.17	3.6
ASP	10.46	11.13	-6.3	9.65	10.42	-7.9
CYS	1.43	1.45	-1.4	2.37	2.22	6.5
GLU	11.25	11.39	-1.3	12.94	13.40	-3.5
GLY	5.01	4.98	0.6	4.67	4.53	2.9
PRO	2.00	1.97	1.2	2.99	2.97	0.7
SER	4.48	5.03	-12.2	5.14	5.43	-5.8
TYR	5.61	5.82	-3.6	5.27	4.83	8.3
Total NEAA	46.93	48.90	-4.2	48.39	49.22	-1.7
Total AA, % of DM	346.6	339.0	2.2	295.0	290.7	1.4

[1]AA composition after 24 h hydrolysis time

[2]AA composition determined from least-squares non-linear regression from multiple hydrolysis times.

Implications for Amino Acid Predictions in Nutrition Models

When considered together, the results from this study and those reported by Rutherfurd (2009) indicate that specific AA could be underestimated in many post-ruminal AA studies when utilizing single time point hydrolysis. This consideration should recognized for when literature values for AA flows are used in development and evaluation of nutritional models that seek to accurately predict AA supply, especially those that utilize mechanistic post-absorptive sub-models. If supply of AA is not accurately predicted, overall model performance and utilization will suffer. In the case of many of the extant models, this can lead to wide variation and unrealistic values in the apparent efficiency of use for individual AA. Depending on which datasets are used for development, spurious relationships might also be elucidated. Given the data presented here and by Rutherfurd (2009), this might be especially true for Ile, Ser, Val, and possibly Met.

In this analysis, Met was determined to contribute more to total AA than has previously been reported. Currently, the CNCPS v. 7 uses a profile that corresponds to approximately 1.1% of total microbial AA as Met. Compared with the current analysis (4.7 % of total AA), predictions of AA supply from the model would be expected to increase more than 2 fold (assuming microbial AA accounts for 50% of total AA). Adoption of these values will likely result in a re-evaluation of many common ratios and relationships currently used to balance essential AA for lactating cattle. This concept plays into an interesting discussion of the importance of NEAA in metabolism. Cysteine and Tyr, which are not considered essential due to the ability to be synthesized from Met and Phe in the liver, are not currently modeled or considered in nutrient requirement tables. However, all animals lack the ability to form carbon skeletons for Met and Phe, which means there is no de-novo synthesis of Cys or Tyr (Wu, 2014). Therefore, this

singular reliance on Met or Phe as a precursor for Cys or Tyr might cause these NEAA to become limiting at high levels of productive output. Most other NEAA can be synthesized using multiple pathways and substrates, thus providing key metabolic flexibility needed during lactation (Lobley, 2007). Therefore, when modeling supply and requirements of individual AA, it might be necessary to consider special cases for some AA, especially Met, Phe, Cys, and Tyr, as their requirements by the animal are of relatively more importance. Mechanistic models of AA requirement and supply will likely need to model these aspects of AA metabolism to establish accurate predictions for energy driven allowances for individual AA.

CONCLUSIONS

Microbial composition and digestibility of individual AA are very important to the accurate predictions in many nutrition models used to feed dairy cattle. Previous literature reports have used incomplete techniques, and new procedures might provide better estimations of key parameters needed to properly characterize metabolizable AA supply. Digestibility of AA in microbial isolations can be measured using a new in vitro technique, and multiple time point hydrolysis can improve the recoveries of certain AA. In this study, Met concentration of microbial isolates was higher than previously reported, which has significant implications for future model use and development.

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CHAPTER 6: Summary and evaluation of a re-parameterized microbial sub-model of CNCPS v. 7.

MODEL EVALUATION

Updates to the Microbial Sub-model and Microbial AA composition

Relationships and information from chapters 3 and 5 were utilized to update elements of the microbial sub-model in CNCPS v.7. In the original model evaluation by Higgs (2014), predictions of total non-ammonia N, microbial N, and undegraded feed N were found to be close to the observed values from omasal studies. Flows of EAA however were generally over-predicted, with some AA predictions closer to observed values than others.

