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Characterization of protein and fat in dairy feeds and implications on digestibility and milk composition

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CHARACTERIZATION OF PROTEIN AND FAT IN DAIRY FEEDS AND IMPLICATIONS ON DIGESTIBILITY AND MILK COMPOSITION

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CHARACTERIZATION OF PROTEIN AND FAT IN DAIRY FEEDS AND IMPLICATIONS ON DIGESTIBILITY AND MILK COMPOSITION

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A variety of feedstuffs are used to comprise dairy rations, each with their own nutritional contributions. In order to best utilize these feedstuffs to maximize production, characterization of their chemical composition is needed. Through the use of both in vitro and in situ methods, not only can the composition of the feed be determined, but also its digestibility. Also, because of the important role they play in human health, omega 3 fatty acids have received increasing attention. Most individuals consuming Western diets do not meet the recommended requirement for omega 3 fatty acids, and one way to improve that is through the enrichment of dairy products by selective feeding high fatty acid feedstuffs to dairy cows.

In the first experiment, three assays were used to determine RUP digestibility, the Mobile Bag (MOB), Modified Three Step (MTS), and Ross (ROS) assays, were compared. Also with this experiment, 10 samples of feather meal from different plants across the United States, five with blood (FMB) and five without blood (FM), were evaluated. Each of the ten samples were subjected to all three assays. The results indicate that while the average initial compositions were different between FM and FMB samples, very little difference was observed in the ruminal or intestinal digestibility of the protein. However, there was differences in values among assays. Assay had a significant effect on rumen dry matter digestibility, RDP, RUP, total tract dry matter digestibility, and total

tract crude protein digestibility with MOB and MTS being the most similar in values. Nonetheless, RUP digestibility did not differ among assay or blood inclusion. Overall, even though values between samples and assays varied, there was no difference in RUP digestibility among blood inclusion and assay.

The second study's goal was to evaluate the effect of the novel fatty acid supplement, Perfect Omega 3 (PO3), on the milk fatty acid profile and energy utilization. Diets ranging in 0 to 20% PO3 inclusion were fed to four multiparous Jersey cows in a 4 × 4 Latin square, and headbox-style indirect calorimeters were used to determine the effect of increasing inclusion on energy utilization. Results show that increasing inclusion of PO3 not only increased the milk fat concentration but also increased the concentration of α-linolenic acid in the milk while decreasing linoleic acid with no difference in milk yield. Gross energy increased with increasing inclusion, but DE and ME did not differ among treatments. Increasing inclusion also had no effect on NDF and energy digestibility. Through this study, increasing inclusion of PO3 not only maintained milk production, but also increased milk fat concentration with favor towards omega 3 fatty acids.

“She is clothed in strength and dignity, and she laughs without fear of the future.”

-Proverbs 31:25

“Believe in yourself and all that you are. Know that there is something inside you that is greater than any obstacle.”

- *Christian D Larson*

“If you don’t go out and get it, you won’t have it.”

- *Rebecca Flicher*

“There is no vocation on God’s green earth that calls for higher elements of character, for deeper research, for grander nobility of nature than that of the farmer.”

- *W.D. Hoard*

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GENERAL INTRODUCTION

Dairy cattle diets are comprised of a variety of feedstuffs that can broadly be categorized as forages, concentrates, and byproducts. Each category has its own unique characteristics, and within each category, feedstuffs can vary in chemical composition. Since these feedstuffs can differ from each other, chemical composition should be well described prior to ration formulation procedures to ensure that animal requirements are met at a least cost (Tran et al., 2020). Protein is an important component of several feedstuffs and can vary in degree of digestibility and thus varies in how it contributes amino acids to the animal (Schwabb et al., 2003). One portion of protein, rumen undegraded protein (**RUP**), supplies amino acids directly to metabolizable protein but can vary in its availability. The digestibility of RUP (**dRUP**) is variable depending on the type of feed and processing (Gargallo et al., 2006). An accurate estimate of dRUP for each feedstuff is necessary for accurately balancing diets for RUP (Schwabb et al., 2003). Another important component of feedstuffs is fat, which is a generic term to describe compounds that contain a high content of fatty acids (**FA**) (NRC, 2001). Mammals are unable to synthesize two polyunsaturated fatty acids (**PUFA**): α-linolenic acid (**ALA**), which is an omega 3 FA, and linoleic acid (**LA**), an omega 6 FA (Markiewicz-Keszycka et al., 2013). While both ALA and LA are essential, omega 3 FA have more of an impact on human health (CAST, 2018).

Several methods exist to determine dRUP, such as use of acid detergent insoluble crude protein, as well as *in vitro* and *in situ* procedures (Schwabb et al., 2003). One of the most commonly used procedures is the Mobile Bag assay, which was first introduced in ruminants by Hvelplund (1985) and takes place almost entirely *in situ*. Because this assay

can be labor intensive, a three-step assay that determines intestinal digestibility *in vitro* was introduced by Calsamiglia and Stern (1995) and later modified by Gargallo et al. (2006). Since both the Mobile Bag and Modified Three-Step assays use porous bags to contain samples throughout the procedure, limiting microbial access to samples is a concern. To eliminate the problem all together, Ross (2013) developed an assay using rumen fluid from a donor and Erlenmeyer flasks to mimic rumen incubation.

Omega 3 fatty acids are PUFA that play an important role in human health. In infants, adequate supply of omega 3 FA is essential for optimal visual, neural, and behavioral development (CAST, 2018). For adults, consumption of omega 3 fatty acids reduces the risk of cardiovascular disease by reducing inflammation, blood triacylglycerol concentrations, and blood pressure (Calder, 2004). Alpha-linolenic acid is commonly found in plant-based sources, and within the human body, can be converted to the other essential omega 3 fatty acids eicosapentaenoic acid (**EPA**) and docosahexaenoic acid (**DHA**), which are commonly found in animal-based sources (CAST, 2018). As essential as omega 3 fatty acids are, individuals consuming a Western diet often do not consume the recommended 1.8 g/d by the American Heart Association (Krauss et al., 2000). An approach to improve dietary consumption is strategic feeding practices that lead to an enrichment of animal products (CAST, 2018). In dairy cattle, supplemental dietary omega 3 fatty acids may improve fertility because ALA is converted to EPA which is a precursor for prostaglandins (Petit et al., 2002), which have positive effects on ovulation, embryo survival, and parturition (Gulliver et al., 2012). However, feeding diets containing high concentrations of fat to dairy cattle may result in some challenges. This is because rumen microbes hydrogenate the bonds in PUFA since they are toxic to

microbes. An intermediate of the process of hydrogenation is conjugated linoleic acid (**CLA**), and certain isomers of CLA have been shown to cause milk fat depression by suppressing milk fat synthesis in the mammary gland (Baumgard et al., 2002).

Previously, comparisons of various assays developed to determine RUP digestibility have been conducted (Schwab et al., 2003; Ross, 2013; Boucher et al., 2009), but no studies have compared the Mobile Bag, Modified Three-Step, and Ross assays within one study. The first objective of this thesis was to compare the Mobile Bag, Modified Three Step, and Ross assays while determining the RUP digestibility of feather meal with and without blood. Also, a large number of studies have evaluated the ability of several feedstuffs to alter the fatty acid profile of milk to be higher in omega 3 fatty acids (Abu-Ghazaleh et al., 2001; Petit, 2002; Wright et al., 2002; Hurtaud et al., 2010, Judy et al., 2019). However, to the author's knowledge, no studies have been conducted to evaluate the effect of a blend of byproducts, not commonly seen in the United States, on milk's fatty acid profile as well as the energy utilization of lactating dairy cows. For this reason, the second objective of this thesis was to chemically characterize a novel high fatty acid supplement containing a high concentration of omega 3 FA and to evaluate its impact on the milk fatty acid profile and energy utilization of lactating cows.

CHAPTER 1

LITERATURE REVIEW

Feedstuff Chemical Composition

Chemical composition varies within and among feedstuffs due to differences in growing and storage conditions as well as processing methods. These differences lead to challenges in ration formulation, especially when relying upon default values of feed composition in feed libraries included in ration software. In order to yield the most precise predictions, models, such as the Cornell Net Carbohydrate and Protein System (**CNCPS**), require accurate estimates of feed composition generated from lab analysis (Tedeschi et al., 2002). Any over- or under-predictions of nutrients and energy can lead to not only poor production but also potential illness, mortality, and economic losses (Fox et al., 2004). Assays to determine the various nutritional components of feedstuffs have been developed and refined over the past several years to yield accurate values.

Protein

Digestion. Proteins are large molecules found in the cell walls and contents that vary in size, function, and amino acid composition (NRC, 2001, Schwab et al., 2003). These differences influence the structure of the protein and may affect degradability within the digestive tract. Protein digestion in ruminants is complex system that is largely the result of enzymatic activity of rumen bacteria (NRC, 2001; Bach et al., 2005). As sizeable as the contribution of microbes is, protein digestion should be viewed as two phases: microbial action in the rumen and post-rumen digestion by secreted enzymes (Santos et al., 1984). Over the last 25 years, the focus when balancing rations for protein has shifted from crude protein (**CP**), which is defined by the NRC (2001) as the percent

nitrogen (**N**) content of feed × 6.25, to rumen degraded protein (**RDP**) and rumen undegraded protein (**RUP**) fractions as well as metabolizable protein (**MP**) (Bach et al., 2005; Eastridge, 2006).

The portion of CP that is degraded in the rumen is known as RDP. This portion of protein is essential as it provides precursors necessary for microbial growth, activity, and synthesis of microbial protein (Schwab et al., 2003). Non-protein nitrogen (**NPN**) and any protein that is easily and believed to be rapidly soluble in rumen fluid – such as free AA, nucleic acids, and amines (Schwab et al., 2003) – are considered RDP. The soluble protein and NPN are rapidly converted to ammonia (**NH_3**) by rumen microbes (Pichard and Van Soest, 1977). This portion of protein is known as Fraction A in the NRC (2001).

Rumen degradation of true protein, which according to the NRC (2001) includes the CNCPS B_1 and B_3 fractions, begins with the attachment of rumen bacteria to feed particles (Bach et al., 2005), and these organisms secrete a variety of proteases, peptidases, and deaminases that begin the process of breaking down protein (NRC, 2001). The process, known has proteolysis, involves hydrolysis of peptide bonds, which yields oligopeptides (NRC, 2001) that can be further broken down into peptides and free amino acids (**AA**). Those amino acids are then taken up by rumen microbes, and through deamination and decarboxylation, result in a-keto acids that are used to produce volatile fatty acids (**VFA**), methane (**CH_4**), NH_3, and carbon dioxide (**CO_2**) as well as heat (Tamminga, 1978; Ahmed Mohammed, 1982). These products are then released back into the rumen and are absorbed by the animal. The microbes themselves are able to use free peptides, after further hydrolysis, and AA as well as the produced NH_3 for synthesis of microbial crude protein (**MCP**) (Russell et al., 1992) The resulting MCP can then pass

out of the rumen and to the hind gut where they contribute to MP. Microbial CP supplies 40 to 80% of daily AA requirements to the small intestine (Sniffen and Robinson, 1986).

Protein that escapes rumen degradation and passes small intestine to be digested, absorbed, and utilized by the cow is known as RUP (NRC, 2001); RUP includes the B_2 and C Fractions. This protein portion as well as MCP that passes onto the intestines comprise MP. Protein first is exposed to abomasal digestion when exposed to pepsin. Pepsin hydrolyzes approximately 15 to 20% of dietary protien to AA and small peptides by hydrolyzing the peptide bonds between AA with phenyl groups (tyrosine, tryptophan, and phenylalanine) and a dicarboxylic acid (aspartate and glutamate) (Meisfeld and McEvoy, 2017). Protein then passes onto the small intestine where it is further exposed to and degraded by the proteases trypsin and chymotrypsin. Trypsin is known to break down peptides by cleaving the peptide bonds on the carboxyl side of arginine and lysine, and chymotrypsin cleaves peptide bonds on the C-terminal side of the aromatic amino acids tyrosing, tryptophan, and phenylalanine (Meisfeld and McEvoy, 2017). The C Fraction of protein consists of proteins that are associated with lignin, tannins, and heat-damaged, such as by Maillard reactions, are termed unavailable as they cannot be degraded by microbial and mammalian enzymes. This Fraction doesn't supply any AA post-ruminally because they are believed to be unavailable to the animal (Sniffen et al., 1992).

Difference among sources. Several factors such as harvesting, ensiling, and processing methods can alter the availability of protein. An example of processing methods affecting protein availability is byproducts produced by the rendering industry, such as hydrolyzed feather meal and bloodmeal. During the rendering process, raw material is ground to a uniform size and then cooked in a continuous-flow or batch

system. During the cooking process, most of the moisture and fat is removed. Steam is used to heat the material to 115 to 145 °C and these temperatures are held for 40 to 90 minutes; the extent of time depends upon the type of material (Meeker and Hamilton, 2009). The time and temperature at which material is cooked may influence protein degradability in the animal. For example, a study conducted in rats found that intestinal protein digestibility varied from 17.0 to 94.6% depending on the drying method, time in drier, and temperature of the drier (Moughan et al., 1999).

Hydrolysis is a process in which feathers and hair are heated to high temperatures under pressure to break keratin bonds within the material, and this increases digestibility (Meeker and Hamilton, 2009). While this process may improve the digestibility, the availability of AA decreases since some AA can be modified or destroyed during hydrolysis (Moughan, 2003), which can lead to a reduction in milk protein (Meeker and Hamilton, 2009). When blood is added to feather meal, whether blood is added before or after hydrolysis has a large effect on protein availability. When blood was added from 10 to 38% of the total product prior to hydrolysis, protein digestibility ranged from 46 to 85%. When 10 to 15% blood was added following hydrolysis, digestibility was less variable and ranged from 63 to 68% (Contach et al., 2007) The length of hydrolysis has little effect on digestibility. A longer hydrolysis increased DM digestibility (54.8 to 57.5%), but true protein digestibility was not observed to be not different in sheep (Blasi et al., 1990).

Impact on production. Using a model derived from 393 measurements obtained by 82 protein studies, the NRC (2001) states that increasing CP content of a diet from 15 to 16 percent can result in an expected increase in milk yield of 0.75 kg/d and an increase

from 19 to 20 percent would have an increase of 0.35 kg/d. Using a different model derived from 17 protein studies with production records for 625 cows, an increase of 1.8 kg/d in milk yield would be expected when increasing diet CP from 12 to 16 percent. Also to be noted is that a diminishing response was observed as dietary CP concentration increased due to a decrease in dry matter intake (DMI) (Roffler et al., 1986).

Studies examining increasing amounts of RUP to early lactation cows report conflicting responses in milk production. In some studies, milk production is increased (Cunningham et al., 1996; Greenfield et al., 2000) while others have reported no response (Henson et al., 1995; Davidson et al., 2003). The lack of a response seen in early lactation cows could be caused by a change in the protein reaching the small intestine. Using a model derived from 17 published lactation studies consisting of 625 individual cow production records, MCP synthesis and flow to the small intestine decreased while non-ammonia and non-microbial N increased when soybean meal, which is one of the most commonly used sources of protein in lactating cow diets and is known to be low in RUP, was replaced by a high RUP source. The combination of these responses resulted in no change in the flow of protein to the small intestine and milk yield (Roffler et al., 1986). While the amount of protein supplied to the small intestine did not change, the amount of protein that can be digested as well as the AA profile likely did (Henson et al., 1995). Santos et al. (1998) compared the AA profile of milk protein to the AA profiles of several feedstuffs as well as MCP and found that MCP provided a more well-balanced source of essential AA than other options. Considering those results, when RUP is increased at the expense of RDP for MCP synthesis without balancing for essential AA, a lack of a response in milk yield is likely.

However, when RDP is high, there is also a high ammonia concentration within the rumen as a result of microbial degradation. The ammonia is then absorbed and transported to the liver through the bloodstream where it is converted to urea. High concentrations of circulating urea have negative effects on the animal such as decreased pregnancy rates (Aboozar et al., 2012). Also, even though MCP has been shown to be a well-balanced source of essential amino acids (**EAA**), adequate dietary RUP needs to be fed with the appropriate AA balance in mind to complement the MCP synthesized to provide the desired protein to energy ratio to the animal (Schwab, 1995). When fed increasing concentrations of available dietary RUP, milk production increased. This response was attributed to an increased availability of AA in the small intestine to be used by the cow for milk production and milk protein synthesis (Waltz, 1989).

Methods of Measuring Degradability and Digestibility of Feed Protein

Current dairy cattle feeding systems rely upon feed characterization values obtained via *in vitro* and/or *in situ* procedures to determine the availability and supply of nutrients. There are three major assays used to determine protein quality, the fraction of protein that escapes rumen degradation and its digestibility, of feedstuffs: the Mobile Bag (Paz et al., 2014), Modified Three-Step (Gargallo et al., 2006), and Ross (Ross et al., 2013) assays. While these 3 assays all determine the same parameters of protein, they all vary in how they attempt to mimic the activity of the dairy cow's digestive tract. In the case of the Mobile Bag assay digestibility is estimated entirely *in situ* while the Ross assay is completely *in vitro*.

The mobile bag assay. This method involves placing a small feed sample into a N-free, porous bag with a pore size small enough to retain the feed sample but large

enough to allow microbial access. The technique of estimating rumen digestion was first estimated by measuring disappearance of feedstuffs from a silk bag placed into the rumen by Quin et al. (1938) in sheep. In 1979, Orskov and McDonald introduced mathematical tools to aid in calculating the effective protein degradability (**EPD**) which enabled the *in situ* technique to be more extensively used. The equation is as follows:

$$EPD = a + b\,[c/(c + k)] \qquad [1]$$

where,

a = fraction of immediately degradable (soluble) protein;

b = fraction of not soluble, but degradable, protein;

c = the fractional rate of degradation of fraction of fraction b

k = fractional outflow rate from the rumen

In 1983, the mobile bag method for estimating intestinal protein digestibility was introduced in pigs by Sauer et al. Shortly after, the method was adapted for ruminants, largely using the work and calculations by Quin et al. (1938) and Orskov and McDonald (1979). Since then, attempts have been made to standardize the procedure. The bags used are recommended to now be made of polyester or nylon with a pore size of 40 to 60 µm and width to length ratio of 1:1 to 1:2.5 (NRC, 2001). Bags should be washed using an automatic washing machine for 10 to 15 minutes and animals used for incubation should be fed at or just above maintenance (Hveplund and Weisbjerg, 2000). With these standardizations in mind, below is the modernized procedure, as outlined by Paz et al. (2014).

The steps of the Mobile bag assay are shown in Figure 1.1. To start, approximately 1.5 g of sample is weighted into 10 5 × 10 cm N-free polyester bags

(R510, Ankom Technologies, Macedon, NY) with a mean pore size of 50 µm. Bags are then heat-sealed using an Ankom Heat Sealer (Ankom Technologies, Macedon, NY) before being placed in mesh bags (46 × 38 cm) that contain secured 100 g weights to prevent bags from floating in the rumen mat. Mesh bags are then inserted into the rumen through a rumen cannula and positioned in the ventral sac before incubating for 16 h. Following incubation, mesh bags are removed and washed in a domestic washing machine using the washing procedure of 5 cycles of 1 min wash and 2 min agitation. After incubation, 4 bags of each sample are gently rinsed with distilled water to force residue to the bottom of the bag, rolled, and dried in a 45 °C oven for 24 h. The remaining 6 bags are then incubated in a pepsin-HCl solution comprised of 1 g of pepsin per L of 0.01 N HCl for 3 h in a 39 °C water bath with occasional stirring to stimulate abomasal digestion.

After incubation in the pepsin-HCl solution, bags are removed and rinsed with distilled water to wash out the solution and force residue to the bottom of the bag. The upper portion of the bag is then rolled before being inserted into the duodenum using a duodenal cannula of the cow in which it was rumen incubated; bags are inserted at a rate of 1 every 5 minutes. Following passage through the remainder of the digestive tract, bags are recovered from the manure from the time of first bag appearance (approximately 8 h following insertion) until 24 h after first insertion. Bags are then rinsed lightly with cold water to remove fecal material and placed on ice to halt any further degradation. Once all bags are recovered or 24 h has passed, bags are once again washed using the washing procedure, rinsed with distilled water to force residue to the bottom, rolled, and

placed in a 45 °C oven to dry for 24 h. Following drying, all bags are weighed to determine the weight of the remaining residue (Paz et al., 2014).

The RDP content of samples is determined as portion of the CP that disappeared from the nylon bag following the *in situ* incubation. Rumen undegraded protein is then calculated as 100 – RDP. Total tract CP digestibility is calculated by subtracting the indigestible protein following transit through the digestive tract from 100. The digestible portion of the RUP was assumed to be the percentage of the CP escaping ruminal disappearance but not recovered in the residue following intestinal incubation and was calculated as 100 – (total-tract indigestible protein/RUP).

This assay is completed almost entirely *in situ* with the only step in the procedure completed *in vitro* being the pepsin bath. The purpose of a pepsin-HCl bath is to subject samples to conditions similar to abomasal digestion. The HCl solution creates an acidic environment that is at a pH level commonly seen in the abomasum of cattle. Addition of pepsin in the solution recreates the further degradation of proteins in the abomasum. While the necessity of this step was questioned, Hvelplund and Weisbjerg (2000) validated that this step is necessary based on intestinal digestibility values obtained after a pepsin-HCl pretreatment, or lack of, in a study completed by Hvelplund in 1985.

One of the major advantages of this assay is that most steps are conducted *in situ*, thus most test materials are exposed to the actual digestive environment. Unfortunately, the requirement of animals that are both ruminally and duondenally cannulated is not only costly but also labor intensive. Exposure to the actual digestive environment is possible because of the use of N-free nylon bags, which also comes at a cost. The ability of these bags to expose contents to rumen microbes without causing extensive washout

has been evaluated and sometimes criticized. Both Voigt et al. (1985) and Vanhatalo and Ketoja (1995) evaluated bag pore sizes ranging from 9 to 70 µm and found that there was no difference in the digestibility or disappearance of samples. As noted by Vanhatalo and Ketoja(1995), the more important aspect of the bags is that a large enough free surface area (> 5%) is maintained. With the use of bags, there is also risk for bacterial contamination, which would decrease the estimate of RDP (Alexandrov, 1998). Bags are washed to attempt the removal of bacteria from feedstuffs but contamination may still occur. To correct for bacterial contamination, purines can be used as a marker but any purines in the feedstuffs can alter the accuracy. Paz et al. (2014) explored the use of microbial DNA markers as a way to estimate bacterial contamination and found that bacterial contamination estimates were lower than when using purines, which could be because DNA markers are not present in all microbial species.

The modified three-step assay. Current feeding systems have a need for quick and accurate feed analysis to be used in ration formulation. As noted, a disadvantage of the *in situ* analysis is the costs related to cannulating and maintaining cows. To better accommodate the need for rapid analysis, there is a need for assays that allow for fast and affordable results while still being accurate and reliable (Calsamiglia et al., 2006). To meet his need, the modified three-step assay lessens the cost and labor intensity by moving the intestinal digestion from *in situ* to *in vitro*.

In 1995, Calsamiglia and Stern developed a three-step in vitro assay (**TSP**) to estimate the intestinal digestion of proteins that was affordable, reliable, and inexpensive while still simulating the physiological conditions of the ruminant. While the rumen incubation and incubation in a pepsin solution of 1 g pepsin/L 0.1 N HCl was left

unchanged from the mobile bag assay, a modification of the pancreatin procedure introduced by Akeson and Stahmann (1964) was used to simulate intestinal digestion; the new pancreatic solution consisted of 3 g pancreatin/L of solution, 50 mg/kg thymol to prevent microbial growth, and 0.5 M KH_2PO_4 as a buffer that was adjusted to pH 7.8 using NaOH. Samples were rumen incubated in polyester bags in the rumen, but following incubation, samples were pooled and then 15 mg of residual N were subjected to the pepsin and pancreatin incubations; undigested protein was precipitated using trichloroacetic acid and separated by centrifucation (Calsamiglia and Stern, 1995).

In 2006, the TSP assay was modified to optimize the pepsin-pancreatin portion of the procedure. The pepsin used in the original development of the assay had a high enzymatic activity and was expensive; there was, however, a less purified pepsin that was more affordable and just as effective once concentration was increased. Also, the entire pepsin and pancreatic incubation portions of the assay were adapted to the Daisy^{II} incubator (Ankom, Fairport, NY) by increasing the amount of solutions needed as well as keeping residues in bags following rumen incubation. The Modified Three-Step (**MTS**) assay as outlined by Calsamiglia et al. (2006) is outlined below and diagramed in Figure 1.2.

Similar to the mobile bag assay, approximately 1.5 g of sample is weighted into 10 5 × 10 cm N-free polyester bags (R510, Ankom Technologies, Macedon, NY) with a mean pore size of 50 µm that are then heat-sealed using an Ankom Heat Sealer (Ankom Technologies, Macedon, NY) before being placed in mesh bags (46 × 38 cm) that contain secured 100 g weights to prevent bags from floating in the rumen mat. Mesh bags are then inserted into the rumen through a rumen cannula and positioned in the ventral sac.

Mesh bags are incubated for 16 and then are removed and washed in a domestic washing machine using the washing procedure of 5 cycles of 1 min wash and 2 min agitation. Four bags of each sample are then gently rinsed with distilled water to force residue to the bottom of the bag, rolled, and dried in a 45 °C oven for 24 h. The remaining 6 bags are then separated by cow and placed in incubations bottles, with no more than 30 bags per bottle and incubated in a pepsin-HCl solution (1 g of pepsin/L of 0.01 N HCl) for 1 h at 39 °C with constant rotation in a Daisy^{II} incubator (Ankom, Fairport, NY).

Following incubation, bags removed from the bottle and lightly rinsed before being returned back to their designated incubations bottles, with no more than 30 bags per bottle once again. Two L of a pancreatin solution (0.5 M KH_2PO_4 buffer, adjusted to pH 7.75, containing 50 ppm thymol and 3 g/L pancreatin) are added to each jar. Bottles were then placed in a Daisy^{II} incubator (Ankom, Fairport, NY) and incubated for 24 h at 39 °C with constant rotation. After 24 h, bags are removed from the jars and rinsed with tap water to wash out the pancreatin solution and to force the residue to the bottom. Bags are then rolled and dried in a 45 °C oven for 24 h. Following drying, bags are weighed to determine the weight of the remaining residue (Gargallo et al., 2006).

The original protocols outlined by Gargallo et al. (2006) called for a 12 h incubation time. In a book chapter, Hvelplund and Weisbjerg (2000) stated that an incubation time of 16 h best simulates the influence of rumen metabolism on a feedstuff before proceeding further along the digestive tract. Another study conducted by Calsamiglia and Stern (1995) did not identify differences in the estimate of true digestibility of proteins between 12 and 18 h of rumen incubation. Because of this evidence, a rumen incubation time of 16 h was adopted.

The largest challenge when developing an *in vitro* assay is accurately simulating the conditions of the ruminant digestive tract. In the small intestine, the pancreas secretes a number of enzymes that aide in digesting substrates in the intestine. For *in vitro* assessments, pancreatin, a mixture of amylase, lipase, and protease, is used to simulate the effects of the pancreatic enzymes that would typically be present (Calsamiglia et al., 2000), and a 24 h incubation in the pancreatic solution is used to maximize protein digestion (Calsamiglia and Stern, 1995). To validate the modified TSP, Boucher et al. (2009) compared the RUP digestibility values obtained from the assay to values obtained *in situ* and found them to be highly correlated.

The Ross assay. While the MTS is less labor intensive, more affordable analysis, there is still concern about the use of bags. The use of bags can create a barrier between the sample and microbes, which slows microbial attachment and causes a lag in digestion. There is also possibility of loss of highly soluble particles from the bag prior to digestion and throughout digestion. When looking at other in vitro assays, there is a lack of uniformity in the pancreatic enzymes used as the units are dependent upon the substrate being hydrolyzed as well as the activity of that specific commercially-produced enzyme (Ross, 2013). In response to this, Ross et al. (2013) developed a new *in vitro* procedure, which standardizes the enzymes used without the use of bags. Without the use of a cow's actual digestive environment, the Ross assay also provides some flexibility by creating an anaerobic environment similar to the rumen utilizing an oxygen scrubber, shaker bath, and rumen fluid from a donor cow. This flexibility and lack of maintaining a cow on site makes the Ross assay the most commercially common assay to estimate intestinal digestibility of ruminant feeds. Even though the Ross Assay is conducted in

vitro, careful consideration has gone into making sure that it best recreates the environment of the dairy cow's digestive tract.

