THE EFFECT OF AN EXOGENOUS AMYLASE ON PERFORMANCE AND TOTAL TRACT DIGESTIBILITY IN LACTATING DAIRY COWS

by

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ABSTRACT

This thesis consisted of 2 experiments. The objective of Experiment 1 was to determine performance and digestibility response of lactating dairy cows to a reduced starch diet containing a commercial amylase product. The objective of Experiment 2 was to determine the effect of various levels of amylase on in vitro starch digestibility of 3 substrates. In Experiment 1, 19 multiparous (86 ± 46 DIM) and 5 primiparous (93 ± 8 DIM), were blocked by parity and DIM and assigned to treatments in a 3 × 3 Latin square design, with 28 d periods. Treatments were a normal starch TMR (NS), a reduced starch TMR (RS), and a reduced starch TMR with (351 KNU/ kg TMR DM) exogenous amylase added to the concentrate (RSE). The hypothesis was that reducing ration starch content would decrease milk production and diet digestibility compared to NS due to a decrease in available energy, and that RSE would alleviate some of this decrease by increasing nutrient digestibility. Rations were 41% concentrate and the NS TMR contained 12.8% corn grain, 2.9% soyhulls, and 2.9% citrus pulp. The RS and RSE TMR contained 6.0% corn grain, 6.9% soyhulls, and 6.9% citrus pulp. Starch concentrations in NS, RS, and RSE TMR were 27.5, 23.2, and 22.4%, respectively. Data were analyzed using a mixed model containing the fixed effects of treatment, week, period, and their interactions, and the random effects of cow and block. Feeding a RS diet compared with a NS diet resulted in decreased milk, FCM, milk protein yield, milk lactose yield, and increased MUN and NDF digestibility. Feeding the RSE diet resulted in increased milk protein percentage and increased DM, NDF, and CP digestibility. Exogenous amylase decreased milk lactose

yield and tended to decrease milk yield and 3.5% FCM yield. In Experiment 2, NS and RS grain samples and corn starch were pre-incubated (18 h prior to start of in vitro) or co-incubated (during in vitro) with 4 levels of liquid amylase (0, 382, 1274, 3833 KNU/ kg substrate DM) and 7 h in vitro starch digestibility was measured. Data were analyzed using a mixed model including the fixed effects of substrate, amylase, pre-incubation, day, and all multi-way interactions. Pre-incubation of amylase with substrate for 18 h prior to in vitro resulted in increased starch digestibility compared to co-incubated samples. The starch digestibility for co-incubated samples was greatest at amylase application of 383 and 1274 KNU/kg substrate DM. While the addition of exogenous amylase increased in vitro starch digestibility as well as increased the digestibility of some nutrients during the lactation trial, this did not result in improved animal production performance.

Chapter 1

INTRODUCTION

Feed costs currently represent 35 to 50% of operating costs for dairy farmers in the United States. Dairy producers invariably strive to minimize feed costs in order to maximize production efficiency; however, the reduction of feed costs becomes especially important when milk prices are low.

The main energy sources in dairy rations are forage and non-forage carbohydrates. The majority of non-forage carbohydrates come from cereal grains, including corn, sorghum, wheat, barley, and oats. While many cereal grains are fed, corn grain is typically the primary energy source fed to high producing dairy cows in the United States. The main energy source in corn grain comes from starch.

In dairy rations corn grain represents a sizable portion of the ration cost, and feeding a reduced starch diet may present one means of reducing high feed costs. Currently, reducing dietary starch content without the addition of supplemental fat, results in less available energy for the cow and can lead to reduced milk yields (Oba and Allen, 2003). Some research that has been conducted in the Midwestern United States has shown that feeding a reduced starch diet with exogenous α-amylase may reduce feed costs without negatively affecting milk production (Gencoglu et al., 2010). Exogenous amylase appears to enhance ruminal carbohydrate digestibility in

cows fed rations with increased concentrations of by-product feeds (Klingerman et al., 2009; Gencoglu et al., 2010). However, the effect of α-amylase on reduced starch rations containing by-products typical of an Eastern United States diet has not yet been determined.

The objectives of this thesis were: 1) to determine the effect of an α-amylase product on dry matter intake, milk production, and apparent total tract digestibility when fed as part of a reduced starch diet to lactating Holstein cows, and 2) to determine effect of amylase on in vitro starch digestibility of various substrates.

Chapter 2

REVIEW OF THE LITERATURE

RUMINANT DIGESTION

High producing dairy cows are constantly challenged to meet their energy requirements, and energy intake continues to be the chief limiting factor on milk yield for high producing cows (Allen, 2000). Carbohydrates can account for more than 65% of dry matter (**DM**) in the diet of dairy cows; however, the extent of carbohydrate digestion is extremely variable among feedstuffs (Allen, 1997).

Carbohydrate chemistry

Carbohydrates in plant cells serve as either storage carbohydrates (sugars and starch) or structural carbohydrates (cellulose and hemicellulose). The main functions of carbohydrates in the diet of dairy cattle are to provide energy for rumen microbes and for the cow, as well as to stimulate cud chewing and saliva production to buffer the acids that are produced in the rumen (NRC, 2001).

Carbohydrates can be separated into fiber, or structural carbohydrates and non-fiber, or nonstructural carbohydrate components. Nonstructural carbohydrates (**NSC**) are made up of sugars, starches, organic acids and other carbohydrates, and will be discussed further in a later section of this review.

Structural carbohydrates, which include celluloses and hemicelluloses, are found in plant cell walls (NRC, 2001). Celluloses are made of β-1, 4 linked linear

glucose chains and provide strength to the plants (Van Soest, 1973). Hemicelluloses are mainly composed of xylose but contain a mixture of complex polysaccharides. Hemicelluloses are made of branched chain polysaccharides and can be covalently bound to lignin, gluing the cell wall polysaccharides together (Knudsen, 1997). Both cellulose and hemicellulose are digestible by rumen microbial populations. Pectin is sometimes classified with hemicelluloses because they contain some of the same sugars, but pectin contains galacturonic acid and is present in cell walls and intracellular spaces (Van Soest, 1982). Pectin is considered to be a soluble fiber and is highly digestible. Lignin, while technically not a carbohydrate, strengthens the cell walls of the plant and facilitates water movement. However, lignin is indigestible to the ruminant animal because the rumen microbes are unable to break down the phenolic compounds present in lignin. The increase in lignin content is a main reason for the reduced animal digestibility of mature forages (Miller, 1979).