To address the issues with AA supply predictions, elements of the protozoal sub-model were updated to reflect relationships discussed in Chapter 3 and microbial AA profiles determined in Chapter 5. The previous version of the model assumed that protozoa lyse at ½ the rate of passage, and that no selective retention occurred in the rumen. Slight changes were made to reflect retention, where protozoa pass at 0.9 x the solids $k_{p,}$ which effectively results in a selective retention of 10 %. The relationship between dilution rate and microbial turnover depicted in Wells and Russell (1996) was used to re-evaluate the lysis rate used in the model. A lysis rate of ½ the rate of particle passage in a lactating dairy cow results in a fractional lysis rate of ~ 0.03 h^{-1}; which corresponds to turnover of the protozoa pool of ~50 % (Figure 3 in Wells and Russell, 1996). This would require protozoa to have a true growth rate 2x the rate of passage, or approximately 0.10 to 0.12 h^{-1}. Given that this is very close to the actual rate of carbohydrate availability, a lower lyses rate (25% of the passage rate) was assigned to the protozoal pool to be consistent with data presented in Chapter 3. Amino acid composition of rumen bacteria and

protozoa was determined from the results of Chapter 5. A compromise between calculated and literature values was used, as this was considered to be a more conservative approach. An example of this can be seen with Met, as most literature reports Met values of ~4 % of EAA compared with ~8 % reported in Chapter 5. Values used for each EAA are presented in Table 6.1.

Evaluation Method

To evaluate the effect of the updated parameters in the microbial sub-model of CNCPS v.7, a model evaluation was performed similar to that described by Higgs (2014) and (Van Amburgh et al., 2015). The database used for analysis was the same one used for previous evaluations of the CNCPS (Higgs, 2014; Van Amburgh et al., 2015). Sixteen published studies representing 61 treatment means were used to evaluate microbial N (**MN**), rumen undegraded feed N (**RUN**), total non-ammonia N (**NAN**) flows at the omasal canal. For individual AA, 11 publications with 43 treatment means were used to evaluate predictions of individual AA at the omasal canal. Full descriptions of the criteria used to select and enter the studies into the database have previously been reported by Higgs (2014). Table 6.3 from that report is reproduced as Table 6.2 here.

Table 6.1. Essential AA composition used for simulations

AA, % of EAA	Bacteria	Protozoa
MET	5.0	4.0
LYS	15.8	17.0
ARG	12.2	12.9
THR	10.8	10.1
LEU	10.9	12.8
ILE	8.7	8.9
VAL	10.2	8.5
HIS	4.5	6.0
PHE	9.0	11.4
TRP	12.9	8.5
EAA, % of AA	53.0	52.0
TAA N, % of N	60.0	70.0

Table 6.2. Omasal sampling studies used to evaluate model N and AA flows[1]

Study	Amino acid flows reported
Ahvenjärvi et al. (1999)	
Ahvenjärvi et al. (2002)	x
Ahvenjärvi et al. (2006)	
Brito et al. (2006)	x
Brito et al. (2007a)	x
Brito et al. (2007b)	x
Brito et al. (2009)	x
Broderick and Reynal (2009)	x
Choi et al. (2002)	
Korhonen et al. (2002)	x
Colmenero and Broderick (2006)	
Owens et al. (2008a)	
Owens et al. (2008b)	
Reynal and Broderick (2003)	x
Reynal and Broderick (2005)	x
Vanhatalo et al. (2009)	x

[1]Table reproduced from Higgs (2014)

Analysis of the data from the simulations was performed as described by (Higgs, 2014) to provide relevant comparisons of the updated model. Briefly, a mixed model using the restricted maximum likelihood estimation method of SAS v. 9.3 (SAS Institute, Cary, NC) was used to evaluate the dataset. The model contained terms for the overall intercept across studies, the random effect of study, overall slope of the regression across all studies, and the effect of the repetition of the continuous variable within study. Squared sample correlation coefficients reported were based on the BLUP (R^2_{BLUP}) or model predictions using a mean study effect (R^2_{MP}). The inclusion of the random effect of study is important for ensuring parameter estimates remain un-biased (St-Pierre, 2001). Additional adequacy statistics are reported to provide more information on the accuracy and precision of the model. The root mean square prediction error (RMSPE) indicates accuracy, while decomposition of the mean squared prediction error (MSPE) gives estimations for the source of model error (Tedeschi, 2006, Higgs, 2014). The concordance correlation coefficient (CCC) is also provided for simultaneous evaluation of the accuracy and precision of the prediction.