Figure 1.3 describes the steps of the Ross assay. To retrieve rumen fluid, a pump, constructed using a silicone hose with a 10 mm diameter and attached rubber bulb inserted in a 1m long plastic probe with drilled holes and rounded edges, is used. The pump is warmed with hot water, with the bulb filled, up until collection. To start the collection, a handful of rumen contents are placed in the bottom of a warmed 2 L thermos. The plastic probe is then inserted into the ventral sac of the rumen. With the bulb compressed, the hose is inserted into the probe; the bulb is then released to draw up rumen fluid, and then transferred to the thermos. The process is repeated until approximately 2 L of rumen fluid is collected. Another handful of rumen contents is added to the thermos prior to closing to reduce airspace, prevent sloshing, and provide material to aid in filtering. Prior to use, rumen contents are filtered through 4 layers of cheesecloth, glass wool, and a nylon screen in a Büchner funnel. Filtered fluid is collected in a 4 L Erlenmeyer flask incubated in a 39 ºC water bath with constant infusion of CO_2.

Four 125 mL Erlenmeyer flasks are then filled with 0.5 g of sample along with 40 mL of Van Soest rumen buffer and 10 mL of rumen fluid. Flasks are incubated in a water bath at 39 °C for 16 h under continuous CO_2 to maintain anaerobic conditions. Following incubation, 2 flasks are set aside to be used to determine rumen degradation. Flask contents are filtered using 2-piece glass filter holders (90 mm) and manifold through 1.5 µM Whatman 934-AH glass filters with boiling water. Filters are then dried at 105 ºC for 24 h in a drying oven.

The remaining 2 flasks are acidified with 2 mL 3M HCl to reduce the pH to 2 and allowed to shake in a shaking water bath at 39 ºC for 1 min. Flasks are then incubated for another 1 h after the addition of 2 mL of pepsin and 0.013 M HCl. Following incubation, the pepsin reaction was neutralized with the addition of 2 mL 2M NaOH. A combination of 10 mL 1.8 M KH_2PO_4 and an enzyme mix (168, 140, 705, 28 units per mL KH_2PO_4 of trypsin, chymotrypsin, amylase, and lipase, respectively) are added to the flask and incubated for 24 h in a 39 °C shaking bath. After this incubation, samples are filtered through the same system as the rumen-incubated samples and dried for 24 h at 105º C (Ross et al., 2013).

Attempting to replicate the environment created by the dairy cow's digestive tract is challenging. The lack of commercial availability of some enzymes and the room for error in mixing concentrations and pH only add to the challenge. In a production setting, diets contain buffering agents as well as balanced to help control rumen pH and promote rumination so saliva, which has buffering capicity, is produced. Diets are also balanced for various macro- and microminerals along with AA to meet rumen microbe needs. During fermentation in the Ross assay, Van Soest Buffer, which is comprised of a macromineral and micromineral solution as well as tryptone and resazurin, is included to support an environment ideal for microbes. The first component, the macromineral solution, includes sodium phosphate dibasic, potassium phosphate dibasic, and magnesium sulfate heptahydrate – also known as epsom salt – dissolved in distilled water. The micromineral solution includes calcium chloride dihydrate, manganese chloride tetrahydrate, cobalt chloride hexahydrate, and ferric chloride hexahydrate also dissolved in distilled water (Goering and Van Soest, 1970). Tryptone, which is an array

of peptides resulting from the digestion casein by trypsin, is included to provide a source of amino acids and fosters an environment that is proactive for microbes (Fraser and Powell, 1950). Lastly, resazurin is included to minimize the amount of oxygen present so that an anaerobic environment is maintained since it has a high redox potential (Ross, 2013).

To simulate hind-gut digestion, pancreatin remains an option, but a combination of amylase, lipase, trypsin, and chymotrypsin are offered as alternatives. Ruminants typically produce high concentrations of amylase and lipase in their saliva to aide in the digestion of starch and fat, but since the feedstuffs tested aren't exposed to the saliva, amylase and lipase are included in the enzyme mix. Trypsin and chymotrypsin, which are proteases, are included to further degrade protein. In the animal, the zymogen for trypsin, trypsinogen, is secreted by the pancrease after it is stimulated by cholecystokinin. The enzyme enteropeptidase or active trypsin activates trypsinogen by cleaving the peptide bond between the 15^{th} residue and lysine which leads to a reconfiguration of the structure. Chymotrypsinogen is the zymogen of chymotrypsin and is activated by trypsin (Meisfield and McEvoy, 2017).

Lipids

Omega 3 fatty acids. Chemically, omega 3 fatty acids (**FA**) are polyunsaturated fatty acids (**PUFA**) with a double bond at the third carbon from the methyl end of the linear chain of carbons that comprise a fatty acid structure. The three main omega 3 FAs are alpha-linolenic acid (**ALA**, 18:3*n*-3), eicosapentaenoic acid (**EPA**), and docosahexaenoic acid (**DHA**) (CAST, 2018; Figure 1.4). Alpha linolenic acid is found in plant foods including walnuts, canola, and flaxseed as well as some legumes and leafy,

green vegetables. Alpha-linolenic acid is the precursor to EPA and DHA (Stark et al., 2008). Figure 1.5 illustrates the steps in the process of converting ALA to EPA and DHA. Through a series of chain elongations and desaturation processes that take place in the endoplasmic reticulum, ALA is first converted to EPA before it is elongated and desaturated further as well as undergo a round of β-oxidation in the peroxisomes to form DHA (Barcelo-Coblijn and Murphy, 2009; Palmquist, 2009). Outside of being synthesized in the body, DHA and EPA are found in seafood, especially fatty fish, as well marine algae, which synthesize EPA and DHA and pass it up the food chain (CAST, 2018). Alpha linolenic acid is considered an essential FA, and since mammals are unable to synthesize omega 3 FA *de novo*, they must be supplied in the diet (Barcelo-Coblijn and Murphy, 2009).

Omega 3 FA serve a variety of purposes in the human body by playing an important role in reducing the risk of cardiovascular disease by lowering blood pressure, inflammation, and blood triacylglycerol concentrations (Calder, 2004). This anti-inflamatory effect is achieved by the conversion of omega 3 fatty acids to eicosanoids and docosanoids, oxygenated metabolites, that act both at the site of synthesis as well as systematically (Palmquist, 2009; CAST, 2018). Studies completed with over 80,000 participants have shown that an increase in plasma phospholipid omega 3 FA levels is associated with a decrease in coronary heart disease (Stark et al., 2008). Both DHA and EPA play a role in brain function by aiding in cell growth, neural signaling, and gene expression (Milte et al., 2012) In infants, DHA is essential for visual, neural, and behavioral development (CAST, 2018). In children diagnosed with attention-deficit/hyperactivity disorder (**ADHD**), supplementation of DHA improved literacy while

lessening the symptoms of ADHD (Milte et al., 2012). In addition to cardiovascular and neural benefits, in some instances, DHA and EPA can slow cancerous tumor growth (Palmquist, 2009; CAST, 2018).

In dairy cattle, supplementary omega 3 FA have shown to have positive effects on fertility (Gulliver et al., 2012). Since omega 3 FA are precursors to eicosanoids, that means they are also a precursor to prostaglandins (**PG**), which play a role in ovulation, embryo survival, and parturition (Gulliver et al., 2012). Specifically, EPA is converted to 3-series PG, such as $PGF_{3\alpha}$ (Petit et al. 2002), which are known to be less inflammatory than 2-series PG, such has $PFG_{2\alpha}$ (Gulliver et al., 2012). A reduction in $PFG_{2\alpha}$ levels can improve fertility by reducing luteolysis (Thatcher et al., 1995). In a study where cows were fed different sources of omega 3 FA, cows fed higher levels of omega 3 fatty acids had lower concentrations of PGFM (13, 14-dihydro-15-keto $PFG_{2\alpha}$), which is the inactivated metabolite of $PFG_{2\alpha}$. This in turn lead to larger corpora lutea (CL), which has potential to improve conception rates (Petit et al., 2002).

Omega 6 fatty acids. Like omega 3 FA, omega 6 FA are also considered as PUFA but are characterized by having at least two double bonds with the first being on the sixth carbon from the methyl terminus (Harris et al., 2009). The most notable omega 6 FA is linoleic acid (**LA**; Figure 1.6), which is primarily found in vegetable oils such as corn, sunflower, and soy (CAST, 2018). Just like ALA, LA cannot be synthesized by mammals, so it must be supplied through the diet. Linoleic acid is the precursor to arachidonic acid (**ArA**, Figure 1.6), the substrate for various eicosanoids. The process of the conversion of LA to ArA is also shown in Figure 1.5. After consumption, LA is desaturated and elongated to form dihomo-γ-linolenic acid, which is then converted to

ArA (Harris et al., 2009). Arachidonic acid has several fates. It can be converted to PGE_2, which has been associated with carcinogenesis as well as promoting growth of cancerous cells (Aronson et al., 2001). Also like omega 3 FA, ArA can be converted into PG, however, instead of being of 3-series like those synthesized from EPA and DHA, ArA-originated PG, such as $PFG_{2\alpha,}$ are 2-series, which are inflammatory (Gulliver et al., 2012). While an inflammatory response is essential to survive, chronic inflammation leads to tissue damage and can cause chronic diseases such as arthritis, diabetes, and chronic heart disease (**CHD**) (Kapoor and Huang, 2006).

Dietary omega 3 and omega 6 fatty acid imbalance. As essential as omega 3 fatty acids are, many individuals who consume Western diets do not consume their daily requirements (Milte et al., 2012). Historically, this lack of consumption hasn't always been the case, but as humans have evolved, the type and amount of EFA in the diet have changed (Simopoulos, 2009). In a typical, modern Western diet, individuals consume approximately 0.15 to 0.25 g/d of omega 3 FA (Scorletti and Byrne, 2013). However, the American Heart Association recommends consuming 1.8 g of omega 3 fatty acids per day for healthy individuals (Krauss et al., 2000). Furthermore, for those who have cardiovascular illness, 2 to 4 g/d is recommended. Omega 6 FA, while potentially harmful in excess, humans still have a minimum requirement of 1 to 4 g/d. It is recommended, however, that consuming 12 g/d for women and 17 g/d for men is optimal (Harris et al., 2009). The intake of LA in a typical Western diet is more than 85% of the total fatty acids consumed (Aronson et al., 2001), and in a meta-analysis of 25 studies that evaluated omega-6 FA consumption, 25% of participants consumed 12% or more above their energy needs. To be clear, consuming omega 6 FA above one's needs is not

harmful, but when high consumption of omega 6 FA raises the ratio of omega 3 to omega 6 FA, there is potential for tissue damage, which can lead to CHD (Harris et al., 2009).

Within mammalian bodies, omega 3 FA are unable to be converted to omega 6 FA and vice versa, which makes them distinct both metabolically and functionally. This often leads to opposing physiological functions. The two EFA compete for the same desaturation enzymes, which are needed to convert them to their beneficial intermediates. Fortunately, two of the enzymes prefer omega 3 FA over omega 6 FA, but high LA levels interfere with the enzymes' action with ALA. Although ArA-originated eicosanoids are only active in small quantities, large amounts can lead to inflammation, which can develop allergic and inflammatory disorders and even cell proliferation (Simopoulous, 2009). By decreasing the EFA ratio, more omega 3 FA are available to outcompete omega 6 FA, thus there is less conversion of omega 6 FA to metabolites (Palmquist, 2009). The ideal ratio of omega 3 FA to omega 6 FA is 1:1. However, the current Western diet usually results in a ratio of 15:1 to 20:1 (Simopoulos, 2007). It should be noted that the focus should not be placed on the ratio of omega 3 to omega 6 FA, but rather increasing omega 3 FA consumption. The ratio concept is just an easier and more applicable method for individuals to apply to their own diets (Palmquist, 2009).

To help improve dietary consumption of omega 3 fatty acids, selective breeding and manufacturing procedures may be used as well as selective feeding of high fatty acid feedstuffs (CAST, 2018). In Western diets, milk and milk products are an important food group and provide several beneficial nutrients, such as protein, B vitamins, and Ca. In studies comparing the prevalence of CHD in Iceland compared to other Nordic countries, which are all known for high milk consumption, CHD, as well as type 2 diabetes, where

less common in Iceland. The reason for this occurrence was attributed to the higher omega 3 FA content in milk from Iceland because of the use of fish meal fed to cows (Thorsdottir et al., 2004). Several studies have enriched milk with EFA, and it was reported that participants who consumed the enriched milk had greater circulating concentrations of DHA and EPA. Participants who consumed enriched milk also had lower concentrations of low-density lipoprotein cholesterol, which is associated with CHD (Lopez-Huertas, 2009).

Several studies have abomasally infused or included various feedstuffs with high omega 3 FA contents with the intention of altering milk's fatty acid profile, and these have reported mixed results. In one study where soybean oil enhanced with stearidonic acid, an intermediate in the conversion of ALA to EPA, was ruminally and abomasally infused, omega 3 FA concentration in milk only increased through the abomasal infusion as was observed to be 3.9% of total FA, which was 500% greater than the control treatment. This response can be explained by avoiding PUFA being biohydrogenated in the rumen (Bernal-Santos et al., 2010). Petite et al. (2002) fed different sources of omega 3 FA and once again observed that abomasal infusion, this time with linseed oil, resulted in the highest omega 3 FA concentration with 13.9% of total FA being ALA instead of 1.0% with the control. Both studies demonstrated that milk omega 3 FA concentration can be increased if rumen biohydrogenation can be avoided or reduced. Also in the 2002 study by Petit et al., formaldehyde-treated linseed was fed but did not lead to any difference in the milk FA profile, showing that it is difficult to manipulate the diet to avoid rumen biohydrogenation. In another study, a protected form of flaxseed (the mode of protection was not described) was fed and yielded 6.4% ALA of total FA as compared

to 0.8% with the control (Goodridge et al., 2001). Petit et al. (2001) fed formaldehyde-treated flaxseed as well as untreated flaxseed, and unlike what was observed with treated linseed, an increase in milk omega 3 FA was observed, which could mean it was the flaxseed rather than the treatment that aided in avoiding rumen biohydrogenation. To evaluate this concept, da Silva et al. (2007) compared whole and ground flaxseed in rations and observed that ground flaxseed, while supplying 6.86% less ALA, resulted in 59.1% more ALA in the milk than whole flaxseed. This collection of studies show that in order to avoid the effects of rumen biohydrogenation, selection of effective feedstuffs, treatment methods, or even a combination of both is needed in order to achieve the goal of increasing omega 3 FA in milk.

Fatty acid profile of milk. Cow's milk fat is comprised of around 400 to 500 different fatty acids (Markiewicz-Keszycka et al., 2013). Of the fatty acids that comprise milk fat, approximately 66% are saturated fatty acids (**SFA**), 30% are monosaturated fatty acids (**MUFA**), and only 4.0% are PUFA (Baer, 1990). Oleic acid has the highest content of MUFA, which is typical of all mammals; but, cow's milk is the richest source with oleic acid comprising 24% of MUFA. The majority of SFA in cows milk is palmitic acid. With respect to PUFA, omega 6 FA comprise 2.83% of total fatty acids with LA being the most abundant (2.57% of total FA). Omega 3 FA only make up 0.56% of total FA (Markiewicz-Keszvcka et al., 2013); ALA is the major omega 3 FA as it comprises 0.38% of total FA (O'Donnel-Megaro et al., 2011). Half of the medium-chain (12 to 17 carbons) and all of the short-chain (4 to 10 carbons) FA detected in milk are derived from acetate and B-hydroxybutyrate by epithelial cells in the mammary gland. The remaining medium-chain and almost all of the long-chain FA (18 or more carbons) are synthesized

from diet-originating FA, whether that be circulating FA in the blood or mobilized body fat stores (Baer, 1990).

Digestion. Shortly after being ingested, lipids, in the form of esterified FA and triglycerides, go through the process of lipolysis where they are rapidly hydrolyzed by microbial lipases. Lipases hydrolyze the ester linkages in glycerol-based lipids, resulting in free fatty acids (**FFA**) and glycerol (Jenkins and Harvatine, 2014). Glycerol is further converted into volatile fatty acids (**VFA**), mainly propionate and butyrate (Tamminga and Doreau, 1991). The process of hydrolysis takes place extracellularly within the rumen (Noble, 1981), and lipases from plants, protozoa, fungi, and saliva contribute to hydrolysis of dietary lipids very little (Lock et al., 2006). The extent of hydrolysis is greater than 85% (NRC, 2001; Lock et al., 2006), but it can be limited by factors that limit microbial growth and activity, such as low rumen pH and ionophores, as well as high dietary fat levels (Lock et al., 2006).

Unsaturated FA are then hydrogenated by rumen microbes to saturated end products. The bacteria involved can be split into two groups based on their metabolic pathways (Lock et al., 2006). The first group involved in the process of hydrogenation isomerizes the *cis*-12 double bond in unsaturated FA to a *trans*-11 isomer to create conjugated linoleic acid (**CLA**) (Jenkins, 1993; Lock et al., 2006; Jenkins and Harvatine, 2014); this step cannot take place unless the FA has a free carboxyl group. Next, stearic acid is formed when the *cis*-9 bond is hydrogenated to oleic acid followed by hydrogenation of the *trans*-11 bond to form stearic acid (Jenkins, 1993), which is done by the second group of bacteria that consists of very few species of bacteria (Lock et al., 2006). This last step in hydrogenation is dependent upon rumen conditions (Jenkins,

1993; Lock et al., 2006). Complete hydrogenation is inhibited by the presence of large amounts of LA but is promoted by the presence of feed particles and cell-free rumen fluid (Noble, 1981; Jenkins, 1993). Unsaturated FA are toxic to rumen microbes, so this step serves as a way to protect themselves from possible harm (Jenkins, 1993). The process of hydrogenation results in stearic acid and various isomers of oleic acid, which are the major FA that leave the rumen since little degradation of long-chain FA occurs in the rumen (NRC, 2001). Short-chain fatty acids (**SCFA**) are produced as result of rumen fermentation processes and vary in concentration and proportion based on the diet fed. A majority of the SCFA are acetic, propionic, and butyric acids, which make up 60 to 70, 15 to 20, and 10 to 15%, respectively, of total SCFA. There is also some branched-chain isomers of both butyric and valeric acid present but in small concentrations (Noble, 1981).

There is little to no absorption or modification of long- and medium-chain fatty acids in the abomasum and omasum, so when lipids enter the small intestine, 80 to 90% of it is in the form of non-esterified SFA absorbed on feed particles and the remaining is associated with microbial cells (Tamminga and Doreau, 1991; Bauman and Lock, 2002). After the lipids enter the small intestine, biliary lipids are also added to the mix (Tamminga and Doreau, 1991). Free fatty acids are then solubilized into a micellar solution in order to be absorbed. Both bile and pancreatic secretions are added to the digesta in the duodenum. Bile provides bile salts and lecithin, and pancreatic juice provides the enzymes needed to convert lecithin to lysolecithin as well as bicarbonate to raise pH. The combination of lysolecithin and bile salts desorb the FA from feed particles

and bacteria, which allow for micelle formation so FA can be absorbed (Bauman and Lock, 2002).

Milk fat depression. The occurrence of milk fat depression (**MFD**) has been investigated for years and several different theories for its causation have been developed. The earliest theory was that limited absorption of fatty acids was the cause, which was disproved since MFD can still occur even with high fat diets (Jenkins and Harvatine, 2014). More recently, focus has fallen on VFA and their proportions. One theory looks at the ratio of propionate to acetate. The theory proposed that with low acetate supply, milk fat synthesis is limited, but it was disproven when ruminal infusion of acetate was used during MFD and milk fat content recovered only slightly (Shingfield and Griinari, 2007; Jenkins and Harvatine, 2014). Another theory proposed that increase absorption of propionate lead to higher plasma glucose, which would stimulate insulin secretion. This combination of events would in turn increase lipogenesis instead of lipolysis. This theory was also disproven when insulin was infused and milk fat only decreased slightly (Jenkins and Harvatine, 2014).

A more recent theory, known as the biohydrogenation theory, involves CLA. As previously stated, CLA is produced during the process of biohydrogenation when lipids are digested. Various CLA isomers are produced, and 3 of them are known to cause MFD, the most notable and explored being *trans*-10, *cis*-12 CLA (Baumgard et al., 2002, Peterson et al., 2003; Shingfield and Griinari, 2007). After being producing in the rumen, *trans*-10, *cis*-12 CLA travels in the blood to the mammary gland (Jenkins and Harvatine, 2014). Once in the mammary gland, the CLA isomer reduces lipogenic capacity by lowering the rate at which acetate is incorporated into FA. In conjunction, the isomer also

decreases the expression of the genes responsible for encoding enzymes involved in the uptake, transport, synthesis, and desaturation of FA (Baumgard et al., 2002).

Changes in rumen environment can have an effect on the amount and type of CLA produced. When rumen pH drops, an alteration in the microbial population occurs, which changes the type of CLA produced. In a continuous culture of mixed rumen microbes, the concentration of *trans*-10, *cis*-12 CLA increased with a decrease in rumen pH (Fuentes et al., 2009). This effect can be compounded because a lower pH is also unfavorable to cellulolytic bacteria, who are responsible for acetate production. This reduces the acetate to propionate ratio, which makes acetate less available for milk fat synthesis (Jenkins and Harvatine, 2014).

Energy Utilization

Energy balance. Any energy consumed by an animal has the potential to contribute to the various functions of the body, such as growth, gestation, and milk production. However, not all feedstuffs have the same value of energy, both in amount and availability. Additionally, different bodily functions have different energy use efficiencies. Thus, a system is needed to attribute value to feedstuffs. The net energy (**NE**) system is based off the first law of thermodynamics, which states that energy cannot be created or destroyed (Weiss, 2007). The total amount of energy an animal consumes is defined as gross energy intake (**GEI**). This value is determined by taking the total feed intake and multiplying it by its gross energy (**GE**), as determined through combustion in a bomb calorimeter (Eq. 2). Not all of GE is able to be digested and utilized, so some of it is excreted. Gross energy less the energy lost in feces is defined as digestible energy (**DE**; Eq. 3). The next step in the cascade is metabolizable energy

(**ME**), which is the digestible energy with the energy of urine and gases removed (Eq. 4). Lastly, the net energy of lactation (**NEL**) is determined by taking ME minus heat increment (Eq. 5).Heat increment (**HI**) is the heat generated by the inefficiency of energy transforming from one form to another, and it is not the same as total heat production (**HP**), which can be determined using calorimetry (Weiss, 2007).

$$\text{GE (Mcal/d)} = \text{feed intake} \times \text{feed GE} \quad [2]$$

$$\text{DE (Mcal/d)} = \text{GEI} - \text{fecal energy} \quad [3]$$

$$\text{ME (Mcal/d)} = \text{DE} - (\text{urinary energy} + \text{gaseous energy}) \quad [4]$$

$$\text{NEL (Mcal/d)} = \text{ME} - \text{HI} \quad [5]$$

Calorimetry

Indirect calorimetry. Through the use of indirect calorimetry, heat production can be estimated. Indirect calorimetry is the measurement of energy exchange that takes place within the animal's living tissue and estimates HP by measuring oxygen consumption along with CO_2, CH_4, and urea production (Foth et al., 2015). The values gathered during collection are then used with the Brouwer equation to determine HP (Eq. 6).

$$HP = 3.866 \times O2 + 1.2500 \times CO2 - 0.518 \times CH4 - 1.431 \times N \quad [6]$$

There are two different types of indirect calorimetry systems: closed- and open-circuit. Closed-circuit systems are not typically used with large animals and ruminants because of the cost and the need to remove CH_4 from the system (Blaxter, 1989). Instead, open-circuit systems are used where samples of air entering and leaving the system are taken to determine the concentration of gases consumed and produced (Reynolds and Tyrrell, 2000). One style of open-circuit indirect calorimetry uses headboxes. Headboxes are less expensive to construct than whole-animal chambers since they only involve

placing the head inside instead of the whole body (Johnson and Johnson, 1995). This style also allows for animals to move freely, and with dairy cattle, cows can still be milked while in the headboxes.

SUMMARY

Proteins are large molecules that vary in structure, which influences its degradability. Within the rumen, any protein that is easily and rapidly solubilized along with protein that is digested and utilized by microbes is known as RDP. The microbes use any free peptides, AA, and NH_3 to synthesize MCP. Along with RDP, MCP contributes to MP, which passes onto the small intestine to benefit the animal. Protein that escapes rumen degradation and is passed onto the small intestine to be digested is known as RUP. Both RUP and MCP are further digested to supply AA to the animal. Any protein that is associated with lignin, tannins, or is heat-damaged is considered unavailable and doesn't contribute any AA to the animal. The availability of protein can vary among sources because of processing methods. In the rendering industry, the rendering process can alter protein availability based on the cooking process, which can vary in temperature, time, and drying method. Hydrolysis, while used to improve digestibility, can alter the availability of AA. These differences in protein quality have an impact on milk production. If there isn't an adequate supply of RDP, MCP synthesis decreases and milk yield decreases. However, RUP still needs to be supplied to ensure a balanced supply of EAA for milk production.

To determine the amount and digestibility of RUP, there are several assays that range in the degree in which they are performed *in situ*. The mobile bag assay is completed almost entirely *in situ* and involves placings samples in porous, N-free bag. To

minimize the cost and labor involved in maintaining ruminally and duondenally cannulated cattle, the modified three-step assay was developed. Rumen incubation still takes place *in situ*, but intestinal digestibility is determined by *in vitro* incubation in a pancreatic solution. Samples are still placed in porous bags, which has been criticized to limit microbial access to feedstuffs and inaccurately estimate RDP through loss of highly soluble particles from the bag that may not be digested. The Ross assay, which is completed entirely *in vitro*, was developed without the use of bags to eliminate those problems.

When it comes to human nutrition, omega 3 FA play an important role in cardiovascular health as well as neural development. Omega 6 FA also are important in human health, especially with inflammatory responses to infections, but in excess, can cause lead to tissue damage and chronic diseases such as arthritis. In a typical Western diet, omega 6 FA are overconsumed while omega 3 FA are under consumed. To improve dietary consumption, the fatty acid profile of milk can be altered through selective feeding of feedstuffs with a high omega 3 FA content. The high-fatty acid supplement, Perfect Omega 3, was developed with that intent in mind. The product is comprised of sesame meal, giant kelp, cassava, and sorghum, which are feedstuffs that aren't commonly used across the United States, with the exception of sorghum. Lipids go through the processes of hydrolysis and hydrogenation in the rumen to yield free FA. However, when feeding fats, MFD can be a concern. During hydrogenation, CLA is created as an intermediate. An isomer of CLA, *trans*-10, *cis*-12 CLA, has been known to cause MFD by inhibiting milk fat synthesis in the mammary gland.

The NE system is based off of the first law of thermodynamics as well as commonly used to describe how energy is partitioned within a cow. Gross energy, DE, ME, and NEL are determined by subtracting various losses of energy, including fecal, urinary, and gaseous energy as well has HI. Heat production can be determined using the Brouwer equation, which needs values obtained through indirect calorimetry. In indirect calorimetry, O_2 consumed as well as CO_2, CH_4, and urea produced are quantified. There are two types of indirect calorimetry systems: closed- and open-circuit, and open-circuit is typically used with ruminants because of CH_4 production. Headbox-style calorimeters are ideal for use with dairy cattle as they allow for the cow to still be milked as well as freedom of movement.

Research Objectives

The objectives of this research were to:

1) Characterize the protein quality of feather meal with and without added blood as well as compare three assays used to determine protein quality: the mobile bag, modified three-step, and Ross assays.
2) Determine the effect of the high fatty acid content feedstuff, Perfect Omega 3, on the milk fatty acid profile as well as the energy utilization and digestibility of lactating dairy cows.