Structural carbohydrates

Forages, such as alfalfa hay, alfalfa silage and corn silage, provide the main source of structural carbohydrates in the dairy ration (Kendall et al., 2009). The particle length of these forages is important because physically effective fiber is the fraction of feed that stimulates chewing activity. Chewing, in turn, stimulates saliva secretion. The bicarbonate and phosphate buffers present in saliva neutralize the acids that are produced by microbial fermentation in the rumen. Maximizing forage fiber digestibility is important in increasing dry matter intake (**DMI**) and maximizing milk production because of its ability to increase rumination, saliva buffer production, rumen pH, and ultimately rumen function (Allen, 2000). The microbial fermentation

that occurs in the rumen provides the cow energy via the production of volatile fatty acids (**VFA**). This topic will be discussed in further detail in a later section.

Neutral detergent fiber (**NDF**) is a chemical analysis of dietary forage fiber including celluloses, hemicelluloses, and lignin as the major components, and ratios of these three components impact NDF digestibility (Van Soest et al., 1991). Acid detergent fiber (**ADF**) is a chemical analysis of forage fiber fractions of cellulose and lignin. While both NDF and ADF are reported in the literature, NDF is considered to best express fiber content (NRC, 2001). Neutral detergent fiber digestibility is a good predictor of DMI in dairy cows (Kendall et al., 2009). It is used to calculate the energy content of forage for ration formulation and to estimate the digestibility of a forage (Hall and Mertens, 2008). Research by Oba and Allen (1999) quantified the relationship between NDF digestibility and animal performance and found that a 1 unit increase in forage NDF digestibility correlated with 0.17 kg/d of increased DMI and 0.23 kg/d of increase of 4.0% fat corrected milk.

Nonforage fiber

Nonforage fiber sources (**NFFS**) are plant by-products that are produced following extraction of starch, sugar or other nonfiber components. The NFFS, or by-product feeds, are secondary products that are obtained during the harvest or processing of a commodity, and have value as animal feed because they have little direct value as either human food or use in consumer products. Ruminants are able to use NFFS because of the ability of rumen microbes to breakdown and digest the β-linkages of structural carbohydrates that monogastrics cannot digest. There are a wide variety of by-product feedstuffs, such as whole cottonseed, dried beet pulp, soyhulls,

citrus pulp, bakery waste, and tomato pomace (Grasser et al., 1995). By-products have been used as alternative feeds in many dairy operations based on their price and availability (Firkins, 1997).

By-products can have NDF concentrations similar to forages, but their particle size is typically more similar to that of concentrates (Pereira et al., 1999). As a result, the rate of passage of by-product feeds from the rumen is often more rapid than that of forages (Firkins, 1997). The rate of NDF digestion greatly varies among and within sources of by-product feeds (Firkins, 1997). As is the case with forage fiber, the physically effective NDF content of NFFS is variable, and the ability to stimulate rumination is dependent on size distribution of the fibrous particles and the retention time of NFFS in the rumen (Allen, 1997).

In 2001, feed costs accounted for 35 to 50% of total costs to produce milk (Ipharraguerre et al., 2003). In order to maximize production efficiency dairy producers must attempt to minimize feed costs, especially when milk prices are low (Ipharraguerre et al., 2003). Because by-product feeds are generally less expensive than traditional feeds, they may offer one means of reducing feed costs. In the Eastern United States, soyhulls and citrus pulp are two commonly available by-product feeds that can be incorporated into the ration to reduce purchased feed costs.

Soyhulls. The composition of soyhulls varies widely among processors, but is mainly composed of the pericarp (seed coat) of the soybean (Ipharraguerre and Clark, 2002). This by-product results from the commercial processing of soybeans, which separates the meat from the hulls. The soyhulls have neither value as food for human consumption, nor for industrial use. Soyhulls have an average of 60.3% NDF,

44.6% ADF, 13.9% crude protein (**CP**), 4.9% ether extract, 2.5% lignin, and 4.8% ash (NRC, 2001). However, because soyhulls are high in NDF as well as digestibility (67.3% total digestible nutrients; NRC, 2001), they can be used as a partial replacement for either forage or grain (generally 10 – 15% of TMR DM) in dairy rations where they are available (Ipharraguerre et al., 2002).

Citrus pulp. Citrus pulp is a mixture of peel, insides, and cull fruit of the citrus family (e.g., orange, lemons, and grapefruit) that have been dried into a coarse, flaky product. The nutrient content of citrus pulp is dependent on the source of fruit and type of processing (Fegeros et al., 1995), but on average contains 24.2% NDF, 22.2% ADF, 6.9% CP, 4.9% ether extract, 0.9% lignin, 7.2% ash (NRC, 2001) with the remaining percentage composed primarily of neutral detergent soluble fiber and is predominantly pectin (Hall et al., 1997). Some properties of citrus pulp are similar to forage fiber and promote a relatively high ruminal pH (Fegeros et al., 1995). Citrus pulp is highly digestible and contains a variety of energy substrates for rumen microbial fermentation (79.8% total digestible nutrients; NRC, 2001).

Non-structural carbohydrates

Non-structural carbohydrates are the principle source of energy for the lactating dairy cow (NRC, 2001). They are found inside the cells of plants and are more easily digested than structural carbohydrates (NRC, 2001). The NSC are made up of sugars, starches, organic acids, and other carbohydrates. This section will primarily focus on starch because dairy ration formulations in the United States contain much higher percentages of starch than other NSC.

Starch is a heterogeneous polysaccharide that is composed of two types of α-glucans, amylose and amylopectin (Tester et al., 2004). Specifically, starch is composed of an insoluble linear polymer of glucose bound by α-1,4 linkages with varying degrees of branching resulting from α-1,6 bonds at each branch point. Amylose is a long, linear α-glucan containing around 99% 1,4 α- and 1% 1,6 α-linkages (Tester et al., 2004). Amylopectin is a much larger molecule than amylose and is a heavily branched structure that is made of about 95% 1,4 α- and 5% 1,6 α-linkages (Tester et al., 2004).

Cereal grains are the main sources of starch in the diets of lactating dairy cows and are made up of a pericarp (outer covering), a germ (embryo), and the endosperm. The pericarp and germ regulate water uptake, but contain little starch. The majority of the grain's starch is stored in the endosperm (Kotarski et al., 1992). Starch makes up 50 to 100% of NSC in most feedstuffs; however, the digestibility of starch varies among feedstuffs (NRC, 2001.). Starch provides approximately 50% of the energy found in corn silage and 75% of the energy in corn grain (calculated from NRC, 2001).