Prediction of Non-Ammonia N, Microbial N, and Undegraded N.

Overall non-ammonia N flow was predicted with a high level of accuracy and precision (CCC of 0.97; Table 6.3). Predictions of MN and RUN were fairly accurate; however some bias was present, especially for RUN (RMSPE of 38, with 31 % and 26 % attributable to mean and systematic bias, respectively). These values are slightly greater than those reported by Higgs (2014), and indicated that changes to the protozoal parameters might have introduced additional variance and error into the partitioning between microbial and non-microbial N flows. The studies used in the evaluation generally used bacterial enrichment of ^{15}N predict microbial nitrogen. As discussed in chapter 3, this can lead to an underestimation of the true microbial N

flow, as protozoa enrich at decreased amounts relative to bacteria. As such, the CNCPS might under-predict true microbial N flow more than is indicated with the current analysis. Predicted protozoa N flows for the dataset averaged 58 g of N/d, which represented 20 % of the total microbial N flow. The range of predicted protozoa N as a % of microbial N was 20 to 22% indicating the possibility for poor sensitivity. Unfortunately, a limited number of studies exist that report protozoa flows, limiting the ability to systematically evaluate model predictions of protozoa dynamics. Previous studies that measured flows in vivo were discussed in Chapter 3. Shabi et al. (2000) estimated protozoa flows using a linear programming approach, and found protozoa N to account for 7 to 19 % of microbial N flow. This was a result similar to that estimated by Steinhour et al. (1982) using a differential ^{15}N enrichment approach, although many assumptions were made pertaining to pool size and turnover in that study. Computer simulations by Dijkstra et al. (1998) indicated that protozoa N could account for 10.7 to 26.1 % of microbial N in cattle at 17.1 kg of DMI.