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FIGURES

Figure 1.1. Diagram of the steps of the Mobile Bag assay.

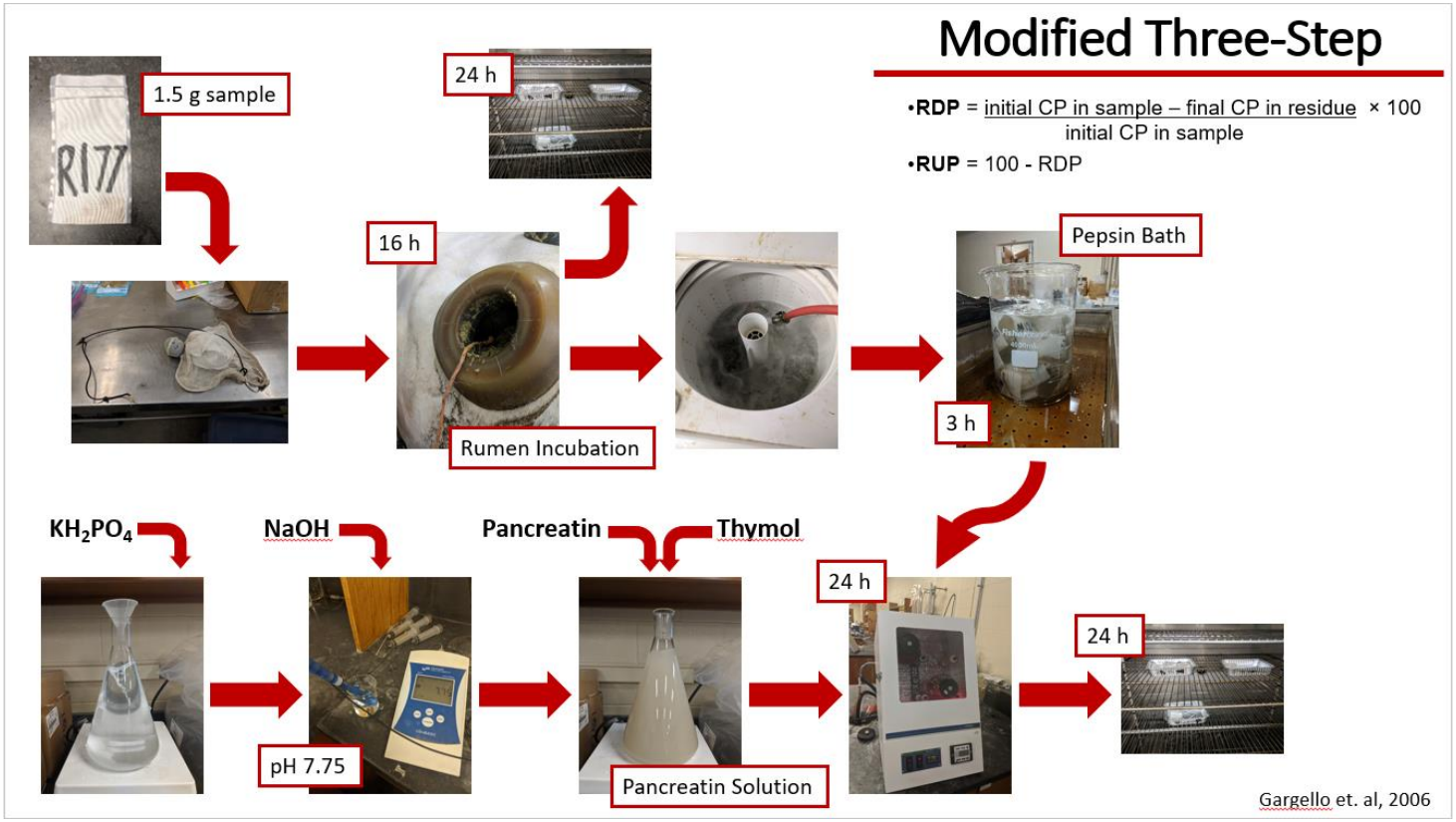

Figure 1.2. Diagram of the Modified Three-Step assay used to determine the intestinal digestibility of rumen undegraded protein.

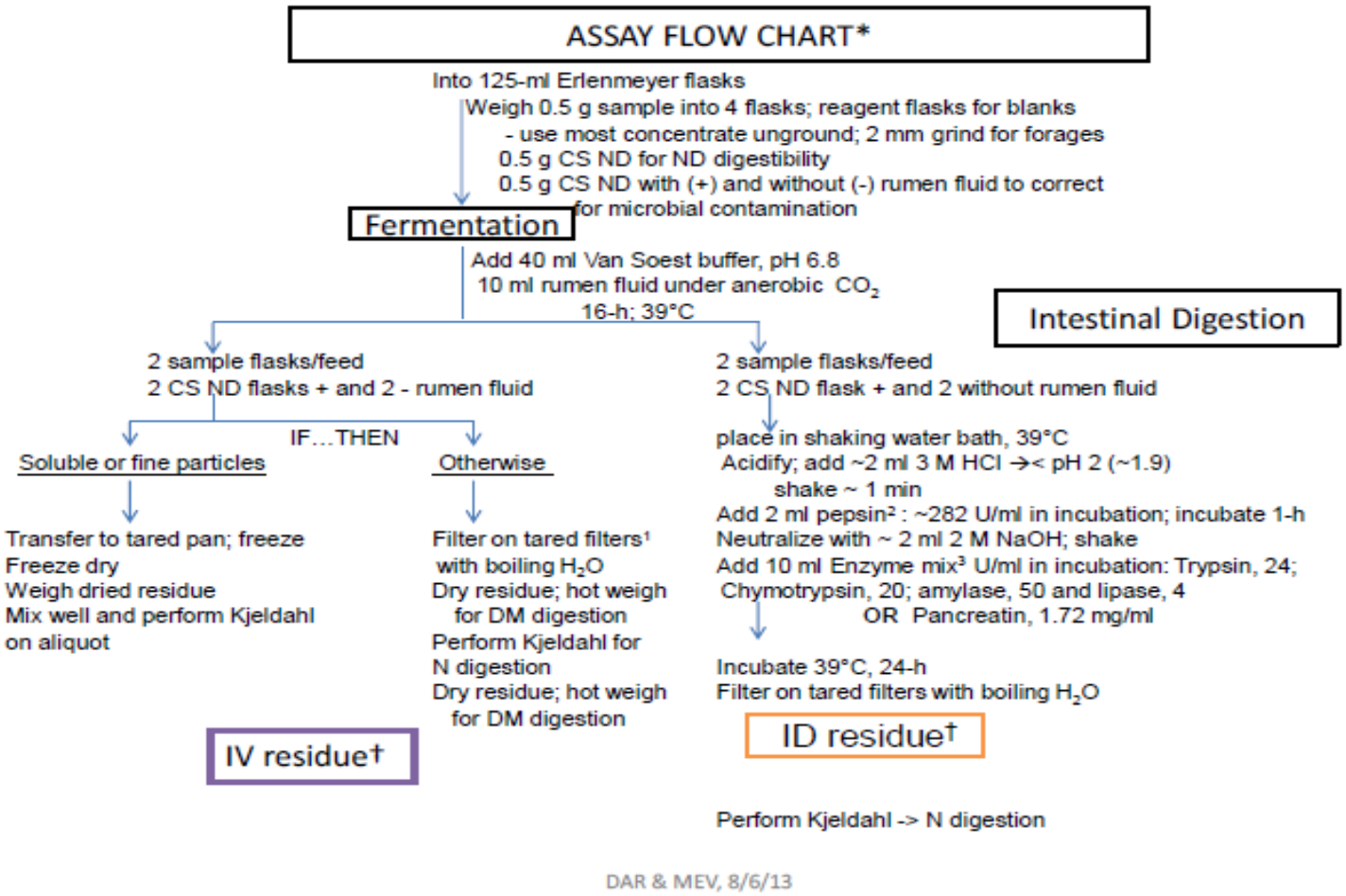

Figure 1.3. Flow chart of the Ross assay (Ross et al., 2013).

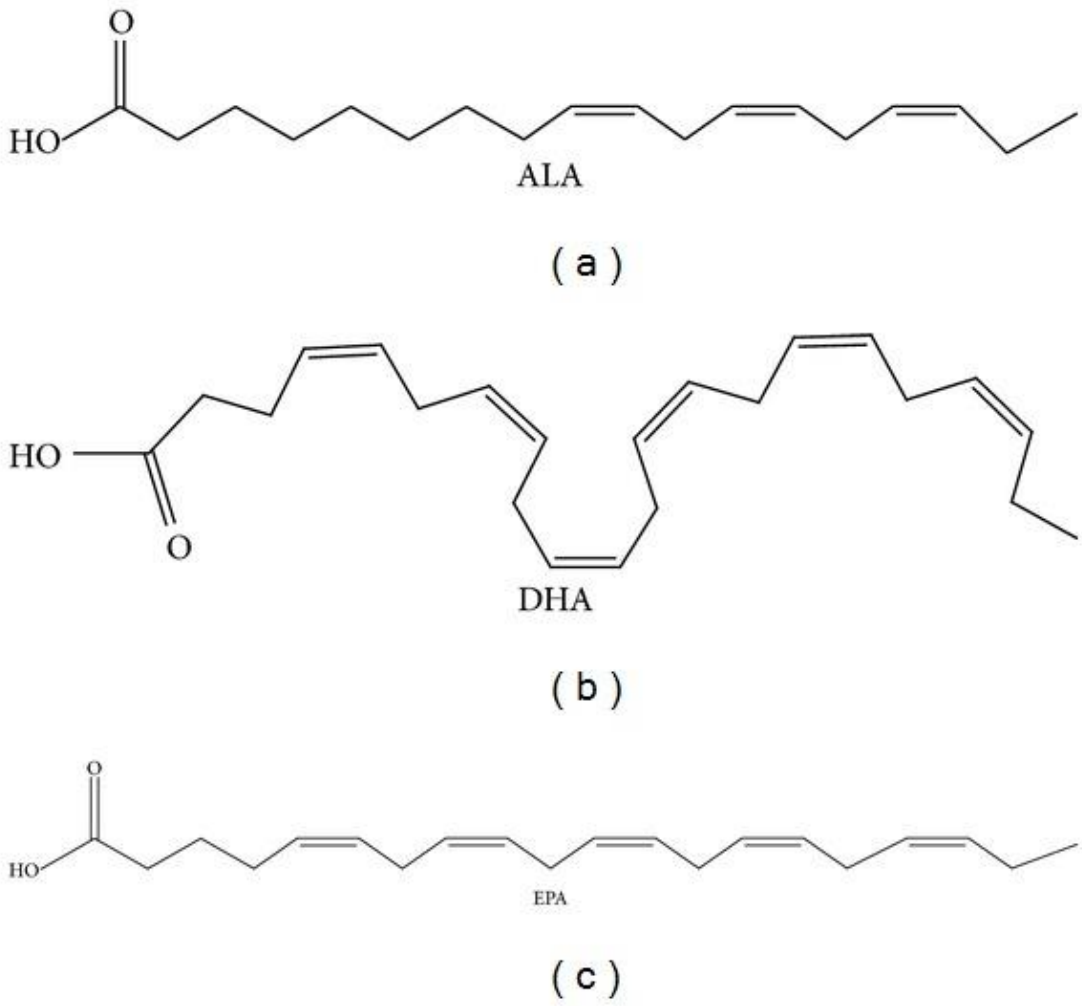

Figure 1.4. The structures of the omega 3 fatty acids α-linolenic acid (ALA; a), docosahexaenoic acid (DHA, b), and eicosapentaenoic acid (EPA, c) (D'Antona et al., 2014).

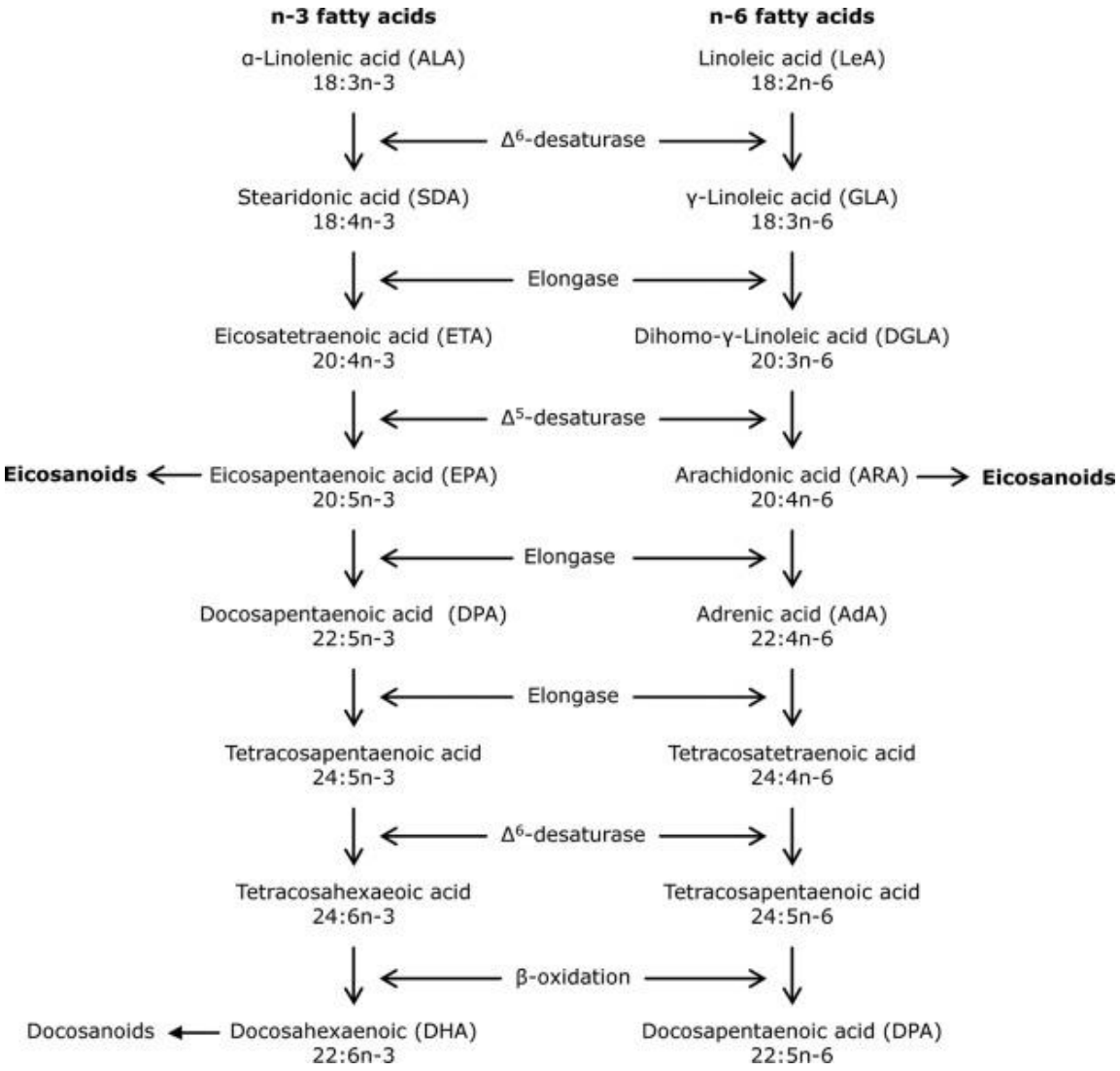

Figure 1.5. The primary steps in the process of converting a-linolenic acid to eicosapentaenoic acid and docosahexaenoic acid within the rumen as well as the major steps of the process of converting linoleic acid to arachidonic acid (van Valenberg et al., 2013).

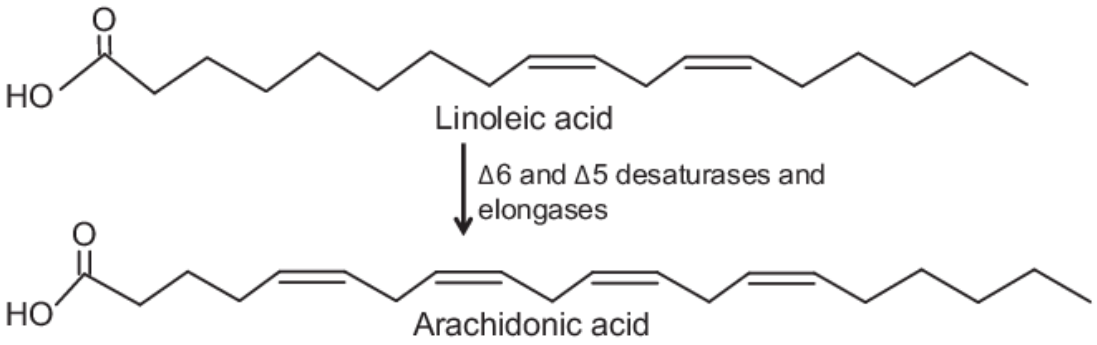

Figure 1.6. The structures of the omega 6 fatty acids linoleic acid and arachidonic acid (Elsherbiny et al., 2013).

APPENDIX A: EQUATIONS

$$EPD = a + b\,[c/(c + k)] \quad [1]$$

$$GE\ (Mcal/d) = \text{feed intake} \times \text{feed GE} \quad [2]$$

$$DE\ (Mcal/d) = GEI - \text{fecal energy} \quad [3]$$

$$ME\ (Mcal/d) = DE - (\text{urinary energy} + \text{gaseous energy}) \quad [4]$$

$$NEL\ (Mcal/d) = ME - HI \quad [5]$$

$$HP = 3.866 \times O_2 + 1.2500 \times CO_2 - 0.518 \times CH_4 - 1.431 \times N \quad [6]$$

CHAPTER 2

INTERPRETIVE SUMMARY. Buse et al. (2020). “Comparison of methods to determine ruminal degradation and intestinal digestibility of protein in hydrolyzed feather meal,” In this article, an experiment designed to evaluate assays used to determine protein digestibility of feedstuffs commonly used in dairy rations using hydrolyzed feather meal with and without added blood. Estimates of rumen digestibility of protein differed among assays but resulted in similar values for intestinal digestibility. A similar response was observed for inclusion of blood with rumen digestibility being greater for feather meal without blood but no difference observed in rumen undegraded protein digestibility. These results suggest that the Mobile Bag, Modified Three-Step, and Ross assays yield similar results in terms of rumen undegraded protein digestibility, and inclusion of blood does not alter the digestibility of hydrolyzed feather meal.

RUNNING HEAD: PROTEIN DIGESTIBILITY ASSAYS

Comparison of methods to determine ruminal degradation and intestinal digestibility of protein in hydrolyzed feather meal

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ABSTRACT

Hydrolyzed feather meal (**HFM**) is a readily available, high bypass-protein feedstuff that can be used as a cost-effective dairy feedstuff. Because the production process may vary, the chemical composition of HFM may also vary. Additionally, some processes may incorporate blood into the final product. To determine the chemical composition of these products, several lab assays can be used with the most common being the Mobile Bag (**MOB**), Modified Three Step (**MTS**), and Ross (**ROS**) assays. While all three assays determine the rumen undegraded protein (**RUP**) digestibility, they vary in the degree in which incubations are done in situ. The objective of this study was to evaluate the ruminal and intestinal digestibility of HFM originating from processes that differ in their inclusion of blood as well as compare the MOB, MTS, and ROS assays. Ten samples of HFM, 5 without blood (**FM**) and 5 with blood (**FMB**), were collected from ten different production plants across the United States and subjected to all three assays. Assay had a significant effect ($P < 0.001$) on rumen dry matter (**DM**) digestibility, rumen degraded protein (**RDP**), rumen undegraded protein (**RUP**), total tract DM digestibility, and total tract crude protein (**CP**) digestibility. A significant effect ($P = 0.007$) was observed in rumen DM digestibility, RDP, and RUP for blood inclusion; no effect was detected for total tract DM digestibility ($P = 0.348$) and total tract CP digestibility ($P = 0.531$). There was no difference in dRUP for both assay ($P = 0.697$) and blood inclusion ($P = 0.859$). There was also no blood inclusion and assay interaction ($P > 0.947$). Results suggest that even though there are differences in chemical composition in HFM associated with the inclusion of blood, little to no differences are observed in ruminal or intestinal digestion of protein. While the assays varied in their estimates of

protein quality and digestibility, MOB and MTS were the similar, and all three assays resulted in a similar dRUP.

Key Words: intestinal digestibility, rumen undegraded protein, assay comparison

INTRODUCTION

Feeding by-products to ruminants has been practiced for centuries (Grasser et al., 1994). The practice is even more valuable in the present day with fluctuating prices for fluid milk and components. Not only are byproducts typically a cost effective source of nutrients (Bradford and Mullins, 2012), they also contribute toward a sustainable industry by using nutrients that would otherwise be disposed of by placing in a landfill (Iriondo-DeHond, et al. 2018). Hydrolyzed feather meal (**HFM**) is a byproduct of the rendering industry and has a high CP content (~85%) of which ~65% is RUP (NRC, 2001). However, very few studies have been completed to evaluate the effect of HFM on dairy cattle production (Harris Jr. et al., 1992; Moss et al., 1995; Grant and Haddad, 1998; Morris et al., 2020b). Several of these studies observed that increasing inclusion of HFM in rations led to a decrease in milk protein yield and concentration. Bloodmeal is another byproduct of the rendering industry that has a similar CP content of 95% and RUP content of 71% to feather meal (NRC, 2001). When fed to dairy cattle, bloodmeal has elicited a response in milk protein concentration and yield similar to ground soybean meal (Pires et al., 1996). Feeding a combination of feather meal and bloodmeal in beef cattle has resulted in greater growth than feeding feather meal alone (Goedeken et al., 1990; Blasi et al., 1991), and in dairy cattle, it has yielded mixed results (Grant and Haddad, 1997; Moss et al., 2019).

The challenge with feeding byproducts is that chemical composition varies among and within byproducts (Ertl et al., 2014) and this leads to challenges in ration formulation. The variation in chemical composition of byproducts often requires regular testing for changes in chemical composition as well as the input of accurate and current values into ration software instead of relying on values given in feed libraries. To estimate protein digestibility, Hveplund (1985) developed the Mobile Bag assay (**MOB**), which was almost entirely *in situ*. To minimize labor and cost, Calsamiglia and Stern (1995) developed a three step procedure (**TSP**) that still included *in situ* rumen incubation but determined intestinal digestibility *in vitro* with a pancreatin solution in centrifuge tubes; the assay was later modified, now known as the Modified Three-Step assay (**MTS**), by Gargallo et al. (2006) to employ the use of a Daisy^{II} incubator (Ankom Technologies, Macedon, NY). A large concern of both the MOB and MTS assays is that the use of nylon bags creates a barrier to microbes, which results in a longer fermentation lag time (Ross, 2013). To address this issue as well as more closely mimic intestinal digestion, Ross et al. (2013) developed an in vitro assay performed in Erlenmeyer flasks with a solution comprised of trypsin, chymotrypsin, amylase, and lipase.

Liebe et al. (2018) compared RUP digestibility (**dRUP**) values obtained through the MOB and MTS assays and observed MOB predicted dRUP 6.2 percentage points greater than MTS. Ross (2013) compared MTS to ROS and observed that rumen N digestibility was 18 percentage points greater in the MTS but that total N digestibility was similar. No research has been conducted to date to compare all three assays. For these reasons, the objectives of this study were to determine protein digestibility as well as the protein quality, the fraction of protein that escapes rumen degradation and its

digestibility, of HFM containing blood while comparing three assays used to estimate these digestibilities. We hypothesized that HFM with blood would have a greater intestinal digestibility than HFM without blood and the MOB and MTS assays would result in similar values with the ROS assay being different.

MATERIALS AND METHODS

Feedstuffs

Feedstuffs evaluated in this experiment included hydrolyzed feather meal without blood (**FM**, Figure 2.1; American Proteins Inc., Cumming, GA; Pilgrim's Pride Corporation, Mt. Pleasant, TX; Pilgrim's, Greeley, Colorado; River Valley Animal Foods, Robards, KY; Simmons Foods, Siloam Springs, AR) and hydrolyzed feather meal with blood (**FMB**, Figure 2.2; Darling Ingredients Inc., Irving, TX; Mountaire Farms, Millsboro, DE; Pet Solutions, Danville, AR; River Valley Animal Foods, Sedalia, MO; Sanimax, Green Bay, WI). During the rendering process, blood can either be removed or allowed to remain with the product (Meeker and Hamilton, 2009). The companies self-identified the samples as containing blood but did not state the concentration of blood within the samples. Five samples of each type of feather meal were collected for a total of 10 samples. SoyPass was used as a standard for all methods. Feedstuffs were analyzed for DM (AOAC, 2000), N (Leco FP-528 N Combustion Analyzer; Leco Corp., St. Joseph, MI 49085), NDF (Van Soest et al., 1991), ADF (method 973.18; AOAC, 2000), sugar (DuBois et al., 1956), ether extract (2003.05; 2006), ash (942.05; AOAC, 2000), and minerals (985.01; AOAC, 2000) by Cumberland Valley Analytical Services Inc. (Hagerstown, MD). The remaining residues from each assay were also analyzed by Cumberland Valley Analytical Services Inc. for DM and N. There was not enough

residue available following lab analysis to determine the amino acid content of each sample.

Mobile Bag Assay

Prior to conducting the experiment all procedures using animals were approved by the University of Nebraska-Lincoln IACUC. Two multiparous Holstein cows (BW of 660 ± 33 kg) fitted with flexible ruminal and proximal duodenal cannulas and averaging 210 ± 17 DIM and 27.3 ± 8.00 kg of milk yield were used for in situ and mobile bag procedures. Cows were housed in tie stalls with continuous access to water and fed a diet listed in Table 2.1 once daily at 1000 h; cows used had an average DMI of 28.3 ± 2.92 kg/d. Ruminal degradations of CP were determined in situ and intestinal digestibilities were determined using the mobile bag technique. For each sample, approximately 1.5 g of sample from each batch were weighed into 10 N-free nylon bags (R510, Ankom Technologies, Macedon, NY) with a mean pore size of 50 µm and a dimension of 5 cm × 10 cm (Figure 2.3). Bags were heat-sealed using an Ankom Heat Sealer (Figure 2.3; Ankom Technologies, Macedon, NY) and then divided into mesh bags (46 × 38 cm) that contained 2 secured 100 g weights, which were used to prevent bags from floating in the rumen mat, as shown in Figure 2.4. Each mesh bag contained 40 to 50 polyester bags. At 1400 h, mesh bags were inserted through the rumen cannula, positioned in the ventral sac, and incubated for 16 h (Figure 2.4). Following rumen incubation, all mesh bags were removed and washed in a domestic washing machine, shown in Figure 2.5, using 5 cycles that consisted of 1 min agitation and 2 min spin. The previously described washing steps will be referred to as washing procedure throughout this paper. After washing, 4 bags of each sample were rinsed with distilled water to force all the residue to the bottom, rolled,

and dried in a 60°C oven for 24 h. The remaining 6 bags were used for the mobile bag procedure according to Kononoff et al. (2007) and are hereafter referred to as mobile bags. Mobile bags were incubated in a pepsin-HCl solution (1 g of pepsin/L of 0.01 M HCl) for 3 h in a 39°C water bath with occasional stirring to simulate abomasal digestion (Figure 2.6).

Following the pepsin-HCl incubation, mobile bags were rinsed with distilled water to wash out the pepsin-HCl solution and to force the residue to the bottom, and then the upper portion of the bag was tightly rolled. Subsequently, mobile bags were inserted through the duodenal cannula of each cow at a rate of 1 mobile bag every 5 min. Mobile bags were inserted in the duodenal cannula of the corresponding cow in which they were ruminally incubated, which is shown in Figure 2.7. Once passing through the cow, mobile bags were retrieved from the manure every 3 h from the appearance of the first bags (8 h after insertion) until 24 h after insertion and then rinsed lightly with cold water to remove fecal material (Figure 2.7). Bags were thawed and washed following the washing procedure. After washing, bags were rinsed with distilled water to force all the residue to the bottom, rolled, and dried in a 60°C oven for 24 h.