Digestion of starch

Amylase. The first site of starch digestion is in the rumen where the starch is fermented by the rumen microbes (Kotarski et al., 1992). The process of starch digestion in the rumen involves α-amylase and isoamylase that are produced by rumen bacteria. The α-amylase randomly cleaves internal α-1,4 linkages of the polymer backbone and releases maltodextrins (low molecular weight oligosaccharides

produced from starch hydrolysis by amylolytic bacteria). Isoamylase cleaves the α-1,6 linkages of the amylopectin branch points (Tricarico et al., 2008).

Rumen bacteria. The rumen bacteria with the greatest capacity for starch digestion are *Ruminobacter amylophilus* and *Streptococcus bovis*, followed by *Prevotella ruminicola* and some *Butyrivibrio fibrisolvens* strains (Tricarico et al., 2008). In order to hydrolyze starch, bacteria must either actively secrete amylase or produce surface associated amylases to hydrolyze starch for transport into the bacterial cell (Kotarski et al., 1992).

Microorganisms are able to utilize hydrolysis products from other species to contribute to ruminal fermentation (Van Soest, 1982; Tricarico et al., 2008). For example, cellodextrins (low molecular weight carbohydrates produced from fiber hydrolysis by cellulolytic bacteria) can be used by non-cellulolytic species (Russel, 1985) and products from xylan hydrolysis can be used by non-xylanolytic species (Cotta, 1993). It is likely that starch in the rumen is hydrolyzed to a variety of products such as glucose, maltoheptaose, and maltodextrins. These starch hydrolysis products may be used as growth substrates by a variety of different rumen microorganisms, including both amylolytic and non-amylolytic species (Tricarico et al., 2008).

Rumen protozoa. While protozoa and fungi are known to contribute to ruminal starch digestion, their roles are still not clearly defined (Tricarico et al., 2008). Ciliated protozoan concentrations tend to increase with an increase in grain feeding, and their populations range from 0 to 10^9/L (Kotarski et al., 1992). In grain fed animals, protozoa can slow overall starch hydrolysis rates by ingesting a sufficient quantity of bacteria to decrease ruminal fermentation rates, as well as by ingesting

starch granules and decreasing the accessibility of these substrates for bacterial fermentation (Kotarski et al., 1992).

Rumen fermentation

The end products of bacterial carbohydrate fermentation are VFA. The primary VFA resulting from rumen fermentation are acetate, propionate, and butyrate, with lactate sometimes produced as an end product during times of excessive fermentation (Allen, 2000). Acetic and butyric acids are the main end products of fiber fermentation in the rumen, while propionic acid is the main end product of starch fermentation. These VFA are absorbed from the rumen into the blood stream and are transported to various tissues, where they are used for energy by the cow.

Carbohydrates have the most variable rates of ruminal degradation among dietary nutrient classes and degradability is impacted by particle size and processing (Allen, 1997). Digestion of starch is dependent on the amount of starch present in the ration. The rate and extent of starch digestion in the rumen in turn influences the composition of VFA that are produced, rumen pH, and the amount of starch available for post ruminal digestion (Kotarski et al., 1992). If starch fermentation rates are slow, the total tract digestion of starch may be reduced, although this is dependent on the amount of starch in the ration. However, if fermentation rates are rapid, the buffering and absorptive capacity of the cow may not be able to compensate for the rapid VFA production by the rumen microbes, leading to acidosis (Kotarski et al., 1992).

Intestinal digestion

Lower tract digestion and absorption of carbohydrates in the cow are relatively low because of the extensive ruminal fermentation and carbohydrate disappearance before digesta enters the hindgut (Van Soest, 1982).

Ruminal starch digestion generally does not limit production in the way that incomplete or slow fiber digestion does (Tricarico et al., 2008) because undigested starch that leaves the rumen has the potential to be digested in the small intestines, whereas fiber can only be broken down by microbial enzymes (Strobel and Russell, 1986). Although starch is digested more efficiently in the small intestines, starch digestion in the rumen is more beneficial than postruminal digestion of starch because ruminal digestion also increases the microbial protein outflow from the rumen where it is absorbed in the small intestines (DeFrain et al., 2005). Therefore, enhancing ruminal starch digestibility can increase microbial protein availability in the hindgut. Because of this, ruminal digestion of starch should be optimized to allow sufficient microbial protein production, where ruminally undigested starch can be later absorbed in the small intestine (Yang and Beauchemin, 2006).

Factors affecting digestion

Of the common grains fed to ruminants, oats are the most digestible and least vitreous grain, followed by wheat, barley, and corn, with sorghum being the least digestible and most vitreous (NRC, 2001). Most grain processing methods increase the rate of starch fermentation and ruminal starch digestibility. Cereal processing methods use heat, moisture, and mechanical methods to break down the endosperm and expose the starch granule, which creates varying degrees of starch gelatinization and increases animal digestibility (Kotarski et al., 1992). Decreasing particle size also

increases the rate of starch digestion (NRC, 2001). The total tract digestibility of starch in dairy cows ranges from 70 to 100% and is affected by grain particle size, processing method, harvest and storage methods, harvest maturity, moisture content and endosperm type, corn silage maturity, chop length, kernel processing, and endosperm type (Johnson et al., 1999).

REDUCING RATION STARCH CONTENT

In recent years high corn prices have increased interest in feeding reduced starch diets. Partially replacing corn grain in the ration with high fiber, low starch byproduct feeds may be a feasible option to decrease costs without negatively affecting animal performance.

Effect of replacing starch with by-product feeds on intake and production

Several studies have evaluated the effect of replacing corn grain with by-product feeds on DMI. In a review by Ipharraguerre and Clark (2003), 15 lactating cow trials were fed diets where soyhulls were used to partially replace cereal grains. Out of 10 studies evaluated that replaced high moisture or dry ground corn with soyhulls, 9 found that there was no significant difference on DMI ($P > 0.10$) between control diets and those diets containing soyhulls. Only one study reported a tendency for 1.9 kg/d greater DMI by lactating dairy cows when high-moisture corn was partially replaced with soyhulls. Beckman and Weiss (2005) reported a tendency for greater DMI for lactating dairy cows fed a 25.4% reduced starch diet where soyhulls and cottonseed hulls partially replaced dry ground corn.