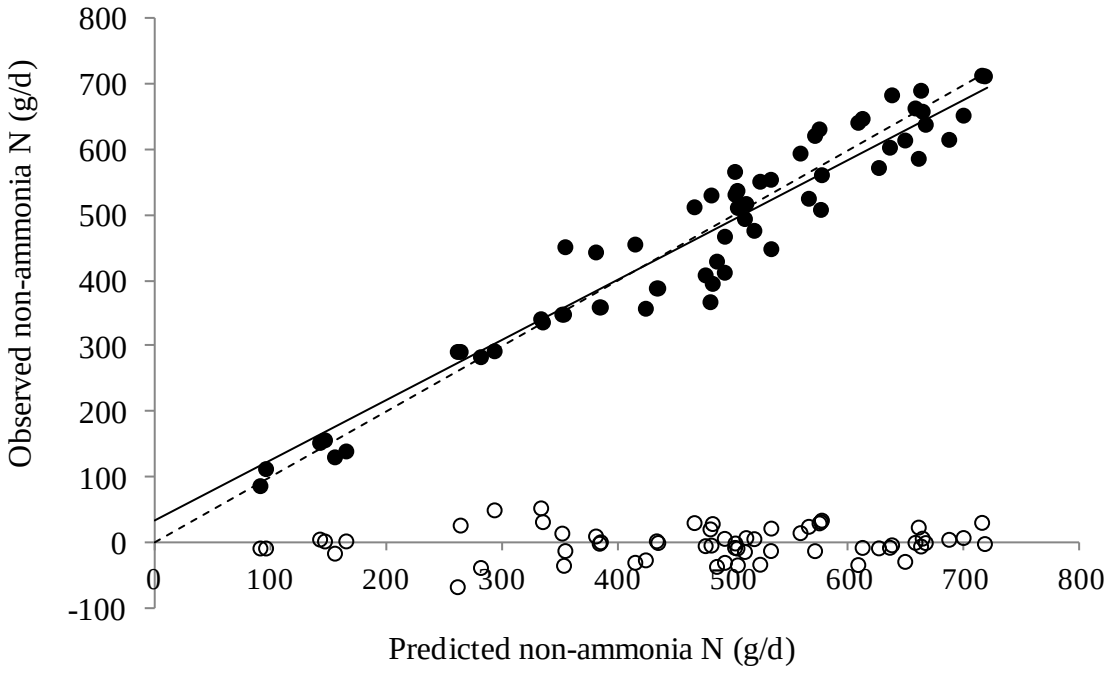


Figure 6.1. Predicted vs. observed omasal flows of total non-ammonia N. Values (●) and residuals (○) from the mixed model analysis, along with lines representing the regression (─) and unity (---) are displayed.

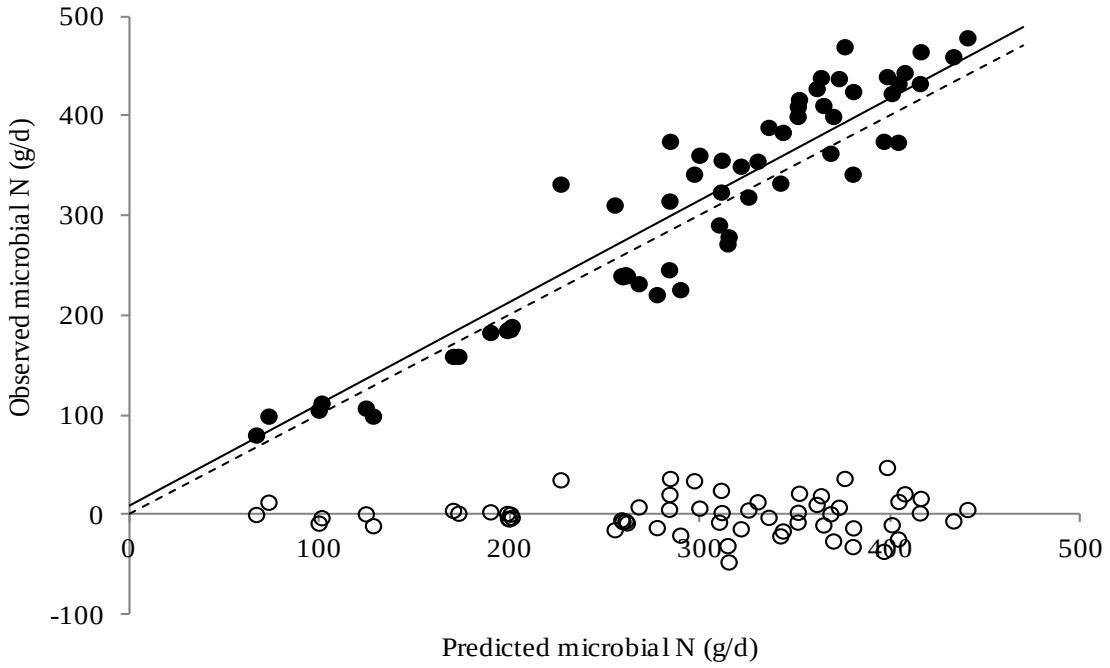


Figure 6.2 Predicted vs. observed omasal flows of microbial N. Values (●) and residuals (○) from the mixed model analysis, along with lines representing the regression (─) and unity (---) are displayed.