Following drying, bags were weighed to determine the weight of the remaining residue. Rumen and mobile bag residues were composited by sample, type, and cow. Composites were then analyzed for DM (AOAC International, 2000) and N (Leco FP-528 N Combustion Analyzer; Leco Corp., St. Joseph, MI 49085) by Cumberland Valley Analytical Services Inc. (Hagerstown, MD).

Modified 3-Step Assay

Prior to conducting the experiment all procedures using animals were approved by the University of Nebraska-Lincoln IACUC. Two dry, multiparous Jersey cows (BW of 482 ± 3 kg) fitted with flexible ruminal cannulas were used for a majority of the ruminal incubation. Due to complications with lab equipment, two incubations were conducted when the cows were 89 ± 11 DIM. Cows were housed in tie stalls with continuous access to water and fed a diet listed in Table 2.1 once daily at 1000 h; cows used had an average DMI of 21.3 ± 0.97 kg/d. Ruminal degradations of CP were determined in situ and intestinal digestibilities of were determined using the MTS assay. For each sample, approximately 1.5 g from each sample were weighed into 10 N-free nylon bags (R510, Ankom Technologies, Macedon, NY) with a mean pore size of 50 µm and a dimension of 5 cm × 10 cm. Bags were heat-sealed using an Ankom Heat Sealer (Ankom Technologies, Macedon, NY) and then divided into mesh bags (46 × 38 cm) that contained 2 secured 100 g weights, which were used to prevent bags from floating in the rumen mat. Each mesh bag contained 40 to 50 polyester bags. At 1400h, mesh bags were inserted through the rumen cannula, positioned in the ventral sac, and incubated for 16 h. Following rumen incubation, all mesh bags were removed and washed in a domestic washing machine using 5 cycles that consisted of 1 min wash and 2 minutes spin. The previously described washing steps will be referred to as washing procedure throughout this paper.

Following washing, all bags were frozen at -20°C to preserve samples until they were subjected to the remainder of the assay. Bags were allowed to thaw at room temperate for 12 h prior to continuation of the assay. Four bags of each sample rolled and

dried in a 60°C oven for 24 h. The remaining 6 bags were used for the MTS procedure according to Gargallo et al. (2006) and are hereafter referred to as 3-step bags. Three-step bags were incubated in a pepsin-HCl solution (1 g of pepsin/L of 0.01 N HCl) for 3 h in a 39°C water bath with occasional stirring to simulate abomasal digestion. Following the pepsin-HCl incubation, mobile bags were rinsed with distilled water to wash out the pepsin-HCl solution and to force the residue to the bottom.

Three-step bags were then separated by cow and were placed in incubations bottles, with no more than 30 bags per bottle, containing 2 L of a pancreatin solution (0.5 M KH_2PO_4 buffer, adjusted to pH 7.75, containing 50 ppm thymol and 3 g/L pancreatin), as shown in Figure 2.8. Bags were incubated for 24 h at 39°C with constant rotation in a Daisy^{II} incubator (Ankom, Fairport, NY; Figure 2.9). After 24 h, bags were rinsed with tap water to wash out the pancreatin solution and to force the residue to the bottom. Bags were then rolled and dried in a 60°C oven for 24 h.

Following drying, bags were weighed to determine the weight of the remaining residue. Rumen and 3-step bag residues were composited by sample, type, and cow, and they were then analyzed for DM (AOAC International, 2000) and N (Leco FP-528 N Combustion Analyzer; Leco Corp., St. Joseph, MI 49085) by Cumberland Valley Analytical Services Inc. (Hagerstown, MD).

Ross Assay

All steps of the procedure were performed at Milk Specialties Global, LLC. (Eden Prairie, MN). Rumen fluid used to quantify rumen degradation was collected from 2 rumen-cannulated multiparous, lactating Holstein cows housed off-site in a tie stall barn with continuous access to water and a TMR ration. A pump, constructed using a silicone

hose with a 10 mm diameter and attached rubber bulb inserted in a 1m long plastic probe with drilled holes and rounded edges (Figure 2.10), was used to collect rumen fluid. The pump was warmed with hot water, with the bulb filled, up until collection. To start the collection, approximately 30 g of rumen contents taken from the rumen mat was placed in the bottom of a warmed 2 L thermos. The plastic probe was inserted into the ventral sac of the rumen. With the bulb compressed, the hose was inserted into the probe; the bulb was then released to draw up rumen fluid, and then transferred to the thermos, shown in Figure 2.11. The process was repeated until approximately 2 L was collected. A second and similar 30 g of rumen contents was added to the thermos prior to closing to reduce airspace, prevent sloshing, and provide material to aid in filtering. Rumen contents were filtered upon arrival at the lab through 4 layers of cheesecloth (100 µm nylon filtration cloth, NC0365403, Fischer Scientific, Hampton, NH), glass wool, and a nylon screen in a Büchner funnel. Filtered fluid was collected in a 4 L Erlenmeyer flask incubated in a 39º C water bath (Precision Model 170, Precision Scientific Co., Chicago, IL) with constant infusion of CO_2. The setup used is shown in Figure 2.12.

Four 125 mL Erlenmeyer flasks were filled with 0.5 g of sample along with 40 mL of Van Soest rumen buffer and 10 mL of rumen fluid, shown in Figure 2.13. Flasks were incubated in a water bath (MW1140A-1, Blue M, Blue Island, IL) at 39 °C for 16 h under continuous CO_2 to maintain anaerobic conditions (Figure 2.14). Following incubation, 2 flasks were set aside to be used to determine rumen degradation. Flask contents were filtered using 2-piece glass filter holders (90 mm) and manifold (C-02923-30 & C-02924-30, respectively, Cole-Parmer Instrument Co., Vernon Hills, IL) through 1.5 µM Whatman 934-AH glass filters (90 mm; GE Healthcare Bio-Sciences Corp.,

Piscataway, NY) with boiling water, which is shown in Figure 2.15. Filters were then dried at 105º C for 24 h in a drying oven (Hotpack Corp. Philadelphia, PA).

The remaining 2 flasks were acidified with 2 mL 3M HCl to reduce the pH to 2 and allowed to shake in a shaking water bath (Precision Dubnoff metabolic shaking incubator, Precision Scientific Co., Chicago, IL; VWR Model 1227 reciprocating water bath, VWR International, Inc., West Chester, PA) at 39º C for 1 min. Flasks were then incubated for another 1 h after the addition of 2 mL of pepsin and 0.013 M HCl. The pepsin reaction was then neutralized with the addition of 2 mL 2M NaOH. A combination of 10 mL 1.8 M KH_2PO_4 and an enzyme mix (168, 140, 705, 28 units per mL KH_2PO_4 of trypsin, chymotrypsin, amylase, and lipase, respectively) were added to the flask and incubated for 24 h in a 39 °C shaking bath. After this incubation, samples were filtered through the same system as the rumen-incubated samples and dried for 24 h at 105º C. Filters were analyzed for DM and N (Leco FP-528 N Combustion Analyzer; Leco Corp., St. Joseph, MI) by Cumberland Valley Analytical Services Inc. (Hagerstown, MD).

Statistical analysis

Data were analyzed using the PROC GLIMMIX function of SAS (9.4). The model included the fixed effects of presence of blood and assay type as well as the interaction of presence of blood and assay type. All data are presented as least-square means ± largest standard error. Significance was declared with a P-value ≤ 0.05.

RESULTS AND DISCUSSION

Hydrolyzed feather meal is a byproduct of the rendering industry and has high CP content of 85%, which is 65% RUP (NRC, 2001). However, previous studies have shown

that increasing inclusion of HFM leads to a decrease in milk protein concentration (Harris, Jr., et al., 1992; Moss et al., 1995; Morris et al., 2020b). Harris, Jr., et al. (1992) showed that increasing the inclusion of HFM in diets resulted in decreasing diet CP digestibility, which also coincides with the results shown by Goedeken et al. (1990). These results indicate that a lack of availability of protein in HFM could in part explain the reduction in milk protein that may occur when cows consume HFM. Goedeken et al. (1990) also evaluated HFM with blood included and observed an increase in DMI and daily gain, in beef cattle, over HFM. Lysine has been determined to be a limiting essential AA in dairy cattle (Schwab et al. 1992), and bloodmeal is a significant source of Lys (8.98% CP; NRC, 2001), which is lacking in HFM (2.57% CP; NRC, 2001). Positive production responses have also been observed in dairy cattle with an increase in milk protein yield and concentration when both HFM and blood meal are included in the diet (Grant and Haddad, 1997).

To quantify the protein quality and availability of these feedstuffs, several techniques exist to determine the protein fractions and digestibility. The Mobile Bag (Hveplund, 1985), Modified Three Step (Gargallo et al., 2006), and Ross (Ross, 2013) assays are three of the most commonly used techniques but vary in the degree in which samples are in situ. Because of the varying degrees in which the feedstuff is exposed to the actual environment of the ruminant digestive tract, estimates for the various protein characteristics can differ.

Feedstuffs

Table 2.2 lists the average chemical composition of the five sources of FM and five sources of FMB. Several of the differences in chemical composition between FM

and FMB are likely due to the addition or absence of blood was well as difference in processing methods across plants (Cotanch et al., 2020). Feather meal samples with blood had a numerically greater DM, CP, and NDF (93.3 ± 1.54, 91.9 ± 1.94, and 28.8 ± 6.06%, respectively) than FM samples (91.9 ± 0.48, 90.5 ± 1.02, and 23.6 ± 3.86%, respectively). Acid detergent fiber and ADICP was lower in FMB samples than FM samples (2.38 ± 1.54 and 4.24 ± 1.07 compared to 3.23 ± 1.13 and 4.96 ± 1.02 %) while NDICP was greater in FMB samples (27.0 ± 5.15 versus 21.7 ± 2.84%). Neutral detergent fiber, ADF, NDICP, and ADICP values for both FM and FM with viscera (blood) are not reported in the NRC. Average crude fat was lower in FMB samples (7.08 ± 1.73%) than FM (8.58 ± 1.51%), and ash was greater in FMB (6.19 ± 3.24%) than FM (2.74 ± 1.13%). Both FM and FMB were greater in crude fat (7.08 and 8.58%) than the NRC values (3.5 and 5.5%). Coinciding with the NRC, FMB had a greater ash content than FM, which can be explained by the significant contribution of Fe with the addition of blood. However, the ash content of FMB is greater than the value reported in the NRC (6.19 vs 5.5%), while FM was lower (2.74 vs 3.5%). The differences observed between FM and FMB could be the result of added blood or it may be the result of, or compounded by, production site. Production sites can vary in how feather meal is hydrolyzed, how bloodmeal is dried, and how much bloodmeal is incorporated into the feather meal, which can alter the chemical composition of the final product (Meeker and Hamilton, 2006).

Digestibility assays

For all measures of chemical composition, no significant effect was observed for the interaction of assay and blood inclusion ($P > 0.40$). The simple means for type of bloodmeal and assay type are presented in tables 2.4 and 2.5.

Differences in chemical composition of FM and FMB after being subjected to the three assays is listed in Table 2.3. Significant differences ($P < 0.01$) of assay type were observed in ruminal DM digestibility (DMD) as well as RUP as a portion of CP. While DMD of MOB and MTS was similar (34.2 and 36.0%, respectively), DMD observed from ROS (17.5%) was lowest. Similarly, the lowest RUP observed was using the ROS assay (77.8, 71.9, and 41.9 ± 1.80% for MOB, MTS, and ROS, respectively). All samples were subjected to a 16 h rumen incubation, but the nature of this incubation varied. Samples for both MOB and MTS were incubated in N-free nylon bags in situ while ROS incubation occurred with samples placed in a flask containing a mixture of Van Soest rumen buffer and rumen fluid under continuous CO_2. The possible reasons associated with incubation could in part explain the observed differences between assays. For example, any soluble components of the samples could escape the bags but not necessarily be digested, which would lead to an over-estimation of rumen digestibility (Ross, 2013). An additional explanation is that the rumen fluid used in the Ross assay could have been exposed to unfavorable conditions, such as change in temperature. While precautions were made to avoid exposure to oxygen and cool temperatures, even short-term exposure can cause a loss in microbial activity (Coleman, 1985). A decrease in microbial activity would lead to less digestion and thus a lower DM digestibility and a greater RUP content. It should also be noted that the presence of fat could have limited microbial activity by having an antimicrobial effect or creating a barrier between the feed particle and microbes in all three assays (Jenkins, 1993).

There was no effect of assay type on estimate of dRUP (average of 60.2 ± 2.90%; $P = 0.70$). Because the primary goal of all three assays is to determine dRUP. Both the

Modified Three Step and Ross assay were developed to provide a rapid, more affordable, less labor-intensive alternative to the Mobile Bag assay, which was originally developed by Hveplund et al. (1985). To simulate intestinal digestion, both assays used constant agitation and a solution of various buffers and enzymes. The Modified Three Step assay still relies upon rumen incubation of samples but uses a buffer-enzyme solution containing pancreatin and thymol in a Daisy^{II} incubator (Ankom, Fairport, NY) (Gargallo et al., 2006). The Ross assay opts for incubating samples in individual Erlenmeyer flasks instead of bags and utilizing an agitating water bath with an enzyme mix including trypsin, chymotrypsin, amylase, and lipase along with pancreatin (Ross, 2013). The similarity among the dRUP values of these assays provided strong evidence that all three are viable options to determine intestinal digestibility of animal-based protein feedstuffs. Digested RUP was significantly different among assays, which is likely due to the difference in estimated RUP content. While the MOB and MTS values are similar for CP total tract digestibility (69.4 and 70.0%), the ROS values are different (94.0%). The differences observed among these assays is likely a residual effect of the differences observed in rumen digestibility.

Feather meal with and without blood

Table 2.4 lists the differences in chemical composition of FM and FMB. A significant (P = 0.01) response was observed for rumen DMD with FMB (32.1%) samples being greater than FM (26.4%). The same significant response (P = 0.01) was observed for RUP as a portion of CP (66.9 and 60.8% for FMB and FM). According to the NRC (2001), feather meal with viscera has a greater true digestibility than feather meal alone (81 vs 78%), which coincides with what was observed for rumen DMD in the

present study. As for RUP, feather meal and feather meal with some viscera both have the same RDP content (65.4%) listed in the NRC (2001).

The exact reason for the observed increase in RUP is unknown. One possible explanation is that the difference could be a residual effect of the assays. With the use of bags, there is a risk for bacterial contamination, which would increase RUP (Alexandrov, 1998). Paz et al. (2014) quantified bacterial CP contamination with the mobile bag technique and found that increasing NDF concentration, which was not fiber but components not soluble in neutral detergent solution, led to greater contamination. While bacterial contamination was not measured in the present study, with an average NDF content for FM of 23.6 and FMB of 28.8, bacterial contamination is a possibility. Processing may also be another explanation as it does affect the digestibility of the sample. Temperature and length of time of the cooking process are both key determinants of quality in rendered products (Meeker and Hamilton, 2006). Longer hydrolysis time increases the digestibility of feather meal (Blasi et al., 1990), and feather meal with blood added before hydrolysis is more digestible than feather meal with blood added after hydrolysis (Contanch et al. 2007). Also during the rendering process, fat is separated from the protein solids, but some residual fat remains with the product, which can affect digestibility (Meeker and Hamilton, 2006). A higher fat content would lead to lower rumen degradation and increase RUP content (Palmquist et al., 1993). The processing methods of the each of the samples used in the present study is unknown, thus we are unclear of differences in processing that could cause the difference in RUP between FM and FMB. We only replicated within FM and FMB and not within a plant, thus we couldn’t test for plant effects.

Similar to what was observed among assays, no difference ($P = 0.859$) in dRUP was observed between FM and FMB (average of 60.5 ± 0.49%). Our observation is lower than the NRC (2001), which lists a dRUP of 70% for feather meal with some viscera and 65% for feather meal. As noted above, rendering process may impact digestibility. The method in which bloodmeal is processed may affect its digestibility. A study conducted in rats showed that intestinal protein digestibility of bloodmeal varied from 17.0 to 94.6% depending on the drying method, time in drier, and temperature of the drier (Moughan et al., 1999). As previously stated, the processing methods of the samples used is unknown, so it is uncertain if processing is the cause of the difference.

The presence of blood had no effect ($P = 0.53$) on the total tract CP digestibility for both FM and FMB samples (74.5 ± 1.34%). The lack of a difference in DM digestibility between FM and FMB is difficult to explain. One explanation is that the DM digestibility is highly dependent upon CP digestibility because animal-based proteins have high CP concentration. While there is a plethora of literature on the DM digestibility of forages, there is a lack of materials on the DM digestibility of animal-based protein sources. However, because the primary focus of animal-based protein sources is the CP digestibility, this lack of information is easily understood. Waltz et al. (1998) determined the CP total tract digestibility of blood meal, feather meal, and a 50-50 blend of blood meal and feather meal using the mobile bag method. Blood meal had a greater digestibility (42.7%) than feather meal (20.8%) as well as the blend (28.2%), which had a digestibility that was approximately the average of blood meal and feather meal combined. In the Contach et al. (2007) study, the N digestibility of samples of feather meal with blood included at varying concentrations was compared. While the drying

method of the blood added also varied in the feather meal with blood samples, total N digestibility ranged from 46.2 to 84.7% with an average of 65.4%; feather meal in the same study ranged from 43.0 to 69.6% N digestibility with an average of 57.6%. Even though the averages of the wide range of samples used in the study were not comparable, the ranges were similar. In the present study, the concentration of blood in the samples as well has processing is unknown, but a varying inclusion level of blood meal could explain the why the total tract CP digestibility of both FM and FMB are not different.

CONCLUSIONS

Even though HFM is an affordable source of RUP, questions remain on the extent to which it is digested in the small intestine. There are multiple assays available to determine this digestibility, but because of the varying degree they are done in situ, estimates can vary. The objective of this study was to compare three of the assays used to determine protein digestibility while determining the digestibility of HFM with and without blood. While there are differences in chemical composition in HFM associated with the inclusion of blood, there are little to no differences observed in ruminal or intestinal digestion of protein. Assays varied in almost all estimates, which includes rumen DM digestibility, RDP, total tract DM digestibility, and total tract CP digestibility. However, there was no difference in dRUP estimates across all assays for both FM and FMB.

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TABLES AND FIGURES

Table 2.1. Chemical composition of each TMR fed to experiment cows for MOB and MTS assays (% of DM)

	Treatment[1]	
Items	MOB	MTS
Ingredients		
Corn silage	37.5	38.5
Alfalfa hay	12.2	14.1
Corn grain, ground	13.7	16.0
Soypass	-	2.82
Corn DDGS[2]	5.94	10.3
Soybean meal	11.3	9.40
Soybean hulls, ground	-	1.79
Molasses, Beet	2.55	1.23
Fat	1.82[3]	1.87[4]
Wheat straw	2.05	-
Expellers soy	4.12	-
Whey protein	2.55	-
Soybean hulls	1.44	-
Bloodmeal	0.89	-
Rumen Protected LYS[5]	0.06	0.41
Rumen Protected MET[6]	0.07	0.11
Mineral-vitamin mix[7]	2.92	3.42
Chemical composition		
DM	60.7 (1.38)	60.5 (1.66)
Ash	8.14 (0.53)	8.65 (0.42)
CP	17.9 (0.76)	17.5 (0.84)
NDF	28.1 (1.34)	26.9 (1.74)
Starch	25.0 (0.73)	30.5 (0.85)

[1]MOB = Mobile Bag assay, MTS = Modified Three-Step assay
[2]DDGS = dried distillers grains with solubles.
[3]Porcine tallow
[4]Energy Booster (Milk Specialties, Eden Prairie, MN).
[5]AjiPro (Ajinomoto Co., Inc., Tokyo, Japan).
[6]Smartamine (Adisseo, Alpharetta, GA).
[7]dd

Table 2.2. Chemical composition of feather meal with and without blood samples prior to being subjected to assays (% of DM unless otherwise stated)

	Treatment[1,2]			
	FM		FMB	
Item	Mean	SD	Mean	SD
Dry matter, % as-is	91.9	0.48	93.3	1.54
CP	90.5	2.14	91.9	1.94
NDF	23.6	1.51	28.8	6.06
ADF	3.23	1.13	2.38	1.54
ADICP	4.96	1.02	4.24	1.07
NDICP	21.7	2.84	27.0	5.15
Crude fat	8.58	1.51	7.08	1.73
Ash	2.74	1.13	6.19	3.24

[1] n = 5.

[2]FM = feather meal without added blood, FMB = feather meal with added blood.

Table 2.3. Chemical composition (% of DM, unless otherwise noted) of hydrolyzed feather meal with and without blood for the Mobile (MOB), Modified Three-Step (MTS), and Ross (ROS) assays
[1]n = 5.

Items[2]	Assay[1]			SEM	*P*-value
	MOB	MTS	ROS		
Rumen DMD2	34.2	36.0	17.5	1.69	<0.001
CP	90.7	90.7	90.7	0.95	1.00
RDP, % CP	22.2	28.1	58.1	1.80	<0.001
RUP, % CP	77.8	71.9	41.9	1.80	<0.001
TTDMD[3]	74.2	71.7	47.1	2.57	<0.001
TTCPD[4]	69.4	70.0	94.0	2.67	<0.001
dRUP, % RUP[5]	61.1	58.1	62.2	3.53	0.697
RUP digested	42.9	38.0	23.4	2.28	<0.001

[2]Rumend DMD = rumen dry matter digestibility

[3]TTDMD = total tract dry matter digestibility

[4]TTCPD = total tract crude protein digestibility

[5]dRUP = rumen undegraded protein digestibility

Table 2.4. Chemical composition (% of DM, unless otherwise noted) of hydrolyzed feather meal with (FMB) and without blood (FM)

Items[2]	Feedstuff[1]		SEM	*P*-value
	FM	FMB		
Rumen DMD2	26.4	32.1	1.38	0.007
CP	90.5	90.4	0.78	0.667
RDP, % CP	39.2	33.1	1.47	0.007
RUP, % CP	60.8	66.9	1.47	0.007
TTDMD[3]	65.8	62.9	2.10	0.348
TTCPD[4]	75.4	73.5	2.18	0.531
dRUP, % RUP[5]	60.1	60.8	2.88	0.859
RUP digested	32.6	37.0	1.86	0.114

[1]n = 5.

[2]Rumend DMD = rumen dry matter digestibility

[3]TTDMD = total tract dry matter digestibility

[4]TTCPD = total tract crude protein digestibility

[5]dRUP = rumen undegraded protein digestibility

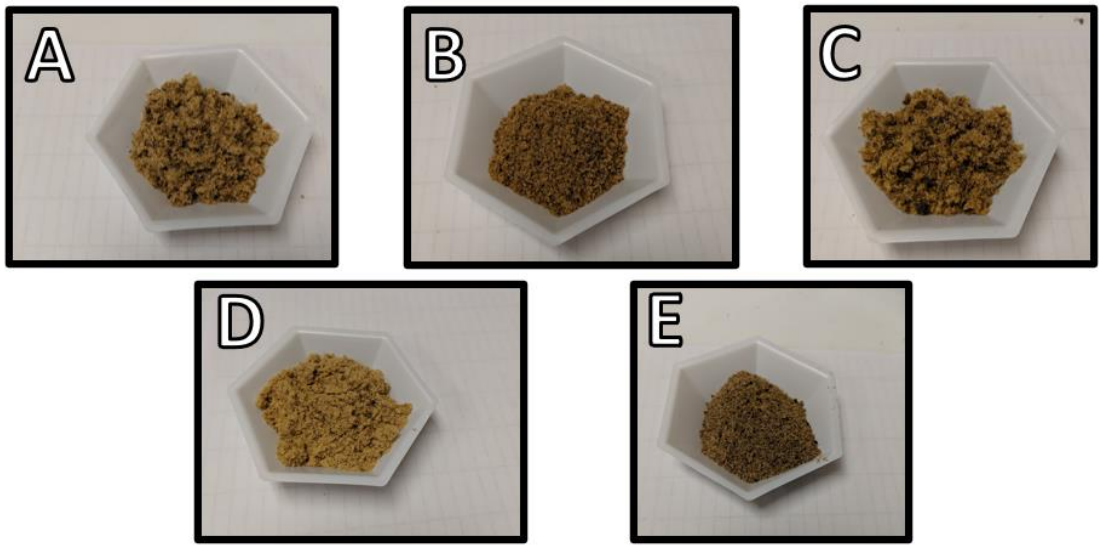

Figure 2.1. Feather meal with out added blood samples from Pilgrim's Pride Corporation (Mt Pleasant, TX; A), American Proteins Inc. (Cumming, GA; B), Pilgrim's (Greeley, CO; C), Simmons Food (Siloam Springs, AR; D), and River Valley Animal Foods (Robards, KY; E).

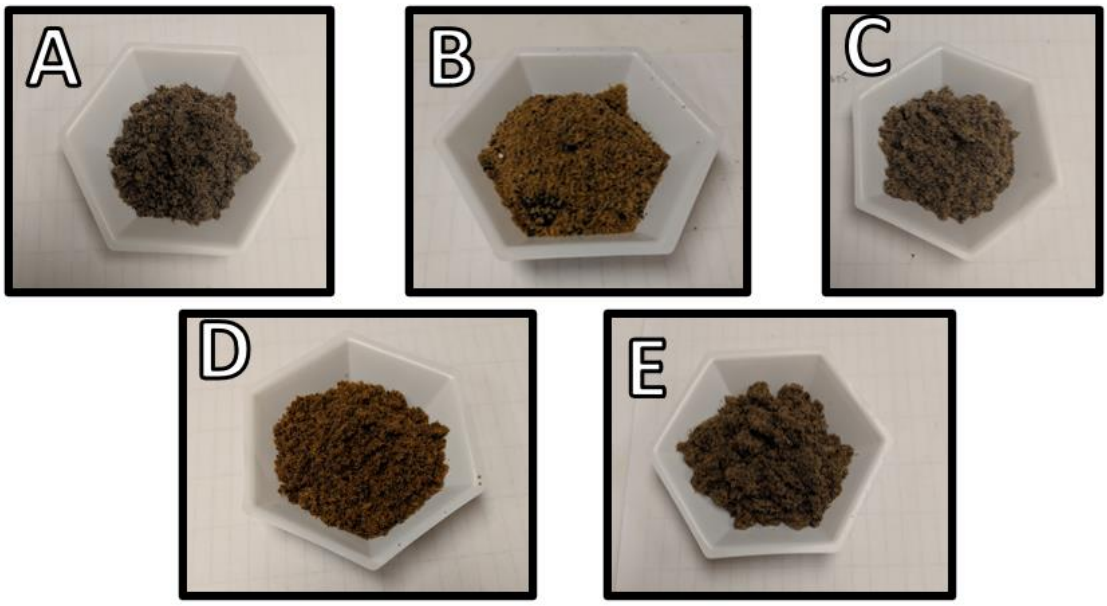

Figure 2.2. Feather meal with added blood samples from Darling Ingredients Inc. (Irving, TX; A), River Valley Animal Foods (Sedalia, MO; B), Pet Solutions (Danville, AR; C), Sanimaxx (Green Bay, WI; D), and Mountaire Farms (Millboro, DE; E).