While the effects of including by-products on DMI have been variable, the effects of replacing by-products for corn grain on milk production and milk

components have also been variable. Solomon et al. (2000) substituted citrus pulp for corn grain in a total mixed ration (**TMR**) fed to lactating dairy cows and found that the cows fed the high citrus pulp diet had lower DMI but similar milk yield, as compared to cows fed a high corn TMR. The review by Ipharraguerre and Clark (2003) similarly found that the partial replacement of grains with soyhulls was not correlated with milk or milk fat yield. However, soyhulls significantly depressed milk protein content in 4 of the 10 trials reviewed, and numerically depressed milk protein content in an additional 5 trials. In the study by Ipharraguerre et al. (2002), when pelleted soyhulls replaced corn at up to 40% of diet DM of, milk yield was reduced at the 40% inclusion rate. However, when included at concentrations of 30% diet DM or less, milk production was not affected, but milk fat percentage and yield were greater than that of the control group. Batajoo and Shaver (1994), Beckman and Weiss (2005) and Gencoglu et al. (2010) similarly reported an increase in milk fat content in response to feeding a reduced starch diet. It was proposed that this increase in milk fat was related to effects of the greater NDF intake and lower starch intake on increasing ruminal acetate concentrations and lowering propionate concentrations to supply more substrate for fatty acid production (Gencoglu et al., 2010). In summary, partially replacing corn grain with by-product feeds (between 10 - 15%) does not appear to negatively affect DMI or milk yield, but has been shown to decrease milk protein and increase milk fat.

Effect of replacing starch with by-product feeds on digestion

Partial replacement of corn grain with by-product feeds has been shown to enhance nutrient digestibility in lactating dairy rations. The review by Ipharraguerre

and Clark (2003) found that replacing cereal grains with soyhulls increased the apparent total tract digestibility of NDF, although different methodologies and soyhull sources resulted in variable estimates of the NDF digestibility among the studies. In the comparison of a high citrus pulp TMR (21% citrus pulp, 9% corn grain) and a high corn TMR (20% corn grain, 10% citrus pulp) fed to lactating cows, the digestibility of NDF and CP were higher in the high citrus pulp group than in the high corn group (Miron et al., 2002).

ADDING EXOGNEOUS AMYLASE TO THE RUMINANT RATION

Amylolytic enzymes

Some exogenous enzymes are resistant to degradation in the rumen and have the potential to increase the digestibility of feeds, and in turn improve animal performance (Klingerman et al., 2009). Because of its hydrolytic action, supplemental α-amylase may increase the availability of starch hydrolysis products in the rumen and alter the ruminal fermentation process (Tricarico et al., 2008). In a study by Klingerman et al. (2009), α-amylase enzyme formulations had a relatively stable α-amylase activity in a 24-h in vitro ruminal fermentation, which suggested that the enzymes were not subject to extensive degradation by rumen microbes. Hristov et al. (1998) reported similar results when the release of reducing sugars following addition of amylolytic enzymes to rumen fluid was used to determine stability.

In vitro experiments

Recent studies summarized by Tricario et al. (2008) suggest that supplemental α-amylase does not necessarily increase ruminal starch digestion, but rather increases hydrolysis of oligosaccharides that can be utilized by non-amylolytic

bacterial species. Experiments with pure cultures of fibrolytic bacteria, such as *Selenomonas ruminantium*, *Megasphaera elsdenii* and *Butyrivibrio fibrisolvens* that cannot grow or grow slowly on starch media alone, have shown rapid growth following supplementation of α-amylase to media containing soluble potato starch as the sole carbohydrate source (Tricarico et al., 2008). In mixed cultures, supplemental α-amylase shifts rumen fermentation to higher molar proportions of butyrate and acetate and modifies rumen microbial populations (Tricarico et al., 2008). Rather than enhancing starch digestion by amylolytic organisms, Tricarico et al. (2008) proposed that the exogenous amylase primarily alters fermentation by increasing release of starch hydrolysis products including maltodextrins and oligosaccharides. The effect of α-amylase on rumen fermentation is believed to be caused by these hydrolysis products providing substrates to non-amylolytic organisms, thereby modifying bacterial populations and VFA production (Tricarico et al., 2008).

Normal starch rations with added amylase

While in vitro data appears to enhance ruminal microbial digestion (Tricarico et al., 2008), in vivo experiments with α-amylase have resulted in variable responses in cow performance and diet digestibility. Feeding an α-amylase product in a normal starch TMR was shown to increase milk production in lactating cows (Tricarico et al., 2005; Harrison and Tricarico, 2007; Klingerman et al., 2009). A concurrent increase in DMI was found in one of these studies (Klingerman et al., 2009) but not in the others (Tricarico et al., 2005; Harrison and Tricarico, 2007). DeFrain et al. (2005) found that exogenous α-amylase improved energy balance in transition cows but did not affect rumen fermentation. However, Hristov et al. (2008)

found no benefit in microbial protein synthesis or nutrient digestion when α-amylase was included in diets containing alfalfa hay or silage as the primary forage.

In finishing beef cattle, dietary supplementation with an α-amylase preparation improved performance in two studies summarized by Tricarico et al. (2008). In both studies, the greatest improvements in average daily gain occurred during the initial 28 d on either a cottonseed hull or high moisture corn finishing diet (Tricarico et al., 2008). However, in another study with feedlot steers fed diets containing either dry rolled or steam flaked corn, supplemental α-amylase had no effect on cattle performance or total tract fiber digestibility (DiLorenzo et al., 2010).

Reduced starch rations with added amylase

A recent study with dairy cows reported improvements in feed efficiency in lactating cows from the feeding of an exogenous α-amylase in a reduced starch ration (21% DM starch) compared to an un-supplemented reduced starch ration where corn grain had been partially replaced by soyhulls (Gencoglu et al., 2010). Additionally, there was an increase in DM, OM, NDF, and CP total tract digestibility for cows fed the reduced starch diet with exogenous amylase over both normal starch without amylase (27% DM starch) and reduced starch without amylase (22% DM starch) control cows. Greater conversion of feed to milk for cows fed a reduced starch diet with exogenous amylase may offer the potential for improving economic performance depending on diet and additive costs (Gencoglu et al., 2010). In a similar study, Ferraretto et al. (2011) fed a normal starch ration (27% DM starch)and a reduced starch ration (22% DM starch) where corn grain and soybean meal had been partially replaced with wheat middlings and whole cottonseed. The low starch ration

was fed with and without additional amylase. However, they found that cows fed the low starch diet with amylase had reduced milk yield and decreased component corrected milk to feed conversions compared to cows fed the normal starch ration. This variation in results along with the paucity of available data on reduced starch rations containing exogenous amylase warrants further study.