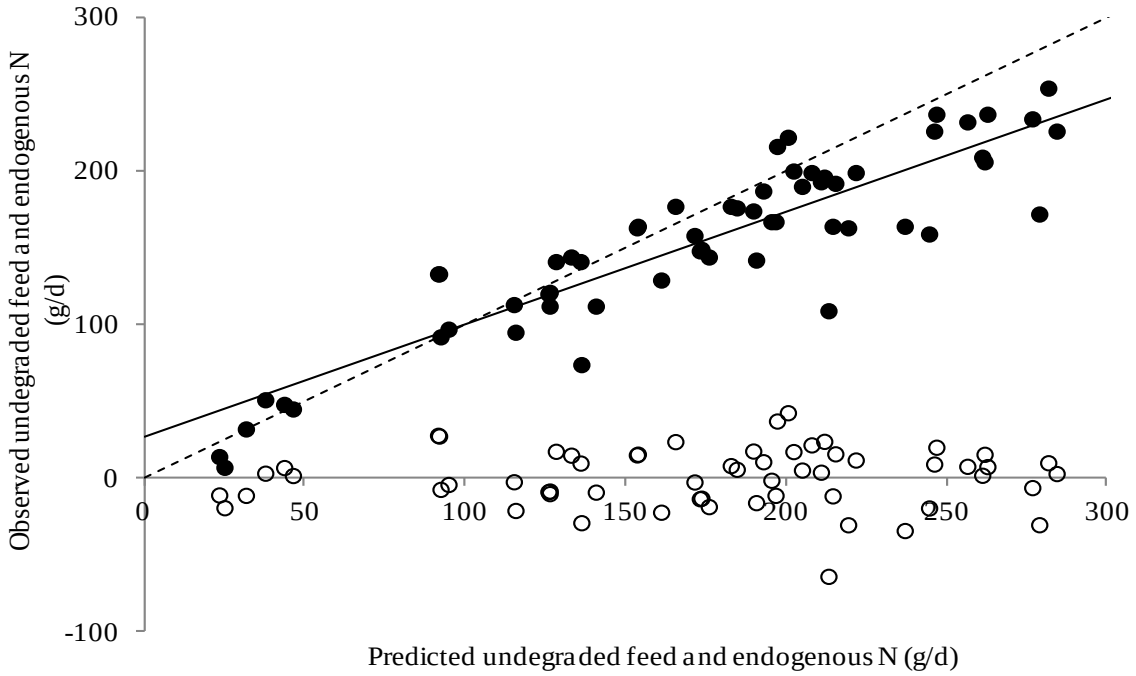


Figure 6.3. Predicted vs. observed omasal flows of non-microbial, non-ammonia N. Values (●) and residuals (○) from the mixed model analysis, along with lines representing the regression (─) and unity (---) are displayed.