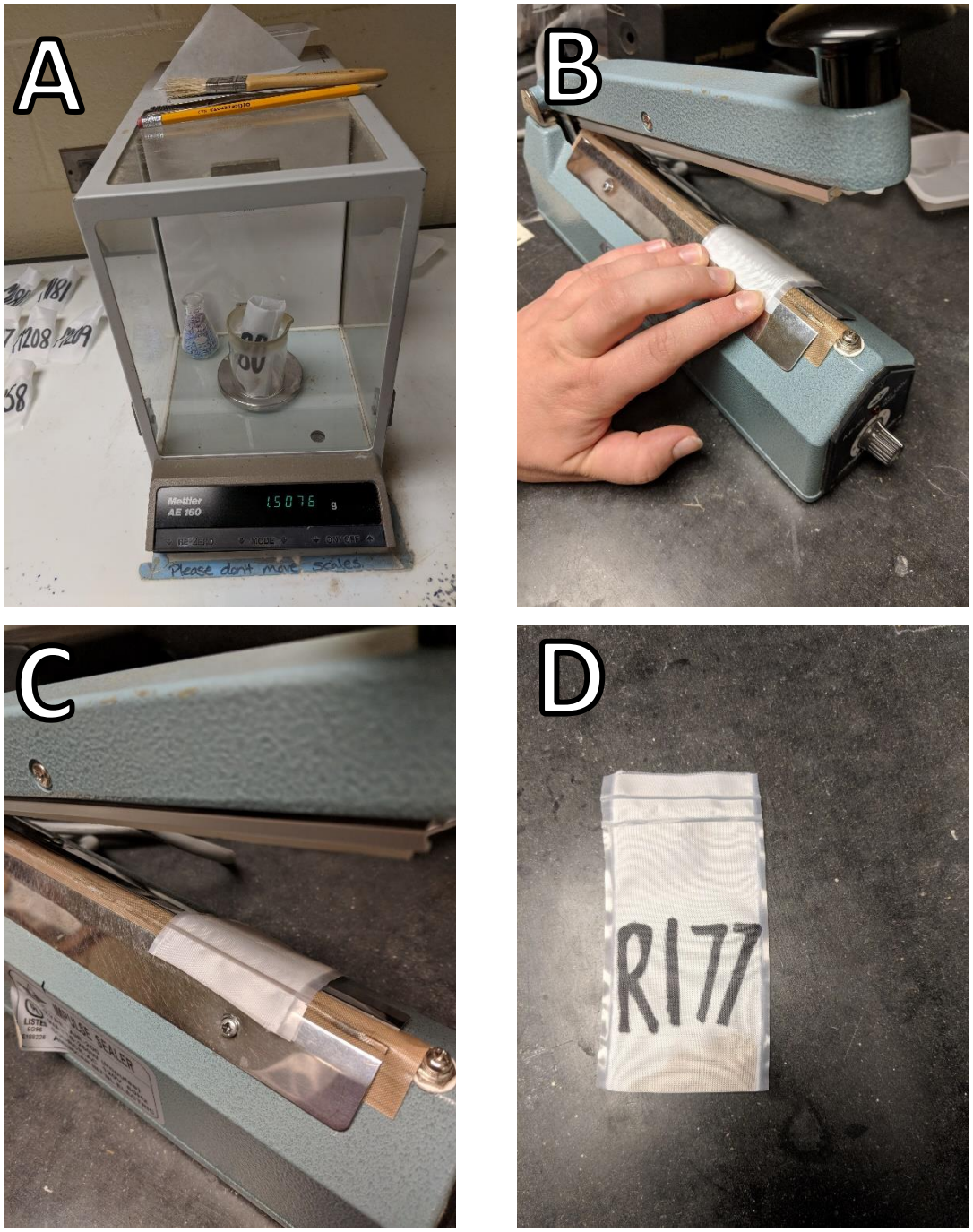

Figure 2.3. To prepare bags for the Mobile and Modified Three-Step assays, approximately 1.5 g of sample is weighed into 5 × 10 cm N-free nylon bags (A) and then sealed using an Ankom Heat Sealer (B) with the first seal placed approximately 1 cm from the open end of the bag (C) and the second approximately 1 cm below the first seal (D).

Figure 2.4. Nylon bags are placed into mesh bags that contain secured weights (A) before being inserted into the rumen through the rumen cannula (B).

Figure 2.5. Domestic washing machine used for the washing procedure of 1 min agitation and 2 min spinning repeated 5 times.

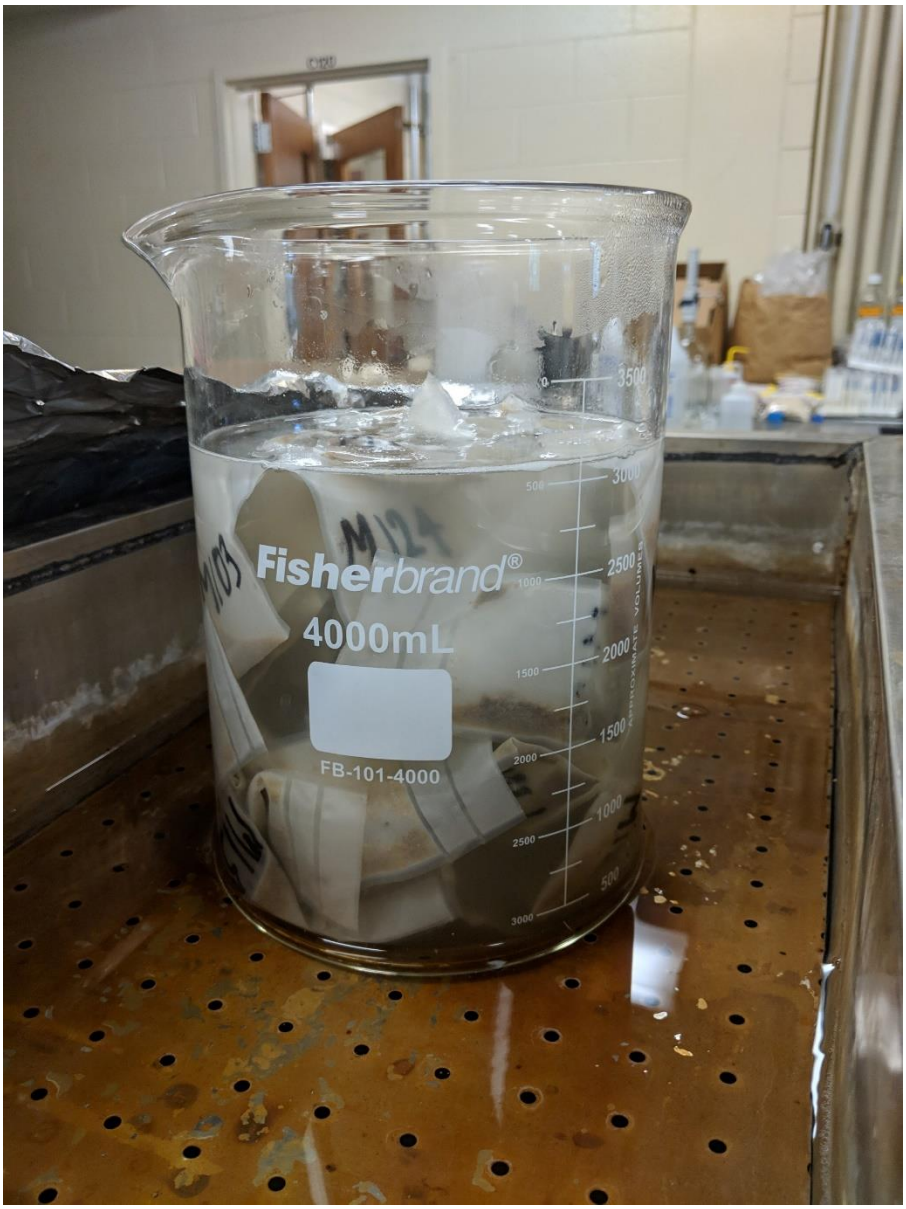

Figure 2.6. Mobile bags incubating in a pepsin-HCl solution of 1 g of pepsin/L of 0.01 N HCl.

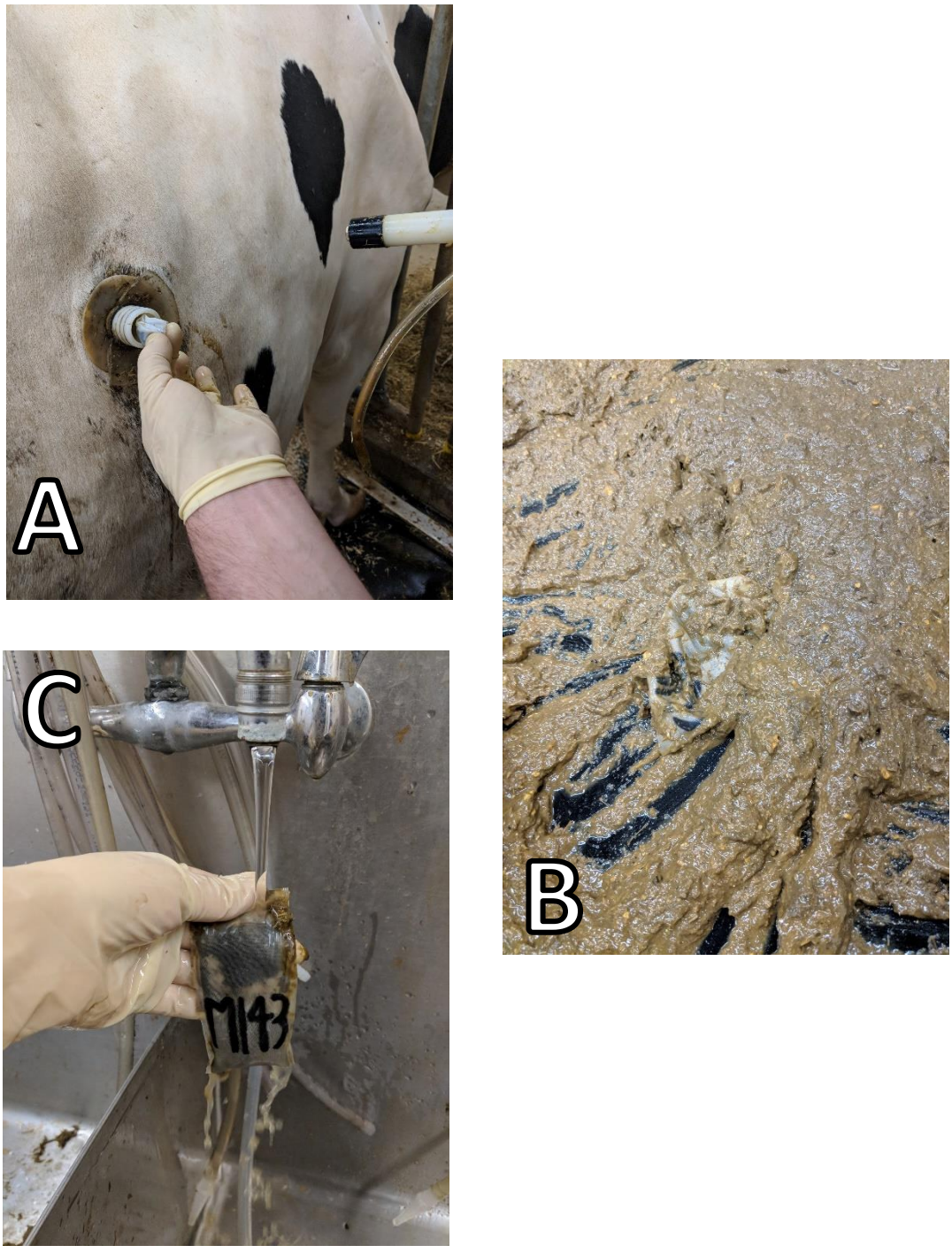

Figure 2.7. Rolled bags are inserted into the duodenum through the duodenal cannula (A). After passing through the small and large intestine, bags are recovered in the feces (B) and then lightly rinsed with cool water to halt microbial digestion (C).

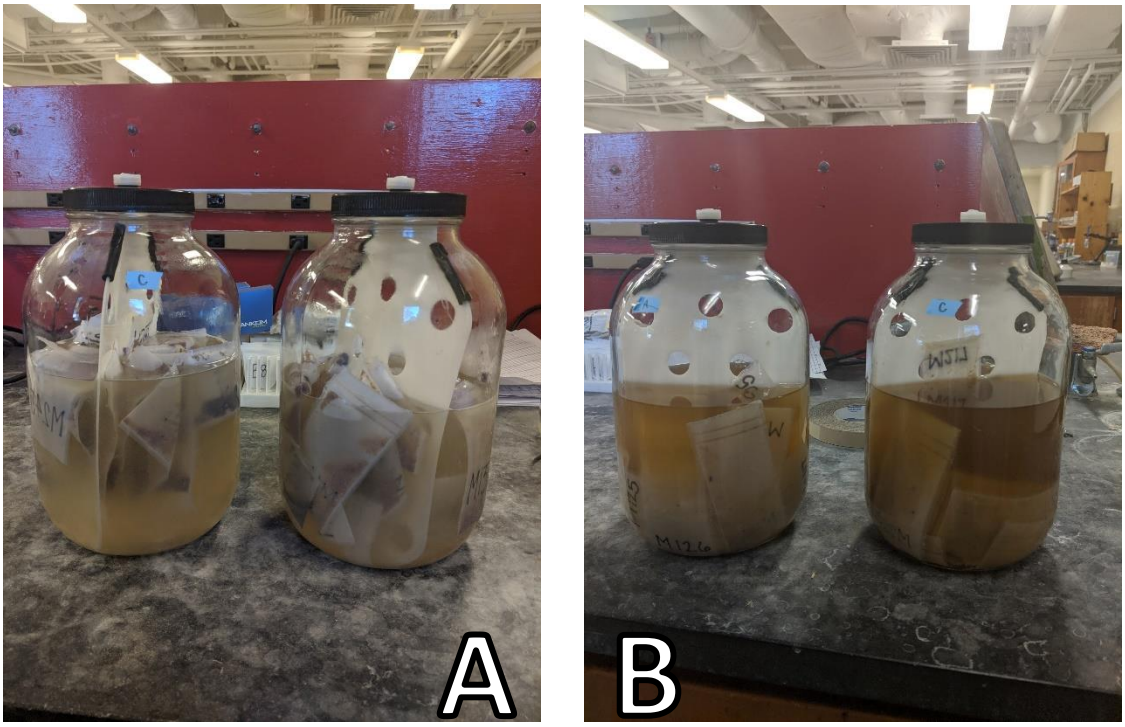

Figure 2.8. Nylon bags in incubation jars with pancreatin solution before (A) and after (B) 24 h incubation in a Daisy^{II} incubator.

Figure 2.9. Incubation jars in a Daisy^{II} incubator.

Figure 2.10. The pump used to collect rumen fluid from donor cows for the Ross assay.

Figure 2.11. Rumen fluid is collected from several locations within the rumen using a hand pump (A) before it is transferred to a pre-heated thermos (B) kept in a preheated container (C) to keep conditions consistent while transporting the rumen fluid.

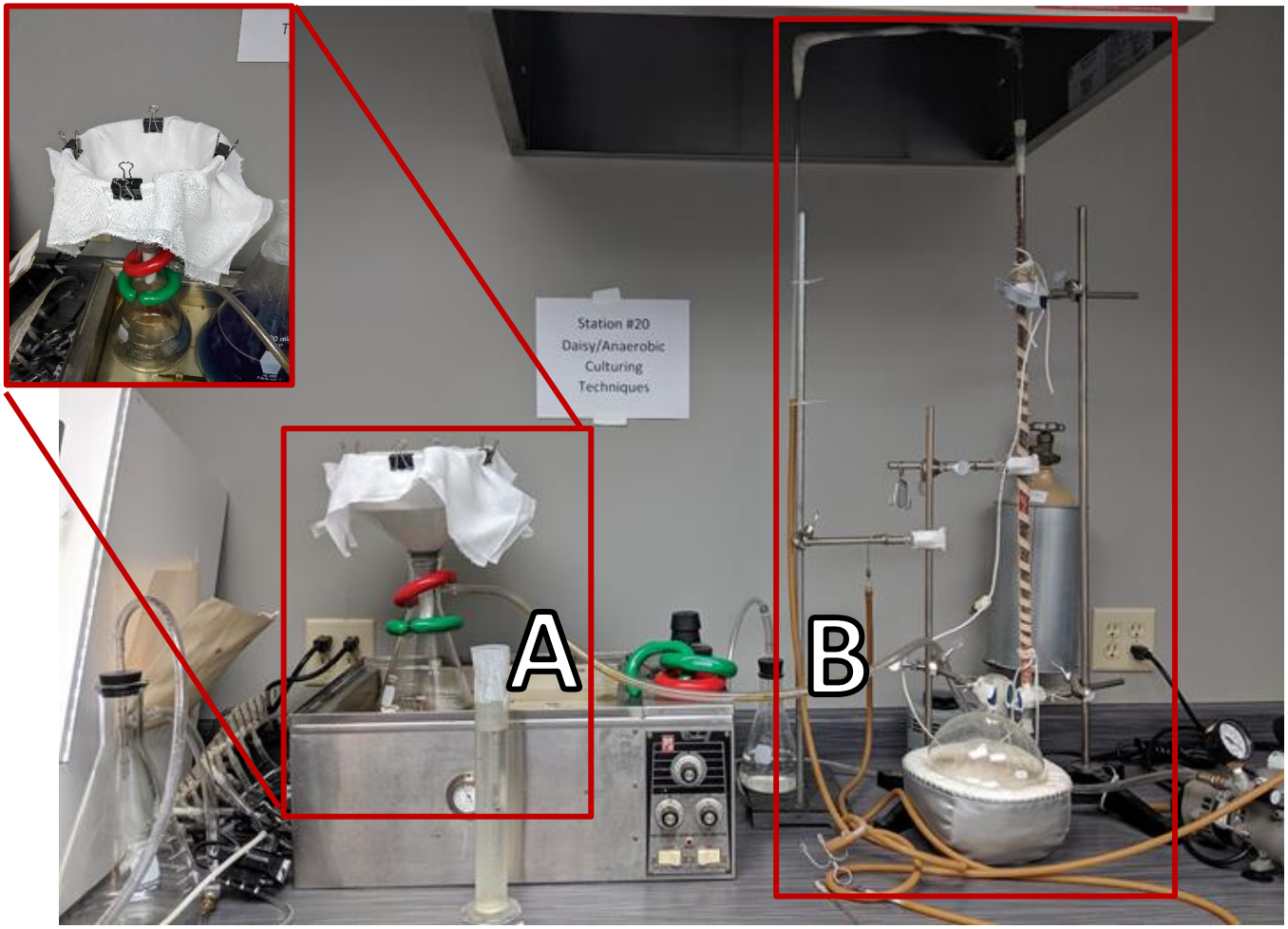

Figure 2.12. The setup used to filter rumen fluid. Fluid is filtered through 4 layers of cheesecloth, glass wool, and a nylon screen in a Büchner funnel (A) with constant infusion of CO_2 through an O_2 scrubbing copper column (B).

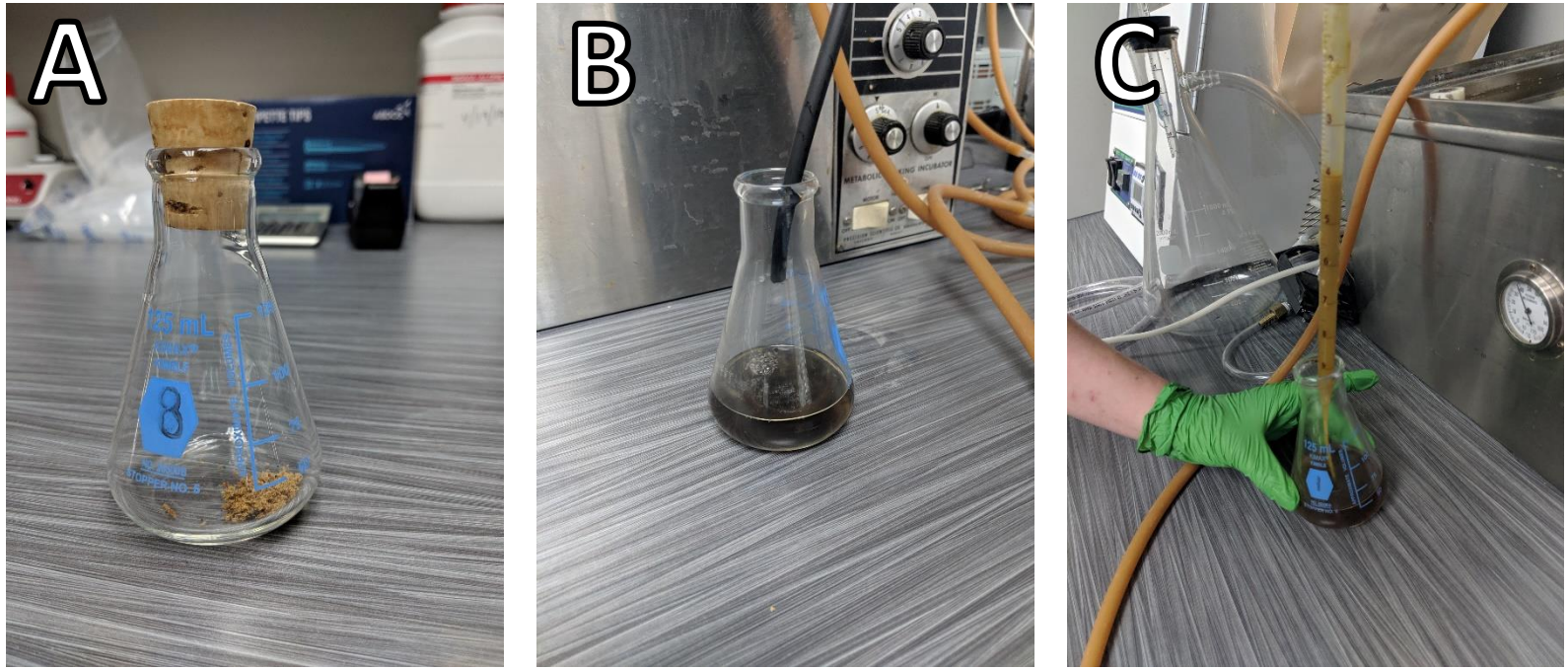

Figure 2.13. Erlenmeyer flasks are filled with0.5 g of sample (A) followed by 40 mL of Van Soest rumen buffer (B) and 10 mL of rumen fluid (C).

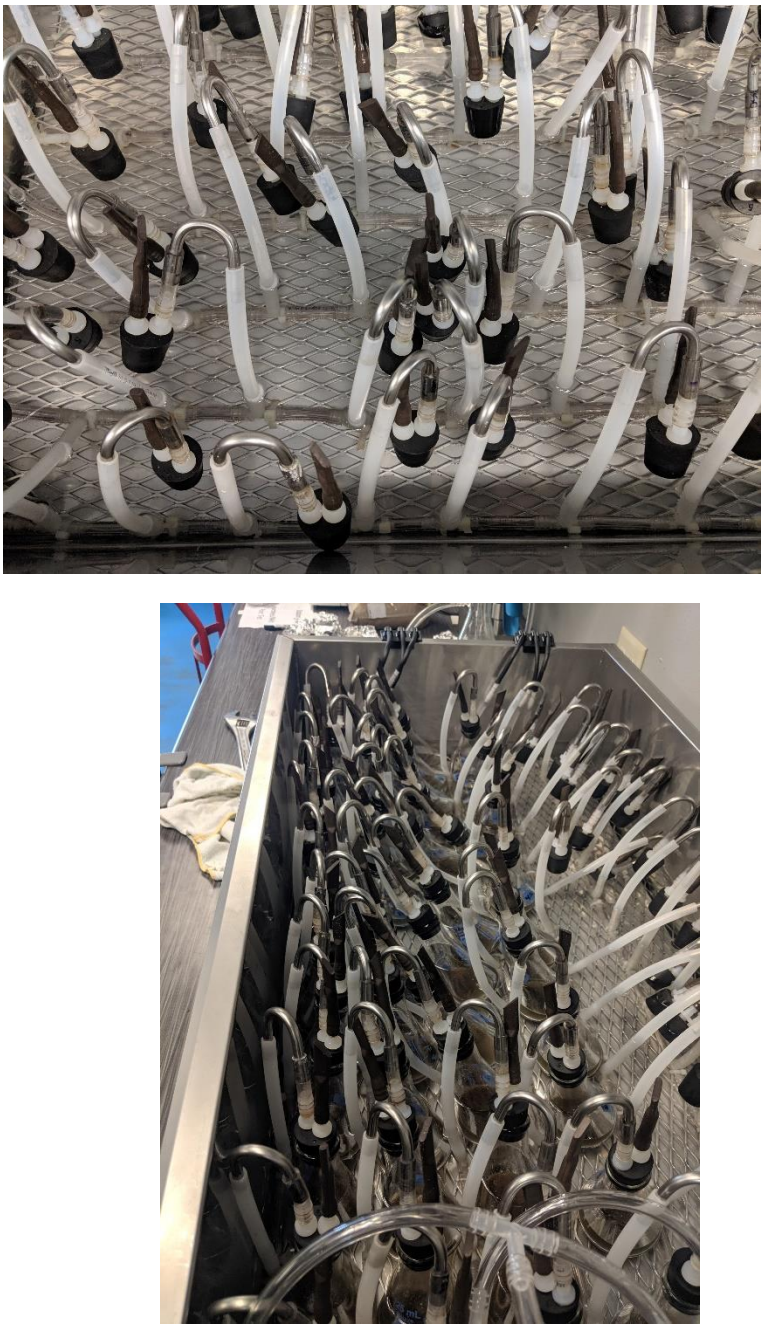

Figure 2.14. The *in vitro* system used to incubate flasks under continuous CO_2.

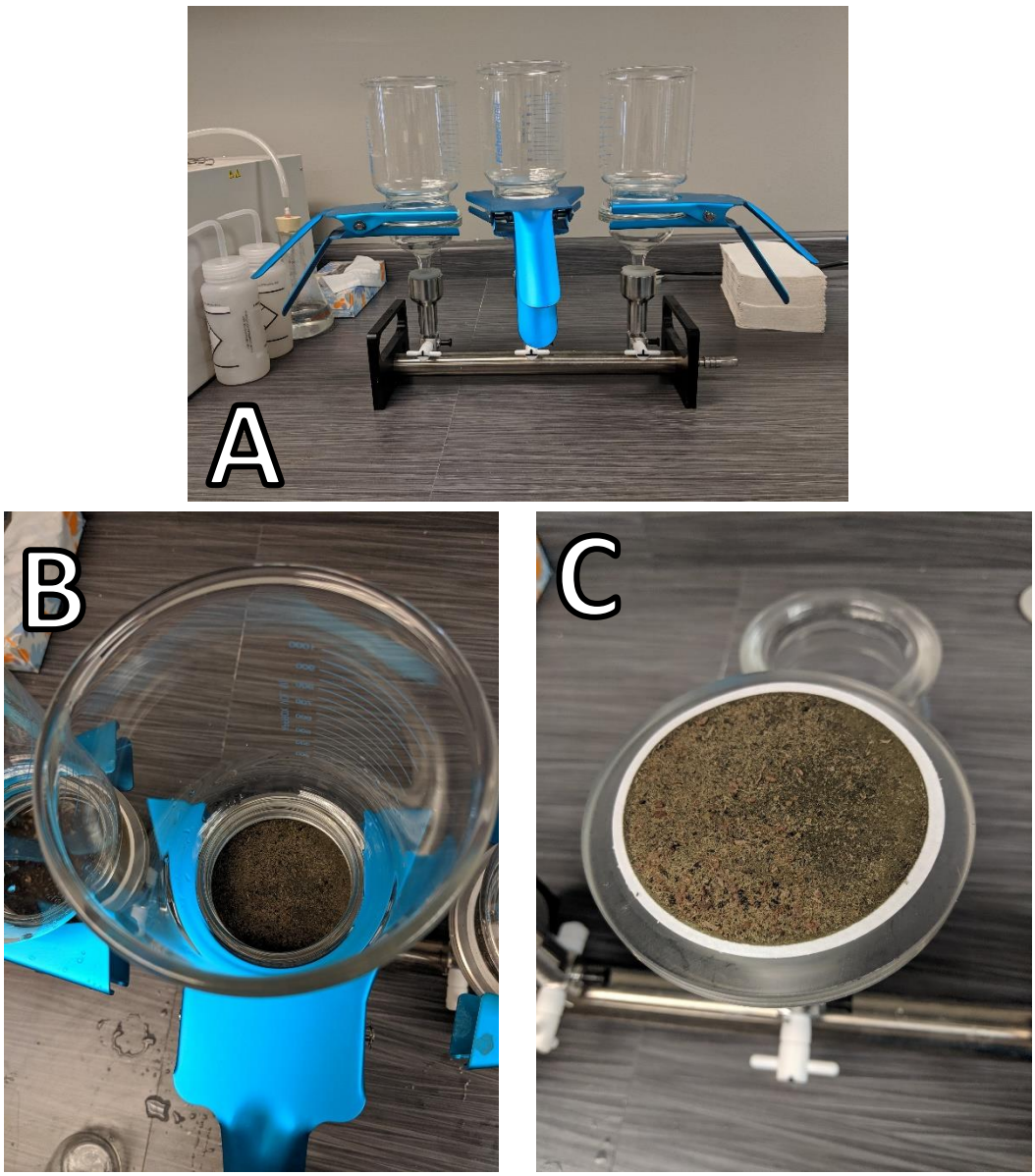

Figure 2.15. The filtration system used to filter samples (A). Filters were placed between the base and the glass, and boiling water was then used to rinse the contents of the flasks into the filter holders (B). Boiling water was used to rinse out any reagents so that only undigested residue was left on the filter (C).