Chapter 3

MATERIALS & METHODS

Experiment 1. Lactation trial

Objective. The objective of this trial was to determine the performance and digestibility response of lactating dairy cows to a reduced starch diet containing a commercial amylase product.

Animals and Treatments. All animal procedures conducted in this experiment were approved by the University of Delaware Agricultural Animal Care and Use Committee. Cows were housed in a sand-bedded freestall barn and were fed individually via a Calan gate system (American Calan, Northwood, NH). Nineteen multiparous (86 ± 46 DIM, 52 ± 21 kg milk/d, 715 ± 97 kg BW at start of trial) and 5 primiparous (93 ± 8 DIM, 41 ± 3 kg milk/d, 565 ± 39 kg BW at start of trial), Holstein cows were used in a replicated 3 × 3 Latin square design experiment with 28 d periods. After a 2-wk adjustment and training period, cows were blocked first by parity (primiparous or multiparous) and secondly by DIM for assignment to the Latin square replicates. The dietary treatments were: 1) a normal starch TMR without exogenous amylase (**NS**), 2) a reduced starch TMR without exogenous amylase (**RS**) and 3) a reduced starch TMR with exogenous amylase (**RSE**). The rations were balanced utilizing the Cornell Penn Miner (**CPM**) ration program. The NS ration was balanced for 40.8 kg/d milk (40.9 kg/d ME allowable milk), 3.8% fat and 3.1% protein

with a predicted DMI of 26.8 kg/d. The reduced starch rations were balanced for the same milk, fat, and protein as the NS ration, although the ME allowable milk was lower (38.5 kg/d). The TMR fed to all cows contained 59% forage and 41% concentrate (Table 1). The NS TMR contained 12.8% corn gain, 2.9% soyhulls, and 2.9% citrus pulp. The RS and RSE TMR contained 6.0% corn grain, 6.9% soyhulls and 6.9% citrus pulp. Sucrose (1.03%) was added to the NS TMR to balance the ration for the higher concentration of sugars present from the citrus pulp in the RS and RSE TMR. The RSE treatment was designed to provide 732 Kilo Novo units (**KNU**) amylase activity per kg grain mix DM and 300 KNU amylase activity per kg of TMR DM. One KNU is the amount of enzyme that releases in a 2-step α-amylase/α-glucosidase reaction, 6 μmol of *p*-nitrophenol per minute from 1.86 m*M* ethylidene-G7-*p*-nitrophenyl-maltoheptaoside at pH 7.0 and 37˚C (Jung and Vogel, 2008). The amylase for the RSE ration was provided in a dry form (Ronozyme RumiStar, DSM, Inc., Basel, Switzerland) and blended into the concentrate grain mix during formulation at the feed mill (Renaissance Nutrition, Inc., Roaring Springs, PA). During this trial all cows were fed ad libitum once daily at approximately 0800 h. Refusals from the previous day were measured and removed prior to feeding. Cows were milked twice daily at approximately 0500 and 1630 h and milk production was recorded automatically via computer.

Sampling and analysis. Silage and TMR samples were collected 3 times a week and stored at -20°C. At the end of each week, frozen samples were thawed and composited. Samples of each concentrate mix and hay were collected once a week. Dry matter of all weekly samples was determined following drying for 48 h in a

forced-air oven at 60˚C, and used for weekly DM adjustments of TMR mixing. Once a period, feed nutrient content was analyzed by wet chemistry methods (Cumberland Valley Analytical Services, Hagerstown, MD). Due to limited storage space, batches of grain mix were produced 3 times during the experiment, with a new batch used each period. Samples of each new batch were collected for measurement of amylase activity. Additional samples were analyzed for amylase activity at the end of each period. At the end of Period 1, these samples were taken from a single feed tub; during Periods 2 and 3, samples of each grain mix were collected weekly and stored at room temperature until composited by period. Amylase activity was measured by DSM Nutritional Products Analytical Services Center (Basel, Switzerland) as described by Jung and Vogel (2008).

Milk samples were taken weekly throughout the trial at consecutive afternoon and morning milkings. During the last week of each period milk samples were taken at 2 consecutive afternoon and morning milkings. Samples were analyzed by Dairy One Cooperative Inc. (University Park, PA) for milk fat, protein, lactose, milk urea nitrogen (**MUN**), and somatic cell count using a Milkoscan System 4000 (Foss North American, Eden Prairie, MN).

Four blocks of 3 multiparous cows were used for nutrient digestibility determination. Fecal grab samples were collected from these cows via rectal palpation during the last 2 d of each period. Samples were collected at 4 time points, which were offset by 6 h at 0900 h d 1, 2100 h d 1, 0300 h d 2, and 1500 h d 2. A portion of each fecal sample was collected and frozen at -20˚C until VFA analysis. The remaining samples were frozen at -20˚C until they were composited (150 ± 20 g, from each time

point) into 1 sample per cow per period. Each TMR and each cow's refusals were sampled daily during fecal sample collection. Fecal composites, TMR, and refusals samples were dried for 48 h in a 60˚C forced-air oven. Refusals samples were used for DMI calculations. Fecal composite and TMR samples were ground through a 2-mm screen using a Wiley Mill (Philadelphia, PA) and analyzed for NDF, N, starch, ash and indigestible NDF. Neutral detergent fiber was determined using sodium sulfite and α-amylase (Goering and Van Soest, 1970) using the Ankom 200 Fiber Analyzer (Ankom Technology, Macedon, NY). Nitrogen was determined using an Elementor Vario Max CN Analyzer (Elementor Americas Inc., Mt. Laurel, NJ). Starch was analyzed by wet chemistry (Hall, 2009; Cumberland Valley Analytical Services, Hagerstown, MD), and ash content was measured following 5 h at 600˚ C in a muffle furnace. Indigestible NDF was used as a marker to calculate fecal output and apparent total tract digestibility (Oba and Allen, 1999). The indigestible NDF was determined after 120 h of in vitro rumen incubation using the Goering and Van Soest (1970) method with modifications. These modifications were weighing the samples into filter bags and incubating them in buffer and rumen fluid for 120 h using a Daisy II incubator (Ankom Technology, Macedon, NY). Rumen fluid was collected from 2 lactating cows being fed the lactating herd ration. After 60 h of incubation, the original rumen fluid and buffer were discarded and were replaced with fresh fluid and incubation continued for an additional 60 h. Analysis of fecal VFA was performed using high phase liquid chromatography on prepared fecal grab samples, as described by Muck and Dickerson (1998).