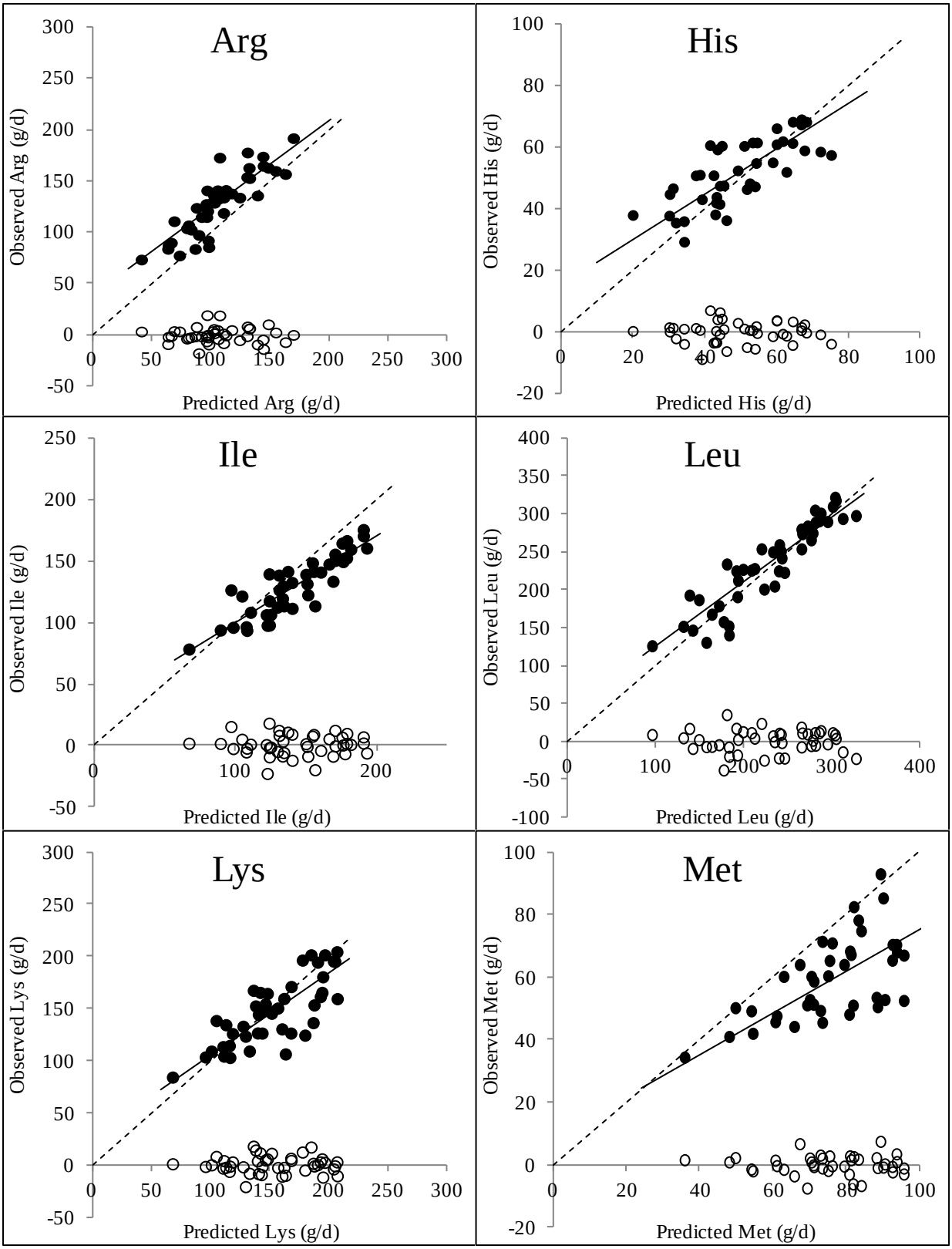
Table 6.3. Model adequacy statistics for the prediction of omasal nitrogen flows and essential AA using from CNCPS v.7 with updated microbial parameters relative to a dataset of omasal sampling studies.

					Var. Component (%)[5]					MSPE Partitioned (%)[8]		
Item, g/d	R^2_{BLUP}[2]	R^2_{MP}[3]	RMSE[4]	Slope	Intercept	Study	Residual	CCC[6]	RMSPE[7]	U_m	U_s	U_r
Total non-ammonia N	0.98	0.93	26	0.92	32	68	32	0.96	45	2	1	97
Microbial N	0.97	0.89	21	1.02	9	74	26	0.92	42	12	8	80
RUN[1]	0.90	0.82	21	0.73	27	28	72	0.84	38	31	26	43
Amino Acids												
Arg	0.94	0.75	16	0.85	38	73	27	0.68	27	66	1	33
His	0.90	0.56	7	0.74	15	70	30	0.71	9	8	34	58
Ile	0.87	0.77	12	0.70	29	40	60	0.77	20	43	22	35
Leu	0.93	0.85	22	0.85	41	48	52	0.92	23	4	6	89
Lys	0.94	0.60	20	0.79	27	81	19	0.76	23	6	21	73
Met	0.95	0.43	10	0.67	8	89	11	0.38	21	66	11	23
Phe	0.91	0.47	23	0.66	22	80	20	0.39	51	68	12	20
Thr	0.92	0.78	14	0.83	14	59	41	0.82	19	40	6	54
Val	0.92	0.75	16	0.76	22	51	49	0.76	23	42	23	35

[1]RUN = Rumen undegraded and endogenous N.
[2]squared sample correlation coefficient based on BLUP.
[3]squared sample correlation coefficient based on model-predicted estimates.
[4]Root mean square error.
[5]Percentage of variance related to the effect of study and random variation.
[6]Concordance correlation coefficient.
[7]Root mean square prediction error.
[8]Mean square prediction error partitioned to: U_m= mean bias; U_s = systematic bias; U_r = random variation. $U_m + U_s + U_r = 100$