APPENDIX A

CALCULATIONS TO DETERMINE PROTEIN DIGESTIBILITY

Mobile Bag. The RDP for all samples was determined as portion of the CP that disappeared from the polyester bag following the in situ incubation. The RUP was calculated as 100 – RDP. The total tract CP digestibility (TTCPD) was calculated as 100 – total-tract indigestible protein. The digestible portion of the RUP was assumed to be the percentage of the CP escaping ruminal disappearance but not recovered in the residue following intestinal incubation and was calculated as 100 – (total-tract indigestible protein/RUP).

Modified Three-Step. The RDP for all samples was determined as portion of the CP that disappeared from the polyester bag following the in situ incubation. The RUP was calculated as 100 – RDP. The total tract CP digestibility (TTCPD) was calculated as 100 – total-tract indigestible protein. The digestible portion of the RUP was assumed to be the percentage of the CP escaping ruminal disappearance but not recovered in the residue following incubation in the pancreatic solution for 24 h and was calculated as 100 – (total-tract indigestible protein/RUP).

Ross. The RDP for all samples was determined as the portion of the initial CP that disappeared following filtering of the samples designated for in vitro rumen incubation. The RUP was calculated as 100 – RDP. Total tract CP digestibility (TTCPD) was calculated as 100 – total-tract indigestible protein. The digestible portion of the RUP was assumed to be the percentage of the CP escaping ruminal incubation but not recovered in

the residue following filtering of the in vitro intestinal digestion and was calculated as 100-(total-tract indigestible protein/RUP)

APPENDIX B

INTERACTION OF INCLUSION OF BLOOD AND ASSAY

Appendix B. Chemical composition (% of DM, unless otherwise noted) of hydrolyzed feather meal with (FMB) and without blood (FM) subjected to the Mobile Bag (MOB), Modified Three-Step (MTS), and Ross (ROS) assays

	Treatment[1]						SEM	P-Value[3]		
	FM			FMB						
Items[2]	MOB	MTS	ROS	MOB	MTS	ROS		BLD	ASY	B×A
Rumen DMD	38.7	38.6	19.1	29.8	33.4	15.9	2.39	0.007	<0.001	0.497
CP	90.5	90.5	90.5	90.9	90.9	90.9	1.35	0.667	1.00	1.00
RDP, % CP	25.9	32.5	59.2	18.4	23.7	57.1	2.55	0.007	<0.001	0.397
RUP, % CP	74.1	67.5	40.8	81.6	76.3	42.9	2.55	0.007	<0.001	0.397
TTDMD	76.3	72.8	48.2	72.1	70.7	46.0	3.64	0.348	<0.001	0.947
TTCPD	71.3	70.4	84.6	67.5	69.6	83.4	3.78	0.531	<0.001	0.910
dRUP	62.0	55.8	62.5	60.2	60.4	61.9	5.00	0.859	0.697	0.794
RUP digested	41.1	33.9	22.9	44.8	42.1	24.0	3.22	0.114	<0.001	0.537

[1] n = 5 for FM & n=5 for FMB.

[2] Rumen DMD = Rumen dry matter digestibility; TTDMD = Total tract dry matter digestibility; TTCPD = Total tract crude protein digestibility; dRUP = rumen-undegradable protein digestibility.

[3] BLD = effect of blood; ASY = effect of assay; B×A = interaction effect of blood and assay.

APPENDIX C

ABSTRACT AND POSTER PRESENTATION FROM ADSA ANNUAL MEETING, 2019

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Department of Animal Science, University of Nebraska-Lincoln, Lincoln, NE 68583

Ruminal degradation and intestinal digestibility of hydrolyzed feather meal with and without blood

Hydrolyzed feather meal (**HFM**) is a readily available, high protein feedstuff that can be used as a cost-effective dairy feedstuff. Because the production process may vary, the chemical composition of HFM may also vary. Additionally, some processes may incorporate blood into the final product. The objective of this study was to evaluate the ruminal and intestinal digestibility of HFM originating from processes that differ in their inclusion of blood. Ten samples of HFM, 5 without blood (**FM**) and 5 with blood (**FMB**), were collected from ten different production plants across the United States. Two multiparous lactating Holstein cows fitted with rumen and proximal duodenal cannulas were used to quantify rumen undegradable protein (**RUP**), and RUP digestibility (**dRUP**) by employing the mobile bag technique. Approximately 1.5 g of each was weighed into 10 N-free nylon bags with a mean pore size of 50 μm and a dimension of 5 × 10 cm and incubated in the rumen for 16 h. A subset of rumen bags were then used to determine RUP. The remaining bags were placed in a pepsin-HCl bath for 3 h and then inserted in the duodenal cannula of each cow. Bags were recovered in the feces and used to quantify dRUP. Data were analyzed as a complete randomized design to test the effect of blood inclusion on RUP and dRUP of HFM. The CP content was similar ($P = 0.57$) between FMB and FM averaging 94.5 ± 0.90%. The RUP content of FMB tended ($P = 0.13$) to be greater than FM (81.6 vs. 74.1 ± 3.19%). The dRUP was not different ($P = 0.77$) averaging 61.1 ± 2.36% across treatments. There was also no difference detected between FMB and FM in total tract DM ($P = 0.40$) and CP ($P = 0.52$) digestibility averaging 74.2 ± 3.34 and 69.4 ± 4.07%. Results of this study suggest that although there are modest differences in chemical composition in hydrolyzed feather meal associated with the

inclusion of blood, very little differences are observed in either ruminal or intestinal digestion of protein.

Key Words: intestinal digestibility, rumen degradation, rumen undegraded protein

Ruminal degradation and intestinal digestibility of hydrolyzed feather meal with and without blood

K. K. Buse, D. L. Morris, P. J. Kononoff
Department of Animal Science, University of Nebraska-Lincoln

INTRODUCTION

- Hydrolyzed feather meal (**HFM**), a byproduct of the poultry industry, has NRC (2001) values of:
  - 92% CP
  - 35% RDP
  - 65% RUP
- Production processes can vary from plant to plant, which leads to variation in the chemical composition of HFM.
- Some plants utilize a processes that can incorporate blood into the final product, which may alter the chemical composition further.

OBJECTIVE

The objective of this study was to evaluate the ruminal and intestinal digestibility of HFM from processes that differ in blood inclusion.

MATERIALS and METHODS

Five sources of hydrolyzed feather meal without blood (**FM**) and 5 sources of hydrolyzed feather meal with blood (**FMB**) were evaluated.

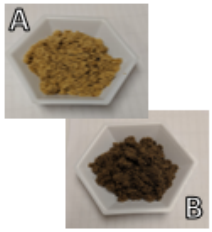

Figure 1. Samples of feather meal without (A) and with (B) blood

Two multiparous lactating Holstein cows fitted with rumen and proximal duodenal cannulas were used to quantify RUP and RUP digestibility (**dRUP**) via the mobile bag technique (Paz et al., 2014).

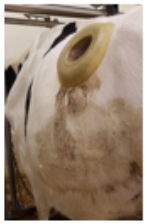

Figure 2. Rumen cannulated Holstein cow

Approximately 1.5 g of each sample was weighed into 10 N-free 5 × 10 cm nylon bags with a 50 µm pore size and then incubated in the rumen for 16 h.

Figure 3. Filled and sealed 5 × 10 cm nylon bag

A subset of rumen bags were then used to determine RUP. The remaining bags were placed in a pepsin-HCl bath for 3 h.

Figure 4. Bags incubating in a pepsin bath

Bags were then inserted in the duodenal cannula of each cow. Bags were recovered in the feces and used to quantify dRUP.

Figure 5. Insertion of bag into a duodenal cannula

RESULTS

Table 1. Chemical composition (% of DM, unless otherwise noted) of hydrolyzed feather meal without blood (FM) and with blood (FMB) subjected to the mobile bag procedure

	Feedstuff[1]			
Items[2]	FM	FMB	SEM	*P*-value
CP	94.9	94.2	0.90	0.57
Rumen DMD	38.7	29.8	2.75	0.05
RDP, % CP	25.9	18.4	3.19	0.13
RUP, % CP	74.1	81.6	3.19	0.13
TTDMD	76.3	72.1	3.34	0.40
TTCPD	71.3	67.5	1.07	0.52
dRUP	62.0	60.2	2.36	0.77

[1]n = 5 for FM and n = 5 for FMB
[2]Rumen DMD = rumen dry matter digestibility; TTDMD = total-tract dry matter digestibility; TTCPD = total-tract crude protein digestibility; dRUP = rumen-undegradable protein digestibility

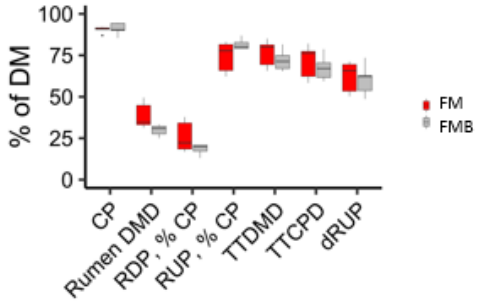

Figure 6. Box plot of the chemical compositions (Rumen DMD = rumen dry matter digestibility; TTDMD = total-tract dry matter digestibility; TTCPD = total-tract crude protein digestibility; dRUP = rumen-undegradable protein digestibility) of FM and FMB samples

CONCLUSIONS

The results of this study suggest that although FMB had a slightly higher RUP value, no differences were observed in rumen degradability and intestinal digestibility between FM and FMB. We can conclude that the addition of blood in hydrolyzed feather meal does not negatively impact intestinal digestibility.

REFERENCES

Paz et al., 2014, JDS 97:6485
NRC, 2001

Acknowledgements

Funded by POULTRY PROTEIN & FAT COUNCIL

Abstract # M14

Nebraska Lincoln

APPENDIX D

JOURNAL OF DAIRY SCIENCE REFLECT STATEMENT

Checklist for REFLECT statement: Reporting guidelines For randomized control trials in livestock and food safety. Bold text are modifications from the CONSORT statement description (Altman DG et al . Ann Intern Med 2001; 134(8):663-694).

Paper section and topic	**Item**	**Descriptor of REFLECT statement item**	**Reported on Page #**
Title & Abstract	1	How **study units** were allocated to interventions (eg, "random allocation," "randomized," or "randomly assigned"). **Clearly state whether the outcome was the result of natural exposure or was the result of a deliberate agent challenge.**	3
Introduction Background	2	Scientific background and explanation of rationale.	4-5
Methods Participants	3	Eligibility criteria **for owner/managers and study units at each level of the organizational structure,** and the settings and locations where the data were collected.	5-11
Interventions	4	Precise details of the interventions intended for each group, **the level at which the intervention was allocated,** and how and when interventions were actually administered.	5-11
	4b	**Precise details of the agent and the challenge model, if a challenge study design was used.**	NA

Objectives	5	Specific objectives and hypotheses. **Clearly state primary and secondary objectives (if applicable).**	5
Outcomes	6	Clearly defined primary and secondary outcome measures and the levels at which they were measured, and, when applicable, any methods used to enhance the quality of measurements (eg, multiple observations, training of assessors).	
Sample size	7	How sample size was determined and, when applicable, explanation of any interim analyses and stopping rules. **Sample-size considerations should include sample-size determinations at each level of the organizational structure and the assumptions used to account for any non-independence among groups or individuals within a group.**	5
Randomization -- Sequence generation	8	Method used to generate the random allocation sequence **at the relevant level of the organizational structure**, including details of any restrictions (eg, blocking, stratification)	5
Randomization -- Allocation concealment	9	Method used to implement the random allocation sequence **at the relevant level of the organizational structure**, (eg, numbered containers ~~**or central telephone**~~**),** clarifying whether the sequence was concealed until interventions were assigned.	5
Randomization --	10	Who generated the allocation sequence, who enrolled **study units,** and who assigned **study units** to their groups **at the relevant level of the organizational structure**.	5

Implementation			
Blinding (masking)	11	Whether or not ~~**participants**~~ those administering the interventions, **caregivers** and those assessing the outcomes were blinded to group assignment. If done, how the success of blinding was evaluated**. Provide justification for not using blinding if it was not used.**	5
Statistical methods	12	Statistical methods used to compare groups for all outcome(s); Clearly state the level of statistical analysis **and methods used to account for the organizational structure, where applicable;** methods for additional analyses, such as subgroup analyses and adjusted analyses.	11
Results **Study** flow	13	Flow **of study units** through each stage **for each level of the organization structure of the study** (a diagram is strongly recommended). Specifically, for each group, report the numbers of **study units** randomly assigned, receiving intended treatment, completing the study protocol, and analyzed for the primary outcome. Describe protocol deviations from study as planned, together with reasons.	11-16
Recruitment	14	Dates defining the periods of recruitment and follow-up.	NA
Baseline data	15	Baseline demographic and clinical characteristics of each group, **explicitly providing information for each relevant level of the organizational structure. Data should be reported in such a way that secondary analysis, such as risk assessment, is possible.**	
Numbers analyzed	16	Number **of study units** (denominator) in each group included in each analysis and whether the analysis was by "intention-to-treat." State the results in absolute numbers when feasible (eg, 10/20, not 50%).	11

Outcomes and estimation	17	For each primary and secondary outcome, a summary of results for each group, **accounting for each relevant level of the organizational structure**, and the estimated effect size and its precision (e.g., 95% confidence interval)	11-16
Ancillary analyses	18	Address multiplicity by reporting any other analyses performed, including subgroup analyses and adjusted analyses, indicating those pre-specified and those exploratory.	11-16
Adverse events	19	All important adverse events or side effects in each intervention group.	11
Discussion Interpretation	20	Interpretation of the results, taking into account study hypotheses, sources of potential bias or imprecision, and the dangers associated with multiplicity of analyses and outcomes. **Where relevant, a discussion of herd immunity should be included. If applicable, a discussion of the relevance of the disease challenge should be included.**	11-16
Generalizability	21	Generalizability (external validity) of the trial findings.	16
Overall evidence	22	General interpretation of the results in the context of current evidence.	16

CHAPTER 3

INTERPRETIVE SUMMARY. Buse et al. (2020). “The effect of a unique high omega 3 fatty acid supplement on milk fatty acid profile and energy utilization of lactating Jersey cows,” This article described an experiment in which increasing inclusion of a high omega 3 fatty acid supplement increased milk fat concentration with the milk fat concentration of α-linolenic acid increasing and linoleic acid decreasing. Increasing inclusion of the product also increased gross energy, but because of decreasing digestibility of fatty acids with increasing inclusion, digestible energy decreased to result in no difference in metabolizable energy. These results suggest that the high omega 3 fatty acid product does shift the profile of milk fat to favor α-linolenic acid, which is healthier from a human health perspective, without adversely affecting metabolizable energy.

RUNNING HEAD: OMEGA 3 FATTY ACID SUPPLEMENTATION

The effect of a unique high omega 3 fatty acid supplement on milk fatty acid profile and energy utilization of lactating Jersey cows

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ABSTRACT

Omega 3 fatty acids are polyunsaturated fatty acids that play an important role in human health by reducing inflammation within the body. To improve dietary consumption of omega 3 fatty acids, enrichment of dairy products through selective feeding of feedstuffs containing a high concentration of omega 3 fatty acids to dairy cows may be an option. Four multiparous Jersey cows (115 ± 36 d in milk) were used in a 4 × 4 Latin square with 4 periods of 28 d (24 d adaptation and 4 d collection) to analyze the effect of feeding a high fatty acid supplement on the milk fatty acid profile and energy utilization. Cows were randomly assigned to 4 different diets ranging from 0 to 20.0% inclusion of Perfect Omega 3 (**PO3**) (Sunseo Omega 3; Chungcheong Buk-Do, South Korea), a feed supplement comprised of sesame meal, giant kelp, cassava, and sorghum. With increasing inclusion of PO3, dry matter intake tended to increase linearly from 21.8 to 24.1 ± 1.41 kg/d, while milk yield did not differ (averaging 31.4 ± 0.37 kg/d). Milk fat percentage increased linearly from 5.30 to 5.82 ± 0.35% with increasing inclusion, and milk fat yield tended to increase linearly from 1.66 to 1.85 ± 0.20 kg/d. Increasing inclusion of PO3 resulted in a linear increase in the concentration of α-linolenic acid in milk fat from 0.24 to 0.72 ± 0.04 g/100 g of milk fat as well as a linear decrease in linoleic acid from 2.61 to 2.23 ± 0.09 g/100 g milk fat. The increasing inclusion of PO3 linearly increased the gross energy content of the diet, but there was no difference among diets in digestible energy and metabolizable (averaging 60.9 ± 2.67 and 53.9 ± 2.57 Mcal/kg of DM). Total fatty acid digestibility linearly increased 57.0 to 67.9 ± 2.40% with increasing PO3 inclusion, but both α-linolenic acid and linoleic acid digestibility linearly decreased. The results of this study indicate that increasing inclusion of PO3

maintains milk production while increasing the concentration of milk fat, with a greater concentration of omega 3 fatty acids.

Key Words: polyunsaturated fatty acids, energy utilization, indirect calorimetry

INTRODUCTION

Omega 3 fatty acids (**FA**) play an important role in human health by lowering blood pressure, inflammation, and blood triacylglycerol concentrations, which reduces the risk of cardiovascular disease (Calder, 2004). In infants, consumption of the omega 3 FA, docosahexaenoic acid (**DHA**), has been deemed essential for normal visual, neural, and behavioral development, and in some instances, DHA and eicosapentaenoic acid (**EPA**) can slow cancerous tumor growth (CAST, 2018). In dairy cattle, supplementary omega 3 FA have shown to have positive effects on fertility (Gulliver et al., 2012). Alpha-linolenic acid (**ALA**), which is a plant-based omega 3 FA is converted to EPA and then to trienoic prostaglandins (Petit et al., 2002). This may decrease pregnancy loss (Dirandeh et al., 2013) and improve embryo quality (Leroy et al., 2013). As important as omega 3 FA are, many individuals who consume Western diets do not meet their daily requirements; The American Heart Association (**AHA**) recommends healthy individuals consume 1.8 g of omega 3 FA per d. This recommendation is greater for those with cardiovascular illness (Krauss et al., 2000). To help improve dietary consumption of omega 3 FA, selective breeding of animals and manufacturing procedures may be used as well as selective feeding of high FA feedstuffs to animals to alter the FA composition of products of the livestock industry (Ashes et al., 1997, CAST, 2018).

The high FA supplement, “Perfect Omega 3” (**PO3**), is comprised of a mixture of sesame meal, giant kelp, cassava, and sorghum. Both sesame meal and giant kelp have

high concentrations of ALA. However, with the exception of kelp, most of these feedstuffs aren't commonly used in the United States. Studies on the effects of these ingredients is limited, and to the authors' knowledge, there is no research on a combination of these ingredients. Also, when attempting to manipulate the FA profile of milk, it is important to understand production responses and changes in energy utilization in response. The objective of this experiment was to evaluate the inclusion of PO3 on the FA profile of milk and energy utilization of dairy cows. We hypothesized that as the proportion of PO3 increased in the ration, the concentration of ALA would also increase in the milk fat.

MATERIALS AND METHODS

Animals and Treatments

Animal care and experimental procedures were approved by the University of Nebraska–Lincoln Animal Care and Use Committee. Four multiparous Jersey cows averaging 115 ± 36 DIM at the beginning of the experiment were used for the study. Cows were housed in individual tie-stalls equipped with rubber mats in a temperature-controlled (20°C) barn at the Dairy Metabolism Facility in the Animal Science Complex at the University of Nebraska–Lincoln and milked at 0700 and 1800 h. Cows used were 96, 99, 102, and 111 d pregnant at the end of the last experimental period. Because all the cows were under 190 d pregnant, fetal energy was assumed to be zero (NRC, 2001).

The experimental design was a 4 × 4 Latin square with 4 28 d periods. The product, Perfect Omega 3, is comprised of 35% sesame meal, 24% giant kelp, 24% cassava, and 17% sorghum. Cows were randomly assigned to one of 4 treatments: Control Diet, 0% Perfect Omega 3 (**CON**); Low Diet, 6.67% Perfect Omega 3 (**LPO3**);

Medium Diet, 13.3% Perfect Omega 3 (**MPO3**); and High Diet, 20% Perfect Omega 3 (**HPO3**). Dietary ingredients for the diets (corn silage, alfalfa hay, and concentrate,) were added to a Calan Data Ranger (American Calan, Inc., Northwood, NH), mixed, and fed as a TMR once daily at 0930 h with a target refusal rate of 5%. Each period included 24 d of ad libitum diet adaptation, followed by 4 d of collection where diets were fed at 100% of the previous 7 d intake to limit refusal.

Sample Collection and Analysis

Individual feed ingredients were sampled daily during collection periods and frozen at −20°C. Corn silage was dried at 60ºC for 48 h, and all feeds were ground to pass a 1-mm screen (Wiley Mill; Arthur A. Thomas Co., Philadelphia, PA). Ground feed samples were analyzed for N (Equipment), NDF with sodium sulfite (Van Soest et al., 1991) and α amylase and corrected for ash contamination (**NDF_{OM}**), and ash. A subsample of ground feed was sent to Cumberland Valley Analytical Services Inc. (Waynesboro, PA) for analysis of starch (Hall, 2009) and FA profile (Sukhija and Palmquist, 1988). Additionally, feed ingredients were analyzed for gross energy (**GE**) content (Parr 6400 Calorimeter, Moline, IL) in the nutrition laboratory of the University of Nebraska-Lincoln and were subjected to the mobile bag assay as outlined by Paz et al. (2014) to determine protein digestibility. Total mixed rations were sampled on d 1 of each collection period and used to determine particle size using the Penn State particle separator (Heinrichs and Kononoff, 2002) on an as-is and DM basis (60ºC for 48 h). During each d of the collection period, refusals were sampled and composited on a weight basis. Refusals were analyzed for N, NDF, NDFom, starch, ash, FA, and GE via the same methods as feeds.

Total fecal and urine output was collected from each individual cow during the collection period for 4 consecutive d. A 137 × 76-cm rubber mat was placed behind the cow to aid in fecal collection. Feces were manually collected by personnel during defecation or were picked up from the rubber mat and deposited into a trash can (Rubbermaid, Wooster, OH) with a trash bag covering the top to minimize N losses prior to subsampling. Daily feces were subsampled (~500 g, as-is), composited on a weight basis, and frozen between collection events. After collections, feces were dried at 60°C for 48 h and ground to pass through a 1-mm screen (Wiley Mill, Arthur H. Thomas Co., Philadelphia, PA). The ground feces samples were analyzed as described for refusals. Total urine was collected by inserting a 30 French foley catheter into each cow's bladder with a stylus. The balloon was inflated to 55 mL with physiological saline. The catheter was drained into a 55-L plastic container via Tygon tubing (Saint Gobain, La Defense, Courbevoie, France). Acid (50% HCl) was added to the urine collection container at the beginning of the collection d to maintain a pH < 5. Urine was subsampled daily and composited on a wet weight basis. Urine samples were frozen (−20°C) until analysis for GE and N as described previously.

Milk production was measured daily, and milk samples were collected during both the morning and evening milking of the collection periods. Milk from individual milkings was preserved with 2-bromo-2-nitropropane-1,3 diol and sent to Heart of America DHIA (Kansas City, MO). Milk samples were analyzed for fat, protein, lactose, SNF, MUN, and SCC using a Bentley FTS/FCM Infrared Analyzer (Bentley Instruments, Chaska, MN). Additionally, milk from each milking event was composited on a weight basis. Composited milks samples were analyzed for gross energy (**GE**) and N as

described previously for urine. An additional 15 mL composite of milk was taken 1 d of the 4 d collection period to be analyzed for FA content. These composites were sent to The Pennsylvania State University and analyzed according to Baldin et al. (2019) with the modification of 1 mL of milk substituted for 1 mL of sodium sulfate solution in the extraction instead of starting with fat cake.

Heat production was determined through the headbox-type indirect calorimeters as described previously (Freetly et al., 2006, Foth et al., 2015). For each cow, a collection period of 23-h was used to measure O_2 consumption as well as CO_2 and CH_4 production. Feed was placed in the bottom of the headbox, and cows were allowed ad libitum access to water from a water bowl placed inside the headbox. Free water intake was measured using a water meter (Model DLJSJ75, Daniel L. Jerman Co., Hackensack, NJ) while each cow was inside the headbox. Within the headbox, temperature and dew point were measured every minute during the 23-h collection interval using a probe (Model TRH-100, Pace Scientific Inc., Moorseville, NC) and recorded using a data logger (Model XR440, Pace Scientific Inc.). Line pressure was measured using a u-tube manometer (Item # 1221–8, Park Supply of America, Inc., Minneapolis, MN) and barometric pressure of the room was measured using a barometer (Chaney Instruments Co., Lake Geneva, WI). Total volume of gas flow through the headbox was measured using a gas meter (Model AL425, American Meter, Horsham, PA) and corrected to standard temperature and pressure (0 ºC, 760 mmHg) with adjustment for moisture content of exhaust air (Nienaber and Maddy, 1985). In addition to volumetric flow meters, mass flow meters were also used (MCW Whisper, Alicat Scientific, Tucson, AZ). From the headbox, continuous samples of incoming and outgoing air were collected into separate

bags (44 L, LAM-JAPCON-NSE; Pollution Measurement Corp., Oak Park, IL) using glass tube rotameters (Model 1350E Sho-Rate "50," Brooks Instruments, Hatfield, PA). Gas bags were analyzed for oxygen (O_2), carbon dioxide (CO_2), and methane (CH_4) using an Emerson X-stream 3-channel analyzer (Solon, OH) according to the method of Nienaber and Maddy (1985). Heat production was estimated as follows (Brouwer, 1965):

$$\text{Heat production (HP, kcal/d)} = 3.866 \times O_2\text{ (L/d)} + 1.200 \times CO_2\text{ (L/d)} - 0.518 \times CH_4\text{ (L/d)} - 1.431 \times \text{Urinary N excretion (g/d)} \quad [1]$$

Respiratory quotient (**RQ**) was calculated using the ratio of CO_2 produced to O_2 consumed. Methane energy was estimated by multiplying CH_4 production by its enthalpy (9.45 kcal/L). Tissue energy was calculated as follows (Freetly et al., 2006, van Knegsel et al., 2007):

$$\text{Tissue energy (TE; Mcal/d)} = \text{ME (Mcal/d)} - \text{HP (Mcal/d)} - \text{Milk energy (Mcal/d)} \quad [2]$$

Statistical Analysis

Data were analyzed using the GLIMMIX procedure in SAS (9.4). The model included the fixed effect of dietary treatment as well as the random effect of cow and period. Linear, quadratic, and cubic effects of concentration of Perfect Omega 3 in the diets were tested. All data are presented as least-squares means ± largest standard error.

RESULTS

Diet Composition

Perfect Omega 3 (**PO3**) contained 88.1% DM, 35.8% CP, 35.9% NDF, and 11.5% starch (DM basis; Table 3.1). The total fatty acids (TFA) in this feed was 7.81% of DM; while 16 carbon FA was 0.82, 18 carbon FA was 6.86, ALA is 1.78, and LA was 2.20% of DM. Perfect Omega 3 has a rumen DM digestibility of 52.2 ± 1.12%, RUP content as a portion of CP of 71.3%, total tract DM digestibility of 78.6 ± 1.05%, 81.4 ± 2.49% total tract CP digestibility, and a RUP digestibility of 73.5 ± 6.55%, as determined by the mobile bag assay (Paz et al., 2014; Table 3.1). Perfect Omega 3 was included at 0%, 6.67%, 13.3%, and 20.0% of the total diet for CON, LPO3, MPO3, and HPO3, respectively, with a portion of dry ground corn, corn DDGS, and soybean meal being replaced. Remaining ingredients were included at similar inclusion rates across diets. Increasing inclusion of PO3 increased CP from 17.5 to 18.1 and NDF from 26.9 to 27.3% DM (Table 3.2). Additionally, with increasing inclusion of PO3, total FA increased from 4.18 to 5.81%, 16 C FA increased from 1.11 to 1.51%, and 18 C FA increased from 2.69 to 3.84%. Both ALA (0.18 to 0.19) and LA (0.95 to 1.15) also increased with increased PO3 inclusion. Diet particle size was not different across diets, as listed in Table 3.2.