Statistical Analysis. Weekly averages for milk yield and DMI were calculated and data from the last week of each period were evaluated. The 3.5% fat corrected milk (**3.5FCM**) was calculated as (0.4324 × kg/d milk) + (0.16216 × % milk fat × kg/d milk). Intake, milk production and milk composition were evaluated using SAS with a mixed model including the fixed effects of treatment, parity, and period, and interactions of treatment × period and treatment × parity. Cow and square were included as random effects. Nutrient digestibility data were analyzed using a mixed model with fixed effects of treatment, period, and their interaction, and random effects of cow and square. Fecal VFA were evaluated using a model including fixed effects of treatment, period, hour, treatment × hour, and treatment × period. Random effects were cow and square. Hour was included as a repeated measure with a heterogeneous autoregressive covariance structure. Pre-planned non-orthogonal contrasts evaluated the effect of starch (NS vs. RS + RSE) and amylase (RS vs. RSE).

Experiment 2. Effect of amylase on in vitro starch digestibility

Objective. The objective of this experiment was to determine the effect of various levels of amylase on in vitro starch digestibility of various substrates.

In vitro treatments. All procedures in this experiment were performed at Cumberland Valley Analytical Services (Hagerstown, MD. For the in vitro starch digestion the substrates were: 1) the NS concentrate grain mix from the lactation trial (23.6% starch), 2) the RS concentrate grain mix from the lactation trial (15.4% starch), and 3) a practical grade corn starch (**CS**; 68.4% starch; S4180, Sigma Aldrich, St. Louis, MO). The NS and RS substrates were ground through a 2-mm screen using a Wiley Mill (Philadelphia, PA). The amylase treatments were: 0, 382 ± 14, 1274 ± 46,

or 3822 ± 139 KNU/kg TMR DM of amylase treatment solution added to each of the three substrates. The 382 KNU/kg TMR DM amylase level was intended to correspond to the 300 KNU/kg target amylase activity of the TMR fed during the lactation trial, although due to a miscalculation the actual amylase levels in this experiment were higher than intended. The substrates (1.000 g) were weighed into Erlenmeyer flasks and all sample and amylase combinations were analyzed in triplicate on each of 2 consecutive days.

Concentrated amylase was diluted to make the amylase treatment solutions. To make the amylase treatment solutions, 0, 0.112, 0.375, or 1.124 mL of liquid Ronozyme RumiStar (providing 302 KNU/mL) was dissolved into in vitro buffer to make a total volume of 100 mL of 0, 0.34, 1.13, or 3.40 KNU/mL amylase, respectively. One mL of amylase solution was added to each flask containing substrate to provide the equivalent amylase concentration of 0, 382, 1274, or 3822 KNU/kg substrate DM.

In vitro assay. The in vitros were performed without pre-incubation (co-incubation) or with pre-incubation. For substrates that were co-incubated, 1 mL amylase treatment solution was added to each flask to provide the equivalent amylase concentration of 0, 382, 1274, or 3822 KNU/kg DM followed by 39 mL of buffer solution, equaling a final volume of 40 mL. This was immediately followed by the addition of 2 mL of cysteine HCl and 20 mL of rumen fluid. The flasks were then incubated under anaerobic conditions for 7 h at 40˚C. Pre-incubation of samples were the same as described above, except that amylase treatment solution (0 or 1274 KNU/kg DM) and buffer were added to the flasks and placed in a 40˚C water bath for

18 h prior to incubation. After in vitro digestion of co-incubated and pre-incubated samples, 15 mL of acetate buffer was added to all flasks and samples were frozen until starch analysis, which was performed as described by Hall (2009).

Statistical analysis. Effects of amylase on in vitro starch digestibility were determined for both co-incubation and for pre-incubation. For the co-incubation, the data set contained only the co-incubation results. The model included the fixed effects of substrate, amylase, day, and all 2 and 3 way interactions. For pre-incubation the data set contained both pre- and co-incubation results for all substrates at the 0 and 1274 KNU/kg DM amylase treatments. The model included the fixed effects of substrate, amylase, pre-incubation, day, and all 2, 3, and 4 way interactions.

Chapter 4

RESULTS

Experiment 1. Lactation trial

All rations were isonitrogenous containing an average of 16.2% CP (Table 2). The NDF contents for NS, RS and RSE rations were 29.9, 33.1, and 32.9%, respectively. The ADF contents for NS, RS and RSE rations were 20.0, 22.8, and 22.7%, respectively. The starch concentrations in NS, RS, and RSE rations were 27.5, 23.2 and 22.4%, respectively.

The manufacturer guaranteed minimum activity of the amylase product was 320 KNU/g, and was measured to contain 93.5% DM, which equates to 342 KNU/g amylase DM. The product analyzed 390 KNU/g amylase activity, 21% above the guaranteed minimum. The expected amylase activity of the grain mixes accounting for this overage is presented in Table 3. Amylase activity analyzed in grain mix samples collected each period was 12% higher than expected in Periods 1 and 2 and 31% lower than expected in Period 3, resulting in measured and expected amylase activity being quite similar when averaged across the 3 periods.

Dry matter intake, milk production and composition, and feed efficiency are shown in Table 4. The treatment × parity interaction was not significant for any measures. A treatment × period interaction was observed for MUN ($P = 0.008$; Figure 1) where there was a dramatic increase in MUN over time with RS, while the increase

over time was less for NS and RSE. Treatment × period interactions tended to occur for milk fat yield, 3.5FCM, and somatic cell score (**SCS**; $P<0.10$, Figure 12, 13 and 14).

There was no effect of treatment on DMI, feed efficiency, milk fat percentage, milk fat yield, milk lactose percent, or SCS. Treatment effects were found for milk yield and 3.5FCM yield. For both measures there was an effect of starch ($P = 0.01$ and $P = 0.04$, respectively), and animals fed the normal starch diet produced 1.9 kg/d more milk and 1.5 kg/d more 3.5FCM than the average of those fed the reduced starch diets. There also tended to be an effect of enzyme on milk yield and 3.5FCM yield ($P = 0.06$ and $P = 0.09$, respectively), with RSE cows producing 1.6 and 1.4 kg/d less than RS cows. The addition of enzyme to the reduced starch ration increased milk protein percentage ($P = 0.006$), but there was no effect of enzyme on milk protein yield. Milk protein yield was affected by starch ($P = 0.01$), with cows fed the NS diet producing 0.06 kg/d more than cows fed the reduced starch diets. The treatment effect on lactose yield was due to both starch and enzyme effects. Cows fed the NS diet had greater lactose yields than cows fed the reduced starch diets ($P = 0.006$), and cows fed the RS diet had increased lactose yields compared to those fed the RSE diet ($P = 0.03$). There was an effect of starch ($P = 0.02$) on MUN, with cows fed the normal starch ration having lower MUN concentrations than cows fed the reduced starch rations. There was also a tendency ($P = 0.10$) for RSE to have a higher SCS than RS.