Prediction of Omasal Amino Acid Flow

Total amino acid flows were predicted with reasonable accuracy and precision (Table 6.3 and Figure 6.4). Key areas of improvement as evaluated by the CCC were Ile, Lys, and Val. Root mean squared prediction error (**RMSPE**) was also improved in His, Lys, and Val. For Lys, the portion of MSPE attributable to mean and systematic bias (6 % and 21 % of MSPE, respectively) was greatly reduced compared with the evaluation by Higgs (2014), who reported mean and systematic bias of 80 % and 9 % of MSPE, respectively. This improvement is largely a result of the decreased microbial Lys content determined using the multiple time point hydrolysis. Higgs (2014) discussed possible discrepancies between reported Lys flows and estimated microbial Lys flow using composition data from Clark et al. (1992). It is possible that the values reported by Clark et al. (1992) might overestimate Lys content through an underestimation of branched chain AA, as these AA are released from the sample matrix at lower rates, while Lys is rapidly hydrolyzed (Chapter 4). Omasal Met flow in the current evaluation was generally over predicted, with a considerable amount of error attributable to mean bias (66% of MSPE). Met analysis is technically challenging, and pre-oxidation recoveries are rarely reported in the literature. It is important to note that reported AA flows are from a single time point hydrolysis, which would likely contribute additional mean and/or systematic bias when values are compared to the predictions from the CNCPS. Overall AA predictions were improved from the previous evaluation of the model. Average reported CCC and RMSPE for the Higgs (2014) evaluation was 0.66 and 28.5, respectively; while the current evaluation averaged 0.69 and 23.8 for CCC and RMSPE, respectively; indicating overall improvement in AA flow predictions. Of the AA considered most often to be first limiting in dairy cattle, Lys and His predictions were improved, while Met predictions were not improved.



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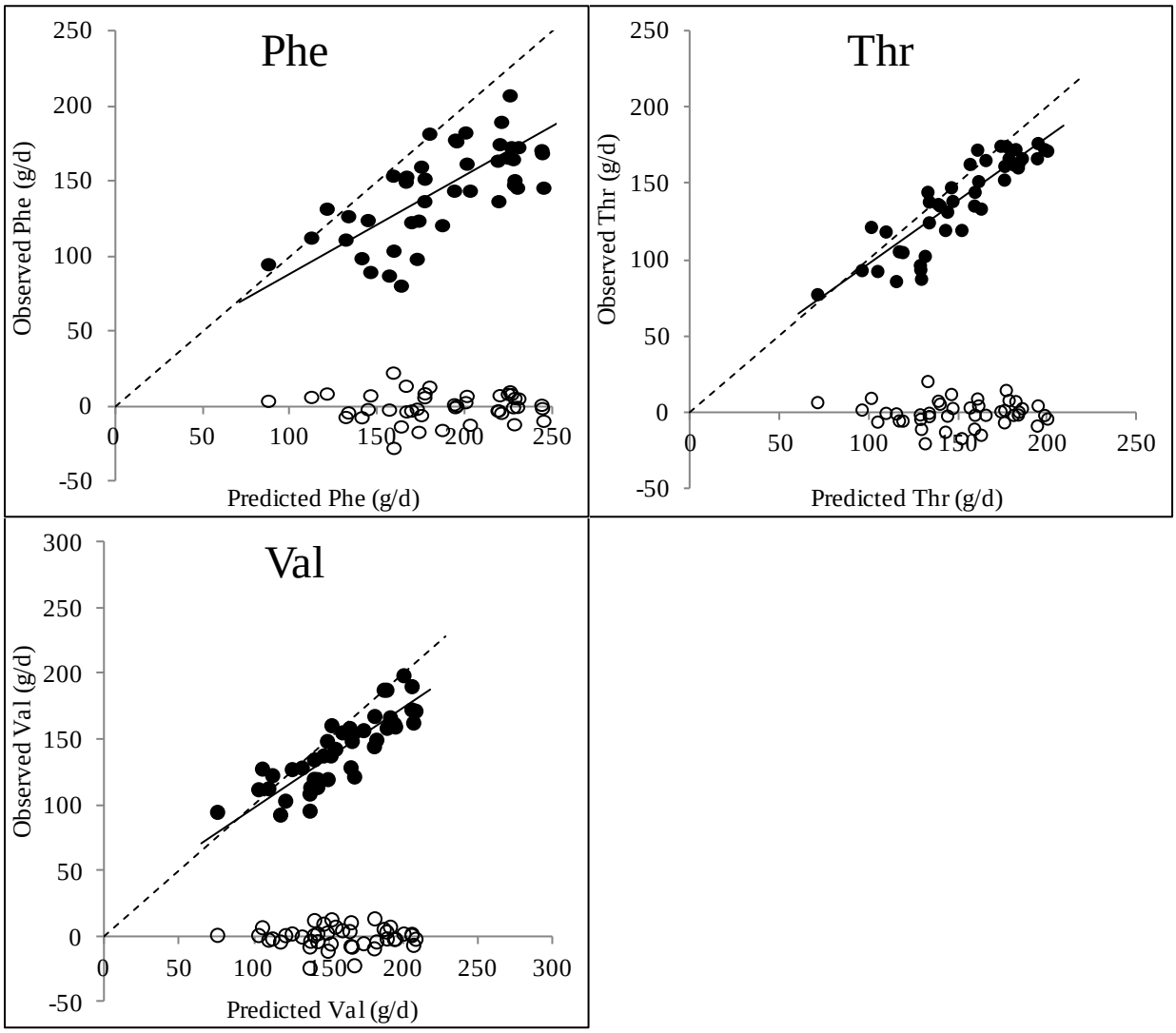