Feed Intake, Milk Production and Composition, and Water Intake

During the last period of the study, one cow was diagnosed with mastitis (4502 SCC, 10^3 cells/ml), so all data for that cow during that period was excluded from analysis. Therefore, 15 out of the 16 observations were used for analysis.

Dry matter intake tended ($P = 0.11$) to increase linearly from 21.8 to 24.1 ± 1.41 kg/d with increasing inclusion of PO3 (Table 3.3). Milk yield did not differ across

treatments ($P > 0.16$, averaging 31.4 ± 0.37 kg/d). Milk fat percentage increased linearly ($P = 0.02$) from 5.30 to 5.82 ± 0.35% with increasing PO3 inclusion, while milk fat yield tended ($P = 0.15$) to increase linearly from 1.66 to 1.85 ± 0.20 kg/d. While LA linearly decreased ($P < 0.01$) with increasing PO3 inclusion from 2.61 to 2.23 ± 0.086 g/100 g of milk, ALA linearly increased ($P < 0.01$) from 0.24 to 0.72 ± 0.036 g/100 g of milk (Table 3.4). No differences were observed in milk protein concentration ($P > 0.30$, averaging 3.73 ± 0.03) as well as milk protein yield ($P > 0.30$, averaging 1.19 ± 0.02).

Energy Partitioning

No differences ($P > 0.26$) were observed in O_2 consumption, CO_2, and CH_4 production with increasing inclusion of PO3. Methane produced per kilogram of DMI, ECM, OM digested, and NDFom digested also did not differ ($P > 0.45$) among treatments. However, a cubic response ($P = 0.07$) in RQ was observed with an increase from CON to LPO3, a decrease to MPO3, and then an increase to HPO3.

Increasing inclusion of PO3 linearly increased ($P = 0.07$) the GE content of the diet from 90.1 to 101 ± 6.04 Mcal/d, but there was no difference ($P > 0.19$) among diets in DE and ME (averaging 60.9 ± 2.67 and 53.9 ± 2.57 Mcal/d; Table 3.5). These same effects are reflected in these measures of energy expressed as Mcal/kg of DM. Total fecal energy excretion linearly increased ($P = 0.02$) with increasing inclusion of PO3 from 32.8 to 38.1 ± 1.52 Mcal/d, but urine energy tended to quadratically ($P = 0.12$) increase from CON (2.57 ± 0.14 Mcal/d) to LPO3 (2.92 ± 0.14 Mcal/d) and then decrease to MPO3 (2.77 ± 0.14 Mcal/d). Milk energy tended ($P = 0.15$) to linearly increase from 28.7 to 29.9 ± 2.64 Mcal/d. There was no difference ($P > 0.20$) in methane (4.17 ± 0.16 Mcal/d),

HP (both Mcal/d and kcal/metabolic BW; 24.6 ± 0.26 Mcal/d and 230 ± 3.30 kcal/metabolic BW), and TE (0.33 ± 2.49 Mcal/d).

With increasing inclusion of PO3 in the diet, the efficiency of ME/DE linearly increased ($P = 0.05$) from 0.88 to 0.89 ± 0.005. A tendency to quadratically ($P = 0.15$) decrease from CON to LPO3 then increase to MPO3 was observed in milk/ME efficiency, and TE/ME had a cubic tendency ($P = 0.15$) to increase from CON to LPO3, then decrease to MPO3, and increase to HPO3. No difference ($P > 0.16$) was observed in HP/ME (0.46 ± 0.02; Table 3.5).

Nitrogen Balance

Increasing inclusion quadratically decreased ($P = 0.02$) fecal output from CON to LPO3 and increased to MPO3 (Table 3.6). Urine output cubically ($P = 0.10$) decreased from CON to LPO3, increased to MPO3, and decreased to HPO3. Nitrogen intake linearly increased ($P = 0.02$) from 589 to 694 ± 36.2 g/d as well as fecal N excreted from 203 to 254 ± 9.89 g/d ($P < 0.01$). Urinary N excreted quadratically ($P = 0.01$) increased from CON to LPO3 and then decreased to HPO3. A cubic tendency ($P = 0.12$) to increase from CON to LPO3, decrease to MPO3, and increase to HPO3 was observed in retained N. No difference ($P > 0.52$) was observed in milk N (averaging 210 ± 1.71 g/d). The proportion of urinary N per unit of N intake linearly decreased ($P = 0.08$) from 34.0 to 29.1 ± 1.83%.

Nutrient Digestibility

No difference ($P > 0.39$) was observed in DM and OM digestibility (averages of 64.9 ± 0.45 and 67.4 ± 0.45%; Table 3.7). Starch digestibility tended ($P = 0.13$) to cubically decrease with increasing inclusion of PO3 from CON to LPO3, increase to

MPO3, and then decrease to HPO3. A linear decrease ($P = 0.05$) from 65.3 to 62.3 ± 1.46% was observed in CP digestibility. There was also no difference ($P > 0.38$) observed in NDF (38.2 ± 1.85%) and NDFom (38.4 ± 4.24%) digestibility. Energy digestibility (average of 62.7 ± 0.65%) was not affected by treatment ($P > 0.35$). Total FA digestibility linearly increased ($P = 0.01$) from 57.0 to 67.9 ± 2.40%. With increasing inclusion of PO3, 16 C FA digestibility linearly increased ($P = 0.01$) from 61.6 to 72.5 ± 2.37%, and 18 C FA digestibility tended to cubically ($P = 0.12$) increase from CON to LPO3, decrease to MPO3, and then increase to HPO3. Both ALA (98.1 to 95.5 ± 0.46%) and LA (97.0 to 95.5 ± 0.49%) digestibility linearly decreased ($P < 0.07$).

DISCUSSION

Long-chain omega 3 FA are known to have an anti-inflammatory effect in humans and may contribute to reducing the risk of cardiovascular disease, but many individuals don't meet the daily recommendation of 1.8 g/d (Krauss et al., 2000; CAST, 2018). To improve dietary consumption, enrichment of dairy products through selective feeding of high FA feedstuffs to dairy cows may be an option (CAST, 2018). The high FA supplement, Perfect Omega 3, is comprised of a mixture sesame meal, giant kelp, cassava, and sorghum. Both sesame and cassava are typically grown in Asia or South America mostly for human consumption, but the byproducts from processing are used as feed for livestock (Carter et al., 1960; Howeler, 2020). On the other hand, the practice of using sorghum and giant kelp in rations is not a foreign concept in the United States. Kelp is often included in organic dairy rations during grazing periods in the Northeast as a source of several macro- and microminerals as well as PUFA and vitamins (Antaya, 2016). Sorghum is an important crop in parts of Africa and Asia (Turhollow et al., 2010),

and in the United States, 6.7 million acres are planted to sorghum with 62% used for livestock feed (Laingen, 2015). Of these feeds includeed in the supplement, both sesame meal and kelp have high concentrations of ALA (0.40 and 0.74% of DM, respectively) (van Ginneken et al., 2011; Feedinamics, 2018). Sesame meal also has a high concentration of CP (45.2% of DM: Feedinamics, 2018), and giant kelp has a high concentration of NDF (53.9% of DM; Antaya et al., 2015). Sorghum also contributes some ALA (0.13% of DM) as well as CP (11.6% of DM; Price and Parsons, 1975). The purpose of this study was to evaluate the effect of the high FA supplement, Perfect Omega 3, on milk FA profile and energy utilization.

Diet composition

Total FA increased with increasing inclusion of PO3. This same response was seen in ALA and LA (Table 3.2). Alpha-linolenic acid is the precursor for EPA and DHA; in contrast, LA is the precursor to arachidonic acid, which is converted to inflammation-causing 2-series PG (Gulliver et al., 2012). Both corn and soybeans have a higher LA content than ALA. According to the NRC (2001), corn has a FA composition of 58.0% LA and 0.7% ALA, and soybeans is 51% LA and 6.8% ALA. Dried corn distillers grains has an LA and ALA content of 49.0 and 1.8%, respectively (Royon, 2012). Increasing the concentration of these ingredients in the diet and replacing them with PO3 shifts the omega 3 to omega 6 ratio to favor omega 3.

Milk fatty acid profile

Several studies have been conducted with the goal of manipulating the FA profile of milk to target increasing FA that are believed to have a positive effect on human health (Palmquist. In the present study, increasing inclusion of PO3 increase ALA from 0.24 to

0.72 g/100 g of milk fat while decreasing LA from 2.61 to 2.23 g/100 g milk fat. Because the formulation of PO3 is unique, we are unable to directly compare our results to other published studies. However, published studies exist that examine the individual ingredients included in the formulation. Qussay et al. (2015) fed sesame meal at 10 and 20% of diet DM in complete replacement of soybean meal as well as displacing a portion of barley, wheat, and corn grain. While the milk FA profile was not reported, increasing the amount of sesame meal in the diet increased milk fat concentration from 3.18 to 3.82% and milk fat yield from 1.66 to 1.85 kg/d. Several factors, such as forage source and type of fat in the diet, could have caused their observed results, but the response was attributed to an increase in the digestibility of ether extract due to the presence of sesame hulls, which decreased the rate of starch digestion in the rumen and lead to a decrease in milk yield. The digestibility of any aspects of the diets in this study were not reported, so it is difficult to determine if observed changes are due to difference in ether extract digestibility. But in the present study, a decrease in starch digestibility with increasing inclusion was observed, but milk yield was not affected, likely because of the increase in TFA digestibility.

In the present study, increasing PO3 from 0 to 20% of the diet DM resulted in an increase of ALA from 3.98 to 13.3 g/d of ALA, respectively. Contrary to this observation, Antaya et al. (2015) supplemented a TMR with a kelp meal top-dress of 57, 113, or 170 g to grazing cattle and observed no difference in ALA content of milk fat. The observed difference of the current study may have been in response to increases in TFA digestibility. Additionally in the current study, LA yield decreased from 80.9 to 71.1 g/d with increasing inclusion of PO3 in the diet. This response is more difficult to

explain, but it may have been due to a dilution effect and the increases in ALA. Studies that observed a decrease in LA concentration in milk (Abu-Ghazaleh et al., 2001;Sulistyowati et al., 2010) also supplied less LA in the diets, which is opposite of the current study since LA supply increased with increasing PO3 inclusion. A possible explanation could be the replacement of corn dried distiller's grains (**DDGS**) in the diet as PO3 inclusion increased. In a study where corn DDGS was fed at 10 or 20% of diet DM, a higher concentration of LA in milk fat was observed with the highest inclusion of DDGS (Anderson et al., 2006). The same response was observed in another study where corn DDGS were fed from 5 to 10% of diet DM (Leonardi et al. 2012). It is possible that the LA in corn DDGS is more protected, so it escapes biohydrogenation within the rumen. In the present study, even though the supply of LA increased, the decreasing concentration of corn DDGS as PO3 concentration increased supplied less protected LA, making less LA available to be incorporated into milk fat. In a finishing study with crossbred steers, Norman et al. (2020) observed that increasing inclusion of PO3 in diets up to 30% of diet DM, with dry-rolled corn being displaced, increased LA concentration by 43.1% in the steak samples, but overall omega 6 concentration decreased by 65.5%.

The AHA recommends an omega 3 FA intake of 2 servings of fatty fish a week or 1.8 g/d. Typically, milk has an omega 3 FA concentration of 0.56% of TFA (Markiewicz-Keszvcka et al., 2013) with ALA being the most abundant at 0.38% of TFA (O'Donnell-Megaro et al., 2011). Using these average values as well as the recommendation of the Dietary Guidelines for Americans (2010) of three servings of dairy per day, if all three servings were consumed as 8 oz (237 mL) of 2% fat milk, only 0.03 g/d of ALA would be consumed. With the values obtained through the present study, 0.03 g/d would also be

provided by CON and 0.09 g/d would be provided by HPO3. Consuming whole milk, which contains 3.5% fat, would increase that amount to 0.06 and 0.18 g/d for CON and HPO3, receptively. To reach the daily recommendation set by the AHA, 14.2 L of 2% milk, 7.11 L of whole milk, 0.30 kg butter (83% fat; Scherr and Ribeiro, 2009), or 1 kg of cheddar cheese (25% fat; Scherr and Ribeiro, 2009) would need to be consumed, if it has the same ALA content as HPO3. Since it is unpractical to meet the daily omega 3 FA requirement by consuming dairy alone, other sources of omega 3 FA, such as fish, should also be included in diets.

Nutrient Digestibility

With increasing inclusion of PO3, CP digestibility decreased from 60.6 to 57.4%. Kelp is a rich source of phlorotannins (Connan et al., 2004), which, like terrestrial tannins, may bind proteins and carbohydrates (Ragan and Glombitza, 1986). When supplemented kelp meal, grazing Jersey cows decreased N digestibility from 70.0% with no supplementation to 67.5% with 113 g/d of kelp meal (Antaya et al., 2019). Because of these results, the decrease in CP digestibility in the present study may have been caused by the presence of phlorotannins in the kelp meal. Another explanation could be that CP in PO3 is less digestible than the CP in the corn DDGS that were displaced. The Mobile Bag assay was used to determine that PO3 has an RUP digestibility of 73.5%. According to the NRC (2001), corn DDGS has an RUP digestibility of 80.0%.

Increasing FA digestibility with increasing PO3 content in the diet was also observed; however, both ALA and LA digestibility decreased. Within the rumen, FA are associated with feed particles and need bile salts to solubilize them before they can be incorporated into micelles and absorbed in the small intestine (Palmquist 1991). This

means that factors such as degree of saturation or protection can affect the solubility of FA and thus their digestibility (Glasser, 2008). Several studies have observed decreased FA digestibility with increasing levels of saturation (Pantoja et al., 1996; Harvatine and Allen, 2006). The response observed in the current study could be caused by an increasing supply of unsaturated FA with increasing concentration of PO3. The inverse response in ALA and LA digestibility is more difficult to explain. Within the rumen, hydrogenation of LA is between 70 and 95%, which increases with increasing concentration (Doreau and Ferlay, 1994). Increasing biohydrogenation leads to decreasing digestibility (Wu et al., 1991). Since the concentration of LA increased in the diet with increasing inclusion of PO3, LA digestibility could have decreased due to increased biohydrogenation. Unlike LA, there is no relationship between the concentration of LA and extent of biohydrogenation (Doreau and Ferlay, 1994). It is possible that the decrease in ALA is a residual effect of increased LA concentration, but the exact cause is unknown.

Energy Partitioning

Increasing the proportion of PO3 in the diet increased the concentration of TFA from 4.18 to 5.81%, which explains the increase in GE in Mcal/kg of DM from 4.14 to 4.20. Also with increasing inclusion of PO3, DE was similar because of decreasing digestibility of TFA. The combination of the responses observed in GE and DE lead to no difference in ME. In a study by Judy et al. (2019) where extruded flaxseed was fed to increase ALA in the diet, no difference was observed in DE because no differences in DMI and digestibility were observed. Increased DMI along with increasing TFA supply

with increasing inclusion lead to the increase in fecal energy while the increase observed in milk energy was the result of the increasing milk fat concentration.

Nitrogen Balance

Nitrogen intake increased with increasing PO3 inclusion, which is attributed to an increase in DMI and diet CP content. The combination of increased N intake and no difference in milk N led to a decrease in milk N efficiency. The observed increase in fecal N was also due to an increase in DMI with increasing PO3 inclusion as well as the combination of increasing diet CP content and reduced CP digestibility. The quadratic response observed in urinary N could partially be due to the cubic response observed in urine output. The increasing proportion of N intake as urinary N could also be an explanation for the observed quadratic response. Limited results on the effect of feeding high FA feedstuffs on N partitioning are available since the primary focus of studying these feedstuffs is not on N. However, flaxseed, when compared to micronized soybeans and another commercial source of dietary fat, had a higher N intake as well as N output in feces, urine, and milk (Petit, 2002). Overall, since PO3 has similar digestibility to the feedstuffs it replaced, the increase in diet N content with increasing PO3 inclusion is the cause of the majority of the observed responses.

CONCLUSION

With the importance of omega 3 FA in human health, there is interest in increasing human consumption through altering milk composition by selectively feeding high omega 3 FA feedstuffs to dairy cows. The objective of this study was to evaluate the effect of the high FA supplement PO3 on the milk FA and energy utilization of lactating dairy cows. With increasing inclusion of PO3, DMI and milk fat concentration increased,

with favor towards increasing ALA while decreasing LA, without altering milk yield. Increasing inclusion of PO3 increased GE but did not affect DE or ME as well as gas consumption and production. Digestibility of FA increased with increasing inclusion, which supported an increase in milk fat concentration and is likely the result of higher concentrations of unsaturated FA.

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TABLES AND FIGURES

Table 3.1. Chemical composition of corn silage, alfalfa hay, concentrate mixes, and Perfect Omega 3 (% of DM)[1,2,3]

	Corn silage		Alfalfa hay		Control Mix		High Mix		PO3	
Item	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
DM, % as-is	38.0	0.01	88.7	0.01	91.5	0.02	91.2	0.01	88.1	-[4]
CP	8.48	0.77	20.4	1.37	20.9	0.95	24.7	0.79	35.8	-
NDF	31.7	0.87	41.3	3.93	18.8	1.84	19.49	5.25	35.9	-
NDFom	30.2	0.88	40.0	3.42	18.0	1.92	17.3	5.18	-	-
Starch	44.8	2.27	1.29	0.21	27.6	0.66	28.6	0.82	11.5	-
Fatty acids										
Total fatty acids	4.71	0.78	1.67	0.12	4.50	0.13	7.94	0.06	7.81	-
16 carbon fatty acids	1.17	0.25	0.43	0.02	1.25	0.05	2.10	0.06	0.82	-
18 carbon fatty acids	3.13	0.50	0.97	0.09	2.84	0.07	5.26	0.07	6.86	-
ALA	0.19	0.01	0.18	0.01	0.17	0.03	0.19	0.04	1.78	-
LA	1.00	0.04	0.59	0.06	1.02	0.07	1.44	0.08	2.20	-
Ash	4.91	0.26	10.6	0.54	10.8	0.91	11.4	0.99	11.2	-
Rumen DMD[5]	-	-	-	-	-	-	-	-	52.2	1.12
RDP, % of CP	-	-	-	-	-	-	-	-	28.7	8.25
RUP, % of CP	-	-	-	-	-	-	-	-	71.3	8.25
TTDMD[6]	-	-	-	-	-	-	-	-	78.6	1.05
TTCPD, % CP[7]	-	-	-	-	-	-	-	-	81.4	2.49
RUPd, % RUP[8]	-	-	-	-	-	-	-	-	73.5	6.55

[1]n = 4 for corn silage, alfalfa hay, control mix, and high mix.
[2]n = 1 for Perfect Omega 3.
[3]Perfect Omega 3 (Sunseo Omega Inc., Chungcheongbuk-do, Korea).
[4]Not determined (-) .
[5]Rumen DMD = rumen dry matter digestibility
[6]TTDMD = total tract dry matter digestibility
[7]TTCPD = total tract crude protein digestibility
[8]RUPd = rumen undegraded protein digestibility

Table 3.2. Chemical composition and particle size distribution of each TMR fed to experiment cows (% of DM)

	Treatments[1]			
Items	**CON**	**LPO3**	**MPO3**	**HPO3**
Ingredients				
Corn silage	38.5	38.5	38.5	38.5
Alfalfa hay	14.1	14.1	14.1	14.1
Perfect Omega 3[2]	0	6.67	13.3	20.0
Corn grain, ground	16.0	15.3	14.7	14.0
Soypass	2.82	2.73	2.65	2.56
Corn DDGS[3]	10.3	6.87	3.43	0
Soybean meal	9.40	7.11	4.84	2.56
Soybean hulls, ground	1.79	1.76	1.74	1.72
Molasses, Beet	1.23	1.23	1.23	1.23
Fat[4]	1.87	1.87	1.87	1.87
Rumen Protected LYS[5]	0.41	0.41	0.41	0.41
Rumen Protected MET[6]	0.11	0.11	0.11	0.10
Mineral-vitamin mix[7]	3.42	3.42	3.42	3.42
Chemical composition				
DM	60.5 (1.66)	61.3 (3.07)	60.4 (1.63)	60.4 (1.26)
Ash	8.65 (0.42)	7.77 (0.36)	7.83 (0.47)	8.49 (0.46)
CP	16.9 (0.58)	17.3 (0.40)	17.6 (0.32)	18.0 (0.42)
NDF	26.9 (1.74)	27.0 (1.32)	27.1 (1.58)	27.3 (2.29)
Starch	30.5 (0.85)	30.7 (0.95)	30.8 (1.06)	31.0 (1.19)
Fatty acids				
Total fatty Acids	4.18 (0.27)	4.72 (0.28)	5.27 (0.29)	5.81 (0.31)
16 carbon fatty acids	1.11 (0.09)	1.24 (0.09)	1.37 (0.09)	1.51 (0.10)
18 carbon fatty acids	2.69 (0.18)	3.07 (0.18)	3.45 (0.18)	3.84 (0.19)
ALA	0.18 (0.01)	0.18 (0.01)	0.19 (0.01)	0.19 (0.02)
LA	0.95 (0.05)	1.02 (0.05)	1.08 (0.05)	1.15 (0.05)
Particle Size				

>19.0 mm, % as-is	1.7 (0.39)	1.3 (0.43)	1.8 (0.47)	1.7 (0.56)
8.0–19.0 mm, % as-is	23.9 (3.90)	25.8 (4.64)	24.9 (4.73)	24.5 (5.06)
1.18–8.0 mm, % as-is	31.4 (2.93)	31.5 (2.91)	31.4 (2.91)	30.6 (3.36)
<1.18 mm, % as-is	21.4 (2.57)	19.5 (2.62)	20.7 (3.28)	19.2 (1.98)
>19.0 mm, % of DM	4.9 (0.30)	4.9 (0.30)	4.9 (0.38)	4.8 (0.38)
8.0–19.0 mm, % of DM	20.6 (2.31)	20.5 (2.19)	20.8 (2.06)	20.5 (2.47)
1.18–8.0 mm, % of DM	33.1 (2.84)	32.9 (2.59)	32.9 (2.61)	31.8 (2.65)
<1.18 mm, % of DM	24.8 (2.90)	24.5 (3.00)	24.1 (3.60)	22.4 (1.83)

[1]CON = Control Diet (0% Perfect Omega 3); LPO3 = Low Diet (6.67% Perfect Omega 3); MPO3 = Medium Diet (13.3% Perfect Omega 3); HPO3 = High diet (20.0% Perfect Omega 3).
[2]Perfect Omega 3 (Sunseo Omega Inc., Chungcheongbuk-do, Korea).
[3]DDGS = dried distillers grains with solubles.
[4]Energy Booster (Milk Specialties, Eden Prairie, MN).
[5]AjiPro (Ajinomoto Co., Inc., Tokyo, Japan).
[6]Smartamine (Adisseo, Alpharetta, GA).
[7]Contained per kilogram of premix: 393 g of CaCO3, 234 g of $NaCO_3$, 179 g of salt, 97 g of MgO, 69 g of CaPO4, 14 g of vitamin premix (14,850 IU/g vitamin A, 3,850 IU/g vitamin D, and 90 IU/g vitamin E), and 14 g of trace mineral premix (180,000 mg/kg Zn, 150,000 mg/kg Mn, 25,000 mg/kg Cu, 2,600 mg/kg I, 2,300 mg/kg Co, 1,000 mg/kg Fe, and 820 mg/kg Se).

Table 3.3. Effects of Perfect Omega 3 on intake, milk production and components, free water intake, BW, and BCS

	Treatment[1,2]				SEM	P-value[3]			
Item	CON	LPO3	MPO3	HPO3		Trt	L	Q	C
DMI, kg/d	21.8	23.7	23.2	24.1	1.41	0.234	0.108	0.513	0.352
Milk yield, kg/d	31.0	31.2	31.4	31.9	2.24	0.709	0.310	0.820	0.858
ECM, kg/d[3]	41.6	41.6	43.9	44.5	4.02	0.456	0.164	0.364	0.640
ECM/ DMI	1.92	1.74	1.83	1.86	0.12	0.164	0.691	0.087	0.209
Fat, %	5.30	5.28	5.64	5.82	0.35	0.097	0.024	0.535	0.463
Fat, kg/d	1.66	1.66	1.83	1.85	0.20	0.397	0.154	0.938	0.556
Protein, %	3.76	3.70	3.73	3.72	0.16	0.991	0.906	0.856	0.849
Protein, kg/d	1.18	1.16	1.21	1.19	0.11	0.896	0.684	0.973	0.532
Lactose, %	4.54	4.52	4.70	4.72	0.15	0.683	0.296	0.885	0.600
Lactose, kg/d	1.42	1.41	1.46	1.51	0.13	0.610	0.300	0.639	0.893
MUN, mg/dL	16.6	15.8	15.9	16.1	1.15	0.773	0.594	0.436	0.850
Free water intake, L/d	98.4	98.5	108	110	3.82	0.031	0.009	0.720	0.077
BW, kg	500	501	500	505	17.5	0.451	0.220	0.542	0.486
BCS	3.35	3.22	3.37	3.32	0.12	0.013	0.566	0.144	0.004

[1]CON = Control Diet (0% Perfect Omega 3); LPO3 = Low Diet (6.67% Perfect Omega 3); MPO3 = Medium Diet (13.3% Perfect Omega 3); HPO3 = High diet (20% Perfect Omega 3).
[2]Least squares means; largest standard error of treatment mean is shown.
[3]Trt = treatment, L = linear, Q = quadratic, C = cubic.