The apparent total tract nutrient digestibility and fecal VFA measure results are shown in Tables 5 and 6. Treatment × period interactions were found for DM ($P = 0.005$), OM ($P = 0.01$), and CP ($P = 0.006$) digestibility (Figures 2, 3, and 4),

and tended to occur for NDF digestibility ($P = 0.09$, Figure 15). The DM digestibility for RSE increased as time progressed, while DM digestibility for NS and RS decreased in period 2 and increased in period 3 (Figure 2). Similar patterns were responsible for the treatment × period interactions observed for OM digestibility (Figure 3). Crude protein digestibility decreased between periods 1 and 2 for NS but increased for RSE, while RS remained relatively constant. There was little change in CP digestibility between periods 2 and 3 for any of the 3 treatments (Figure 4).

There was no difference among treatments for starch digestibility (average of 99.1%). There was an effect of starch ($P = 0.005$) on NDF digestibility, and digestibility was greater for cows fed reduced starch diets than for cows fed the NS diet. There was an effect of enzyme on DM ($P = 0.02$) and CP ($P = 0.05$) digestibility with RSE cows having greater DM and CP digestibility than RS alone. There was also a tendency ($P = 0.10$) for OM digestibility to be greater for RSE cows compared to RS.

Fecal VFA and lactate concentrations are shown in Table 7. Fecal butyrate tended to be greater ($P = 0.06$) for NS cows than for cows fed the reduced starch diets, but no other treatment effects on fecal VFA measures were observed. Time affected all VFA measures except isovalerate. Feeding occurred at approximately 0800 h each morning. The lowest total fecal VFA concentrations were observed at 2100 h and 0300 h, 13 and 19 h after feeding, respectively, while the highest values were observed at 0900 and 1500 h, 1 and 7 h after feeding (Figure 5). Total fecal VFA concentration was plotted against fecal NDF and fecal starch percentages to evaluate whether undigested carbohydrate concentrations affected fecal VFA concentrations (Figures 5

and 6). No relationships were observed between fecal VFA and fecal NDF percentage, or between fecal VFA and fecal starch percentage (R^2 = 0.04 and 0.01, respectively).

Experiment 2. Effect of amylase on in vitro starch digestibility

The in vitro starch digestibility results for co-incubation are shown in Table 8. Interactions of day × substrate, day × amylase, and day × amylase × substrate were not significant for any measure. An effect of day on co-incubation was observed (P<0.001). An amylase × substrate interaction was observed (P<0.001; Figure 9). Amylase addition at 382 KNU/kg DM increased digestibility of CS, 1274 KNU/kg DM increased digestibility of both CS and the RS grain mix, and 3822 KNU/kg DM increased starch digestibility of the NS grain mix.

The pre-incubation results are shown in Table 9. Pre-incubation × amylase and substrate × amylase interactions were not significant. Interactions of day × substrate (P = 0.03), substrate × pre-incubation (P = 0.03), day × amylase × substrate (P<0.001), and day × pre-incubation × substrate (P<0.001) were observed (data not shown). Both pre-incubation and the addition of 1274 KNU/kg DM amylase increased starch digestibility (P<0.001). Samples that were pre-incubated had 3.8 percentage units increased starch digestibility compared to those that were only co-incubated. The addition of 1274 KNU/kg DM amylase increased starch digestibility 7.2 percentage units compared to samples without amylase (Figure 10). An effect of day was also observed (P<0.001).

Chapter 5

DISCUSSION

The third period of the trial had a much lower amylase activity than periods 1 and 2, with an actual activity of 576 KNU/kg concentrate DM and a calculated activity of 236 KNU/kg TMR. We believe that the decrease in amylase activity during this period was due to a mixing error at the feed mill, as the low activity in batch 3 was consistent in all samples taken during period 3. The activity of the amylase product was also very stable, as RSE concentrate samples that were analyzed after 1 yr of storage showed only a 25% decrease in activity (data not shown).

However, the decrease in amylase activity in period 3 did not seem to be responsible for most of the observed treatment × period interactions because period 3 had similar treatment differences as observed in either periods 1 or 2. Milk urea nitrogen, DM digestibility, OM digestibility, CP digestibility, fat yield, and 3.5FCM interactions were caused by a difference in either period 1 or 2 compared to the other 2 periods (Figures 1, 2, 3, 4, 12, and 13, respectively). There is a possible exception for SCS, because treatment effects were similar between periods 1 and 2 but different for period 3 (Figure 14). However, the overall SCS was relatively high on this trial and the increased SCS is not attributed to the treatments differences. Treatment differences

in NDF digestibility were different for all 3 periods (Figure 15). However, RS and RSE behaved similarly except for period 3 where NDF digestibility was actually greater for RSE than RS cows; we conclude that the lower amylase activity in period 3 did not affect the NDF digestibility treatment response.

In the current trial no effect of dietary treatment on DMI or feed efficiency was observed. Similarly, Ferraretto et al. (2011) reported no effect of amylase (324 KNU/kg TMR DM) on DMI when added to a reduced starch (21% starch) diet compared to reduced starch (22% starch) without amylase and normal starch (27% starch) without amylase diets. However, a reduction in feed efficiency for cows fed both of the reduced starch diets compared to the normal starch diet was reported (Ferraretto et al., 2011). In the trial of Weiss et al. (2011), reduced starch diets (26% starch) with amylase (332 KNU/kg TMR DM) and without amylase along with a high starch (31% starch) control diet were fed. Decreased DMI was observed for cows fed the reduced starch diets with no effect of amylase. Gencoglu et al. (2010) found similar DMI between cows fed the reduced starch with amylase diet (21% starch; 332 KNU/kg TMR DM) and those fed the normal starch (27% starch) control diet, while increased DMI was observed for cows that were fed a reduced starch without amylase diet (22% starch). Improvement in feed efficiency with the addition of amylase to the reduced starch ration was also reported (Gencoglu et al., 2010). The addition of amylase to a reduced starch diet does not appear to consistently improve feed efficiency or DMI when compared to a normal starch ration or a reduced starch ration without amylase.