Figure 6.4. Predicted vs. observed omasal flows of non-microbial, non-ammonia N. Values (●) and residuals (○) from the mixed model analysis, along with lines representing the regression (─) and unity (---) are displayed.

SUMMARY AND CONCLUSIONS

Two in vivo studies were performed to evaluate the effects of a commercial fermentation byproduct on rumen nutrient digestion, microbial growth, omasal flows of nutrients, and milk production in lactating dairy cattle. The objectives of the first portion of the first study (Chapter 2) was to evaluate the effects on nutrient digestion in the rumen and total tract, along with flows of total non-ammonia N, microbial N, and non-microbial N. Inclusion of fermentation byproduct decreased dietary nitrogen degradation in the rumen, with no observed detrimental effects on microbial nitrogen flows.

Objectives for the second part of the experiment (Chapter 3) involved investigation of the effects of diet on microbial growth and turnover, partitioning of protozoa and bacterial N, and determination of AA flow at the omasal canal. Cows fed the fermentation byproduct had increased flows of non-microbial AA at the omasal canal. Microbial growth was not affected by diet. Evaluation of the observed results against model predicted fermentation and microbial growth parameters indicated that the rumen sub-model of CNCPS v.7 was adequate to provide accurate estimations of total microbial N. Individual AA flow and partitioning of the microbial N flow between bacteria and protozoa might require additional work to improve predictions.

The second trial (Chapter 4) was performed to evaluate the effects of the fermentation byproduct on cattle performance and milk production in groups of lactating cows. In this study, cows fed fermentation byproduct had increased milk production, energy-corrected milk yield, and protein yield compared with cows fed treated and untreated soybean meal. The estimated economic and environmental characteristics of cows fed fermentation byproduct indicated that Fermenten can be an excellent feed when priced competitively with soybean meal.

In Chapter 5 samples of bacteria and protozoa from the first study, along with several other microbial samples from other studies were used to characterize the EAA composition for use in a dynamic version of the CNCPS. Two methods not previously applied to microbial samples were used to accurately characterize the AA profile and digestibility of bacterial and protozoal protein.

Evaluation of the model updates indicated the CNCPS has a good ability to predict post-ruminal AA flow in lactating dairy cattle. Further work is needed to improve predictions of some AA, especially Met., Re-evaluation of AA ratios and relationships to other dietary or animal parameters used in practical ration formulation will likely need to occur as supply predictions improve. Overall, the methods detailed in this dissertation, including omasal sampling, improved isolation of protozoa, and more accurate determination of post ruminal flows of digestible AA has allowed for a more complete understanding of the value of rumen microbial protein to supply AA to the animal.

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