Table 3.4. The effects of inclusion of Perfect Omega 3 on the fatty acid profile of milk fat produced by lactating Jersey cows

	Treatments[1]				SEM	P-value[2]			
Item, g/100 g of fat	CON	LPO3	MPO3	HPO3		Trt	L	Q	C
C4:0	5.18	5.35	5.32	5.69	0.227	0.023	0.008	0.299	0.186
C6:0	3.00	3.05	3.09	3.15	0.131	0.343	0.087	0.938	0.935
C8:0	1.76	1.75	1.79	1.78	0.113	0.748	0.325	0.792	0.706
C10:0	4.29	4.27	4.43	4.17	0.303	0.709	0.761	0.455	0.394
cis-9 C10:1	0.28	0.30	0.27	0.28	0.024	0.664	0.677	0.833	0.298
C11:0	0.14	0.11	0.10	0.09	0.019	0.103	0.024	0.670	0.789
C12:0	4.81	4.77	4.93	4.53	0.369	0.495	0.399	0.350	0.387
iso C13:0	0.02	0.02	0.02	0.02	0.001	0.068	0.056	0.163	0.079
anteiso C13:0	0.08	0.08	0.07	0.07	0.008	0.351	0.120	0.698	0.596
C13:0	0.17	0.14	0.13	0.11	0.019	0.069	0.015	0.669	0.779
iC14:0	0.07	0.08	0.07	0.09	0.011	0.367	0.235	0.711	0.222
C14:0	11.7	11.6	11.9	11.4	0.295	0.457	0.422	0.320	0.324
C14:1c9	0.75	0.74	0.68	0.66	0.042	0.125	0.032	0.916	0.386
iso C15:0	0.19	0.19	0.16	0.16	0.008	0.012	0.003	0.568	0.080
anteiso C15:0	0.36	0.32	0.30	0.28	0.015	0.012	0.002	0.533	0.825
C15:0	1.23	1.02	0.96	0.85	0.135	0.045	0.011	0.518	0.614
iso C16:0	0.20	0.22	0.18	0.25	0.024	0.219	0.253	0.300	0.127
C16:0	30.8	30.2	29.8	28.6	1.03	0.045	0.009	0.556	0.632
cis-9 C16:1	1.03	0.96	0.88	0.86	0.103	0.016	0.003	0.390	0.691
iso C17:0	0.03	0.03	0.03	0.02	0.003	0.251	0.344	0.414	0.106
C17:0	0.18	0.18	0.18	0.16	0.020	0.253	0.119	0.261	0.777
cis-9 C17:1	0.21	0.18	0.18	0.17	0.017	0.261	0.084	0.518	0.590
C18:0	10.9	11.4	11.7	12.4	0.397	0.018	0.004	0.596	0.612
trans-4 C18:1	0.01	0.01	0.02	0.02	0.003	0.005	0.001	0.418	0.164
trans-5 C18:1	0.01	0.01	0.02	0.02	0.002	0.024	0.005	0.590	0.596
trans-6-8 C18:1	0.26	0.26	0.29	0.30	0.011	0.019	0.004	0.861	0.207
trans-9 C18:1	0.21	0.21	0.23	0.24	0.007	0.021	0.004	0.632	0.351
trans 10 C18:1	0.37	0.35	0.39	0.35	0.020	0.454	0.956	0.638	0.127

trans-11 C18:1	0.88	0.80	0.97	1.02	0.136	0.204	0.100	0.390	0.313
trans-12 C18:1	0.41	0.40	0.44	0.44	0.012	0.102	0.030	0.816	0.208
cis-9 C18:1	14.1	14.7	13.9	15.1	0.335	0.034	0.062	0.296	0.021
cis-11 C18:1	0.60	0.61	0.64	0.64	0.030	0.719	0.303	0.894	0.650
cis-12 C18:1	0.24	0.24	0.26	0.27	0.016	0.353	0.103	0.888	0.528
LA	2.61	2.48	2.41	2.23	0.086	0.013	0.002	0.700	0.568
γ- Linolenic acid	0.13	0.13	0.14	0.14	0.079	0.206	0.066	0.626	0.529
ALA	0.24	0.41	0.56	0.72	0.036	<0.001	<0.001	0.887	0.868
C20:0	0.02	0.03	0.03	0.02	0.004	0.182	0.634	0.060	0.571
cis-11 C20:1	0.07	0.07	0.07	0.07	0.004	0.631	0.865	0.643	0.278
C20:2n6	0.03	0.02	0.03	0.02	0.006	0.383	0.291	0.829	0.196
cis-9, *trans-11* CLA	0.33	0.34	0.37	0.43	0.041	0.137	0.042	0.367	0.948
Total saturated fatty acids	75.1	74.8	75.1	73.9	0.515	0.279	0.142	0.363	0.372
Total unsaturated fatty acids	22.7	23.2	22.8	24.0	0.453	0.196	0.102	0.419	0.240
Omega 6:omega 3	11.6	6.49	4.44	3.34	0.368	<0.001	<0.001	<0.001	0.065

[1]CON = Control Diet (0% Perfect Omega 3); LPO3 = Low Diet (6.67% Perfect Omega 3); MPO3 = Medium Diet (13.3% Perfect Omega 3); HPO3 = High diet (20% Perfect Omega 3).
[2]Trt = treatment, L = linear, Q = quadratic, C = cubic.

Table 3.5. Effect of increasing inclusion of Perfect Omega 3 on gas production and energy partitioning

	Treatment [1,2]				SEM	P-Value[3]			
Item[4]	CON	LPO3	MPO3	HPO3		Trt	L	Q	C
Gas Production									
O_2 consumption, L/d	5335	5558	5626	5314	396	0.696	0.995	0.259	0.830
CO_2 production, L/d	5736	6079	5972	5804	437	0.758	0.933	0.351	0.754
CH_4 production, L/d	476	510	483	490	42.8	0.866	0.910	0.683	0.529
RQ[4]	1.08	1.10	1.07	1.09	0.01	0.279	0.884	0.931	0.069
Components, Mcal/d									
Feces	32.8	36.1	36.8	38.1	1.52	0.070	0.016	0.445	0.588
Methane	4.34	4.22	4.16	3.96	0.39	0.866	0.546	0.617	0.693
Urine	2.57	2.92	2.77	2.76	0.14	0.169	0.363	0.116	0.238
HP[5,6]	24.7	24.8	24.5	24.2	1.99	0.990	0.800	0.875	0.955
Milk	28.7	28.2	29.5	29.9	2.64	0.346	0.154	0.520	0.481
TE	-2.99	2.49	-0.18	1.98	2.79	0.253	0.200	0.441	0.209
Fractions, Mcal/d									
GE	90.1	98.8	97.0	101	6.04	0.165	0.067	0.508	0.333
DE	57.3	62.7	60.3	63.1	5.18	0.382	0.224	0.638	0.332
ME	50.4	55.5	53.6	56.1	1.82	0.359	0.192	0.615	0.360
Fractions, Mcal/kg of DM									
GE	4.14	4.16	4.17	4.20	0.019	0.006	0.001	0.760	0.914
DE	2.63	2.62	2.61	2.59	0.076	0.957	0.698	0.824	0.726
ME	2.31	2.32	2.31	2.32	0.078	0.991	0.875	0.970	0.806
Efficiencies									
ME/DE	0.88	0.88	0.89	0.89	0.005	0.167	0.051	0.330	0.500
Milk/ME	0.58	0.51	0.55	0.54	0.041	0.163	0.425	0.151	0.155
HP/ME	0.49	0.46	0.44	0.44	0.026	0.488	0.158	0.633	0.980
TE/ME	-0.07	0.04	-0.01	0.02	0.054	0.143	0.134	0.306	0.150

[1]CON = Control Diet (0% GG); LOW = Low Diet (6.67% GG); MED = Medium Diet (13.3% GG); HI = High diet (20.0% GG)

[2]Least squares means; largest standard error of treatment mean is shown.

[3]Trt = treatment; L= linear, Q = quadratic, C= cubic

[4]RQ = respiratory quotient, CO2 production/O2 consumption.

[5]HP = heat production, GE = gross energy, DE = digestible energy, TE = tissue energy.

Table 3.6. Effects of increasing Perfect Omega 3 inclusion on fecal and urinary output and N excretion, secretion, and partitioning

	Treatments[1,2]				SEM	P-Value[3]			
Item	CON	LPO3	MPO3	HPO3		Trt	L	Q	C
Output, kg/d (as is)									
Feces	50.8	48.1	50.8	54.1	6.14	0.015	0.017	0.016	0.296
Urine	28.8	28.0	30.7	26.4	1.85	0.201	0.398	0.186	0.102
Mass, g/d									
N intake	589	657	649	694	36.2	0.016	0.005	0.495	0.139
Fecal N	203	221	238	254	9.89	0.006	0.001	0.853	0.977
Urinary N	188	218	218	200	13.3	0.040	0.206	0.011	0.726
Milk N	211	212	208	210	18.4	0.873	0.871	0.524	0.781
Retained N	-12.8	10.4	-13.0	28.6	18.6	0.156	0.120	0.516	0.115
As proportion of N intake, %									
Fecal N	34.7	33.9	36.5	36.7	1.46	0.124	0.051	0.568	0.190
Urinary N	32.3	33.1	33.3	28.8	1.95	0.280	0.209	0.171	0.623
Milk N	35.8	31.6	32.0	30.4	1.93	0.010	0.004	0.122	0.118
Retained N	-2.76	1.46	-2.39	4.04	2.96	0.113	0.090	0.595	0.085

[1]CON = Control Diet (0% Perfect Omega 3); LPO3 = Low Diet (6.67% Perfect Omega 3); MPO3 = Medium Diet (13.3% GG); HPO3 = High diet (20.0% GG).
[2]Least squares means; largest standard error of treatment mean is shown.
[3]Trt = treatment, L = linear, Q = quadratic, C = cubic.

Table 3.7. Effect of increasing inclusion of Perfect Omega 3 on apparent total-tract digestibility (%)

	Treatments[1,2]				SEM	P-Value[3]			
Item	CON	LPO3	MPO3	HPO3		Trt	L	Q	C
DM	65.2	65.4	64.3	64.9	1.32	0.838	0.563	0.827	0.494
OM	67.2	67.8	67.8	66.9	1.24	0.718	0.558	0.679	0.445
NDF	37.9	40.8	37.5	36.5	4.84	0.891	0.707	0.669	0.697
NDF_{OM}	38.6	41.8	40.9	32.4	6.74	0.706	0.492	0.383	0.912
CP	60.6	61.6	61.9	57.4	3.12	0.124	0.501	0.391	0.791
Starch	96.9	96.3	96.6	94.9	0.44	0.029	0.013	0.187	0.133
Fatty acids									
Total fatty acids	57.0	64.3	63.7	67.9	2.40	0.014	0.005	0.367	0.145
16 carbon fatty acids	61.6	68.9	69.6	72.5	2.37	0.018	0.005	0.249	0.319
18 carbon fatty acids	53.7	61.4	60.4	65.5	2.65	0.014	0.005	0.480	0.122
ALA	98.1	97.1	95.6	95.5	0.46	0.003	<0.001	0.311	0.281
LA	97.0	96.6	96.2	95.5	0.49	0.140	0.073	0.203	0.185
Energy	63.4	63.1	62.1	62.2	1.71	0.776	0.350	0.854	0.738

[1]CON = Control Diet (0% Perfect Omega 3); LPO3 = Low Diet (6.67% Perfect Omega 3); MPO3 = Medium Diet (13.3% GG); HPO3 = High diet (20.0% GG).

[2]Least squares means; largest standard error of treatment mean is shown.

[3]Trt = treatment, L = linear, Q = quadratic, C = cubic.

Figure 3.1. Sample of the high-fatty acid supplement, Perfect Omega 3.

Figure 3.2. The collection system for urine, which consists of a Foley catheter, clear tubing, and 55 L plastic container, and feces, which consists of a rubber mat, a large garbage container, and a trash bag.

Figure 3.3. Headbox-style indirect calorimeters used to collect gases from Jersey cows to determine heat production.

GENERAL SUMMARY AND CONCLUSIONS

Accurate estimates of chemical composition of feedstuffs are needed to ensure rations are balanced to meet the needs of lactating dairy cows. The inverse of the extent to which crude protein (**CP**) id digested in the rumen is referred to as rumen undegraded protein (**RUP**) and serves as a major source of metabolizable protein. The intestinal digestibility RUP (**dRUP**) can vary depending upon the type of feedstuff and how it is processed thus a rapid and cost-effective lab procedures are needed to rapidly and routinely quantify these chracteristics. Currently, several assays exist, and each possesses their own peculiarities as they attempt to mimic digestion within the animal. Three of the assays are the Mobile Bag (Paz et al., 2014), Modified Three-Step (Gargallo et al., 2006), and Ross (Ross et al., 2013) assays. Hydrolyzed feather meal (**HFM**) is a high CP feedstuff (85% DM) and possesses a high RUP content (65% of CP) (NRC, 2001). Only a small number of studies have been conducted to evaluate the effect of including HFM in rations on milk production, and many of those studies have reported a decrease in milk protein yield with increasing HFM (Harris Jr. et al., 1992; Moss et al., 1995; Morris et al., 2020b). However, when feeding a combination of HFM and bloodmeal, studies conducted with beef cattle have reported greater growth than HFM alone, and studies completed with dairy cattle have yielded mixed results.

The study in the second chapter was designed to evaluate the Mobile Bag, Modified Three-Step, and Ross assays while determining the protein quality and digestibility of HFM with and without added blood. Five samples of HFM containing blood and five samples of HFM without blood from production sites across the United

States were subjected to each assay. All three assays varied in almost all of the estimates with the Mobile Bag and Modified Three-Step assays resulting in values that were comparable. All three assays yielded similar dRUP values. These results show that all three assays are viable options for determining dRUP of animal-based protein feedstuffs. Even though differences in chemical composition between HFM with blood and HFM without blood were small, HFM with blood had an RUP content of 66.9% of CP while the RUP of HFM without blood was 60.8%. However, the dRUP estimates were similar, averaging 60.5 ± 0.49% (Soypass standard; dRUP = 89.2 ± 1.23%).

With the observed results of the Mobile Bag, Modified Three-Step, and Ross assays producing similar estimates of dRUP for animal-based protein feedstuffs, future research should also be conducted to evaluate other feedstuffs. Plant-based protein feedstuffs as well as rumen-protected forms of amino acids are also commonly used in dairy rations and often need lab analysis to obtain estimates of digestibility. Several of the differences in the initial composition of HFM with blood and HFM without blood could also be a result of differences between production sites as each site varied in the processing methods of byproducts. Future research in evaluating differences across production sites as well as batches within a production site could show how much chemical composition varies between both HFM and HFM with blood.

Omega 3 fatty acids (**FA**) play an important part in human health by reducing inflammation within the body, which reduces the risk of cardiovascular disease and blood pressure (Calder, 2004). However, Western diets often don’t provide enough omega 3 FA to meet the 1.8 g/d requirement set by the American Heart Association but instead are often high in omega 6 FA, which promote inflammation. The omega 3 FA α-linolenic

acid (ALA) and the omega 6 FA linoleic acid (LA) are both considered essential because they are not synthesized by mammals (CAST, 2018). Selective feeding of feedstuffs high in ALA but low in LA may be one way to improve human consumption of omega 3 FA. The effect of a high FA supplement "Perfect Omega 3" (Sunseo Omega 3; Chungcheong Buk-Do, South Korea), a product comprised of 34.5 % sesame meal, 23.6 % giant kelp, 23.5 % cassava, and 18.3 % sorghum, on the fatty acid profile of milk and energy utilization in dairy cattle was evaluated in the study presented in Chapter 3. Increasing the inclusion of Perfect Omega 3 increased the ALA (0.72 g/ 100g milk fat) content of milk while decreased LA (2.23 g/100 g milk fat) content. However, even with an increase in ALA content, other sources of ALA, such as salmon or flaxseed, would need to be consumed in addition to dairy products to meet the recommended daily intake. Dry matter intake also increased but no difference was observed in milk yield. Gross energy increased as the concentration of the supplement increased in the diet, but because of decreasing digestibility of starch, ALA, and LA, led to a decrease in digestible energy. The combination of increasing GE and decreasing DE resulted in no difference in metabolizable energy with increasing the inclusion Perfect Omega 3.

While the FA content of milk prior to processing was measured, future research should be conducted on the FA content of milk following processing into various dairy products to determine if processing affects the FA content of the final products. Even though the requirement for omega 3 FA have been determined for humans, there are no set requirements for dairy cattle. With how important omega 3 FA are for human health, it would be interesting to see how omega 3 FA impact dairy cattle production and determine if there is a minimum requirement. Inclusion of omega 3 FA in dairy cattle

rations has been shown to have positive benefits on reproduction by improving embryo quality and decreasing pregnancy loss (Dirandeh et al., 2013; Leroy et al., 2013) as well as health by improving resistance to diseases (Pike, 1999).

APPENDIX A

EQUATIONS

Heat production (HP, kcal/d) = 3.866 × O_2 (L/d) + 1.200 × CO_2 (L/d) – 0.518 × CH_4 (L/d) – 1.431 × Urinary N excretion (g/d) [1]

Tissue energy (**TE**; Mcal/d) = ME (Mcal/d) – HP (Mcal/d) – Milk energy (Mcal/d) [2]

APPENDIX B

POSTER PRESENTATION FROM ADSA ANNUAL MEETING, 2019

Abstract 82056: The effect of a unique high fatty acid supplement on milk fatty acid profile and energy utilization of lactating Jersey cows

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INTRODUCTION

- Omega 3 fatty acids (**FA**) play an important role in human health by siding in by preventing cardiovascular disease[1] and neural development in infants[2]
- The typical Western diet does not meet the daily requirement of omega 3 FA[3,4]
- Improvement of dietary consumption of omega 3 FA can be done by altering milk FA concentrations through selective feeding of high FA feedstuffs[2]

OBJECTIVES

- Evaluate the inclusion of the high-fatty acid supplement, Perfect Omega 3 **(PO3)**, on the fatty acid profile of milk and energy utilization of dairy cows

MATERIALS and METHODS

- 4 multiparous Jersey cows (115 ± 36 DIM, 501 ± 18 kg BW)
- 4 × 4 Latin square with 4 periods of 28 d
- Cow were randomly assigned to diets with 4 different levels of PO3 inclusions:
  - 0% PO3 (**CON**)
  - 6.67% PO3 (**LPO3**)
  - 13.3% PO3 (**MPO3**)
  - 20.0% PO3 (**HPO3**)
- Measured:
  - DMI, milk production and components (4 d)
  - Milk fatty acid profile (1 d)
  - Urine and fecal output (4 d total collection)
  - O_2 consumption, CO_2 and CH_4 production (1 d headboxes-type indirect calorimeter

- Data analyzed with GLIMMIX procedure of SAS (9.4)
  - Fixed effects:PO3 inclusion; Random Effects: period and cow
  - Tested linear, quadratic, and cubic effects of level of PO3 inclusion

RESULTS

Table 1. Ingredient and chemical composition of the diets fed to all experimental cows

Item (% DM)	CON	LPO3	MPO3	HPO3
Ingredient				
Corn silage	38.5	38.5	38.5	38.5
Alfalfa hay	14.1	14.1	14.1	14.1
Perfect Omega 3	0	6.67	13.3	20.0
Corn grain, ground	16.0	15.3	14.7	14.0
Soypass	2.82	2.73	2.65	2.56
Corn DDGS	10.3	6.87	3.43	0
Soybean meal	9.40	7.11	4.84	2.56
Soybean hulls, ground	1.79	1.76	1.74	1.72
Remaining grain mix[1]	7.04	7.04	7.04	7.04
Composition (mean ± SD)				
DM	95.7 (0.87)	96.2 (0.94)	96.1 (1.21)	96.0 (1.98)
CP	17.5 (0.84)	17.0 (0.39)	18.1 (1.58)	18.1 (0.88)
NDF	26.9 (1.74)	27.0 (1.32)	27.1 (1.58)	27.3 (2.29)
Starch	30.5 (0.85)	30.7 (0.95)	30.8 (1.06)	31.0 (1.19)
Fatty Acids				
Total fatty acids	4.18 (0.27)	4.72 (0.28)	5.27 (0.29)	5.81 (0.31)
C16 fatty acids	1.11 (0.09)	1.24 (0.09)	1.37 (0.09)	1.51 (0.10)
C18 fatty acids	2.69 (0.18)	3.07 (0.18)	3.45 (0.18)	3.84 (0.19)
α-Linolenic acid	0.18 (0.01)	0.18 (0.01)	0.19 (0.01)	0.19 (0.02)
Linoleic acid	0.95 (0.05)	1.02 (0.05)	1.08 (0.05)	1.15 (0.05)

[1]Supplied (% of the total diet): 1.23% beet molasses, 1.87% Energy Booster (Milk Specialties, Eden Prairie, MN), 0.41% Ajipro (Ajinomoto Co., Inc., Tokyo, Japan), 0.11% Smartamine (Adisseo, Alpharetta, GA), 3.42% mineral-vitamin mix.

Table 3. Effects of Perfect Omega 3 on intake, milk production and components, and milk fatty acid profile

	Treatment[1]					P-Value[2]		
Item	CON	LPO3	MPO3	HPO3	SEM	L	Q	C
DMI, kg	21.8	23.7	23.2	24.1	1.41	0.108	0.513	0.352
Milk yield, kg	31.0	31.2	31.4	31.9	2.24	0.310	0.820	0.858
ECM, kg	41.6	41.6	43.9	44.5	4.02	0.164	0.364	0.640
ECM/DMI	1.92	1.74	1.83	1.86	0.12	0.691	0.087	0.209
Fat, %	5.30	5.28	5.64	5.82	0.35	0.024	0.535	0.463
Fat, kg	1.66	1.66	1.83	1.85	0.20	0.154	0.938	0.556
Protein, %	3.76	3.70	3.73	3.72	0.16	0.908	0.856	0.849
Protein, kg	1.18	1.16	1.21	1.19	0.11	0.684	0.973	0.532
Fatty acids								
α-Linolenic acid	0.24	0.41	0.56	0.72	0.036	<0.001	0.887	0.868
Linoleic acid	2.61	2.48	2.41	2.23	0.086	0.002	0.700	0.568

[1]CON = Control Diet (0% PO3); LPO3 = Low Diet (6.67% PO3); MPO3 = Medium Diet (13.3% PO3); HPO3 = High diet (20% PO3).
[2]L = linear, Q = quadratic, C = cubic.

Table 2. Chemical composition of PO3

Item (% DM)	PO3[1]
DM	88.1
CP	35.8
NDF	35.9
Starch	11.5
Fatty acids	
Total fatty acids	7.81
C16 fatty acids	0.82
C18 fatty acids	6.86
α-linolenic acid	1.78
linoleic acid	2.20
Ash	11.2

[1]Perfect Omega 3 (Sunseo Omega Inc., Chungcheongbuk-do, Korea).

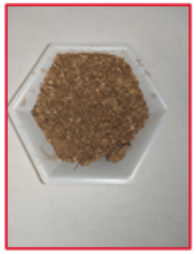

Figure 1. Sample of Perfect Omega 3

Table 4. The effects of increasing inclusion of Perfect Omega 3 on energy partitioning

	Treatment[1]					P-Value[2]		
Item	CON	LPO3	MPO3	HPO3	SEM	L	Q	C
Fractions, Mcal/d								
GE	90.1	98.8	97.0	101	6.04	0.067	0.508	0.333
DE	57.3	62.7	60.3	63.1	5.18	0.224	0.638	0.332
ME	50.4	55.5	53.6	56.1	1.82	0.192	0.615	0.360
Fractions, Mcal/kg of DM								
GE	4.14	4.16	4.17	4.20	0.019	0.001	0.760	0.914
DE	2.63	2.62	2.61	2.59	0.076	0.698	0.824	0.726
ME	2.31	2.32	2.31	2.32	0.078	0.875	0.970	0.806

[1]CON = Control Diet (0% PO3); LPO3 = Low Diet (6.67% PO3); MPO3 = Medium Diet (13.3% PO3); HPO3 = High diet (20% PO3).
[2]L = linear, Q = quadratic, C = cubic.

Table 5. Effect of increasing inclusion of Perfect Omega 3 on apparent total-tract digestibility (%)

	Treatment[1]					P-Value[2]		
Item	CON	LPO3	MPO3	HPO3	SEM	L	Q	C
DM	65.2	65.4	64.3	64.9	1.32	0.563	0.827	0.494
CP	60.6	61.6	61.9	57.4	3.12	0.501	0.391	0.791
NDF	37.9	40.8	37.5	36.5	4.84	0.707	0.669	0.697
Fatty acids								
Total fatty acids	57.0	64.3	63.7	67.9	2.40	0.005	0.367	0.145
C16 fatty acids	61.6	68.9	69.6	72.5	2.37	0.005	0.249	0.319
C18 fatty acids	53.7	61.4	60.4	65.5	2.65	0.005	0.480	0.122
α-linolenic acid	98.1	97.1	95.6	95.5	0.46	<0.001	0.311	0.281
linoleic acid	97.0	96.6	96.2	95.5	0.49	0.073	0.203	0.185
Energy	63.4	63.1	62.1	62.2	1.71	0.350	0.854	0.736

[1]CON = Control Diet (0% PO3); LPO3 = Low Diet (6.67% PO3); MPO3 = Medium Diet (13.3% PO3); HPO3 = High diet (20% PO3).
[2]L = linear, Q = quadratic, C = cubic.

CONCLUSIONS

- Increasing inclusion of PO3 increased DMI and milk fat concentration, with favor towards increasing α-linolenic acid while decreasing linoleic acid
- GE increased with increasing inclusion but did not affect DE or ME
- Digestibility of FA increased with increasing PO3 inclusion

REFERENCES

[1]Calder, 2004. Clin Sci 107:1-11
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[3]Krauss, 2000. Circulation 102:2284-2299
[4]Scorletti and Byrne, 2013. Annu. Rev. Nutr. 33:231-248

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APPENDIX C

JOURNAL OF DAIRY SCIENCE REFLECT STATEMENT

Checklist for REFLECT statement: Reporting guidelines For randomized control trials in livestock and food safety. Bold text are modifications from the CONSORT statement description (Altman DG et al . Ann Intern Med 2001; 134(8):663-694).

Paper section and topic	**Item**	**Descriptor of REFLECT statement item**	**Reported on Page #**
Title & Abstract	1	How **study units** were allocated to interventions (eg, "random allocation," "randomized," or "randomly assigned"). **Clearly state whether the outcome was the result of natural exposure or was the result of a deliberate agent challenge.**	3
Introduction Background	2	Scientific background and explanation of rationale.	4-5
Methods Participants	3	Eligibility criteria **for owner/managers and study units at each level of the organizational structure,** and the settings and locations where the data were collected.	5
Interventions	4	Precise details of the interventions intended for each group, **the level at which the intervention was allocated,** and how and when interventions were actually administered.	5
	4b	**Precise details of the agent and the challenge model, if a challenge study design was used.**	NA

Objectives	5	Specific objectives and hypotheses. **Clearly state primary and secondary objectives (if applicable).**	5
Outcomes	6	Clearly defined primary and secondary outcome measures and the levels at which they were measured, and, when applicable, any methods used to enhance the quality of measurements (eg, multiple observations, training of assessors).	5
Sample size	7	How sample size was determined and, when applicable, explanation of any interim analyses and stopping rules. **Sample-size considerations should include sample-size determinations at each level of the organizational structure and the assumptions used to account for any non-independence among groups or individuals within a group.**	5
Randomization -- Sequence generation	8	Method used to generate the random allocation sequence **at the relevant level of the organizational structure**, including details of any restrictions (eg, blocking, stratification)	5
Randomization -- Allocation concealment	9	Method used to implement the random allocation sequence **at the relevant level of the organizational structure**, (eg, numbered containers ~~**or central telephone**~~**),** clarifying whether the sequence was concealed until interventions were assigned.	5
Randomization --	10	Who generated the allocation sequence, who enrolled **study units,** and who assigned **study units** to their groups **at the relevant level of the organizational structure**.	5

Implementation			
Blinding (masking)	11	Whether or not ~~**participants**~~ those administering the interventions, **caregivers** and those assessing the outcomes were blinded to group assignment. If done, how the success of blinding was evaluated**. Provide justification for not using blinding if it was not used.**	5
Statistical methods	12	Statistical methods used to compare groups for all outcome(s); Clearly state the level of statistical analysis **and methods used to account for the organizational structure, where applicable;** methods for additional analyses, such as subgroup analyses and adjusted analyses.	9
Results **Study** flow	13	Flow **of study units** through each stage **for each level of the organization structure of the study** (a diagram is strongly recommended). Specifically, for each group, report the numbers of **study units** randomly assigned, receiving intended treatment, completing the study protocol, and analyzed for the primary outcome. Describe protocol deviations from study as planned, together with reasons.	9-12
Recruitment	14	Dates defining the periods of recruitment and follow-up.	NA
Baseline data	15	Baseline demographic and clinical characteristics of each group, **explicitly providing information for each relevant level of the organizational structure. Data should be reported in such a way that secondary analysis, such as risk assessment, is possible.**	5
Numbers analyzed	16	Number **of study units** (denominator) in each group included in each analysis and whether the analysis was by "intention-to-treat." State the results in absolute numbers when feasible (eg, 10/20, not 50%).	5

Outcomes and estimation	17	For each primary and secondary outcome, a summary of results for each group, **accounting for each relevant level of the organizational structure**, and the estimated effect size and its precision (e.g., 95% confidence interval)	9-12
Ancillary analyses	18	Address multiplicity by reporting any other analyses performed, including subgroup analyses and adjusted analyses, indicating those pre-specified and those exploratory.	13-19
Adverse events	19	All important adverse events or side effects in each intervention group.	9
Discussion Interpretation	20	Interpretation of the results, taking into account study hypotheses, sources of potential bias or imprecision, and the dangers associated with multiplicity of analyses and outcomes. **Where relevant, a discussion of herd immunity should be included. If applicable, a discussion of the relevance of the disease challenge should be included.**	13-19
Generalizability	21	Generalizability (external validity) of the trial findings.	19
Overall evidence	22	General interpretation of the results in the context of current evidence.	19