We had hypothesized that the addition of amylase to the reduced starch diet would increase the milk yield of RSE cows to equal that of the NS cows. However, the cows on the reduced starch diets produced 2.7 kg/d less milk and 2.2 kg/d less 3.5FCM than NS cows, and milk production of RSE cows tended to be lower than RS cows (Table 4). In previous trials, 1 reported decreased milk yield for cows fed a reduced starch diet compared to a normal starch diet, 1 reported a trend for decrease milk yield, and 2 reported no effect of starch content on milk yield (Gencoglu et al., 2010; Ferraretto et al., 2011; Weiss et al., 2011).

In the current trial the difference in milk yield between the NS and the reduced starch rations was 2.7 kg/d, similar to the CPM predicted difference of 2.4 kg/d. However, when averaged across all 3 treatments, DMI was 1.6 kg/d less than was balanced for and milk production was 5.7 kg/d higher than was balanced for. When the actual average milk yield, DMI, milk fat percentage, and milk protein percentage were put into CPM, the predicted ME allowable milk was 44.3 kg/d for NS, 42.6 kg/d for RS, and 41.0 kg/d for RSE, and all are about 4 kg/d lower than observed milk yields. Cows on this trial appeared to respond to the limited dietary energy. However, during this trial the milk fat was depressed for all treatments. It is proposed that rumen fermentation was altered, and that these alterations resulted in biohydrogenation that lead to a reduction in milk fat synthesis (Bauman et al., 2011). The milk fat depression may have created a rumen environment in which we were not able to see an improvement in performance from amylase treatment even though amylase increased diet digestibility.

Previous studies have evaluated incorporation of amylase into normal starch rations. The addition of amylase to a 26% starch TMR increased milk production and DMI in the trial of Klingerman et al. (2009). Other trials have shown an increase (Tricarico et al., 2005) and a tendency for an increase (Harrison and Tricarico, 2007) in milk yield but not DMI when amylase was fed as part of a normal starch ration. In contrast, DeFrain et al. (2005) observed no effect of amylase on milk or DMI during lactation. In beef cattle, exogenous amylase improved average daily gain in two studies (Tricarico et al., 2008) but had no effect on cattle performance in another (DiLorenzo et al., 2010). Exogenous amylase has been shown to increase milk production and sometimes improve beef cattle performance when incorporated as part of a normal starch ration.

In the current trial, cows fed the RSE ration had a higher milk protein percentage than those fed RS. However, this was not reflected in protein yield because of the decreased milk yields for cow fed the RS rations, and cows fed either of the reduced starch diets had 0.6 kg/d lower protein yield than cows fed NS. In previous studies, reduction in starch content has been reported to decrease protein yield (Ferraretto et al. 2011; Weiss et al., 2011), although one study reported no effect (Gencoglu et al., 2010). The decreased starch content in the RS and RSE rations may have reduced rumen microbial protein production and flow and thus decreased milk protein yield (Oba and Allen, 2003).

In the current trial increased MUN concentrations were observed for cows fed the reduced starch rations compared to those fed the NS ration, but no effect of amylase was observed. Ferraretto et al. (2011) and Gencoglu et al. (2010) similarly

observed increased MUN in cows fed reduced starch compared with those fed the normal starch ration. Weiss et al. (2011) reported no difference among treatments in MUN during both their production and digestibility experiments. The higher MUN concentration and lower protein yield for cows fed the reduced starch rations in the current experiment suggest that there was less microbial protein available to support milk protein yield in the reduced starch rations compared to the NS. Gencoglu et al. (2010) reported an effect of amylase on MUN concentration, where cows fed the reduced starch ration with amylase had decreased MUN concentrations compared to the cows fed reduced starch without amylase. When exogenous amylase was included in a normal starch ration, Tricarico et al. (2005) reported no difference in milk protein or MUN while Klingerman et al. (2009) reported higher protein yield in amylase supplemented cows with no difference in MUN. Although decreasing dietary starch tends to increase MUN, the addition of amylase in either a normal starch or reduced starch ration does not appear to consistently effect MUN concentration.

There was no difference among treatments in lactose percentage in the current trial, although cows fed the RSE ration had decreased lactose yield compared to those fed the NS or RS rations, and cows fed the NS ration had greater lactose yield than cows fed the reduced starch rations due to the differences in milk yields for all 3 treatments. Gencoglu et al. (2010) and the digestibility experiment of Weiss et al. (2011) reported no differences in starch or enzyme treatment for lactose percentage or yield. Weiss et al. (2011) reported that cows fed a diet with reduced starch and amylase treatment had lower lactose percentages compared to cows fed the high starch ration. They also found an effect of starch on lactose yield with cows fed the higher

starch ration yielding greater lactose than those fed the reduced starch rations (Weiss et al., 2011). Ferraretto et al. (2011) similarly observed decreased lactose yield for cows fed the reduced starch diet compared to cows fed the normal starch diet. The decrease in lactose yield observed in previous trials would suggest that there was greater available glucose associated with the rations higher in dietary starch. Across trials, a reduction in dietary starch appears to reduce milk lactose yield, and addition of amylase does not appear to compensate for this reduction.

One proposed mode of action for amylase is that supplemental amylase increases the release of starch hydrolysis products (Tricarico et al., 2008). While the exact mechanism is not known, the effect of amylase on rumen fermentation is believed to be caused by these hydrolysis products providing substrate to non-amylolytic organisms and thereby modifying bacterial populations and VFA production in what is called a cross feeding mechanism (Tricarico et al., 2008). We hypothesized that the addition of amylase would stimulate cross feeding to increase apparent total tract DM, OM, CP, starch and NDF digestibility of the RSE cows compared to RS cows.

Nutrient digestibility data are reported in Table 5 tend to be higher than those previously reported. For example, average DM digestibility in this experiment was 71.1% compared to a mean of 64.9% (range 61.8 to 69.4%) from several studies using lactating cows (Knowlton et al., 2002; Burkholder et al., 2004; Weiss et al., 2011). We believe that fecal output may have been underestimated and therefore inflated digestibility calculations. However, this bias should be consistent among treatments and not hinder interpretation of treatment effects. No difference among

treatments was observed for apparent total tract starch digestibility in the current trial (average of 99.1%). Although Weiss et al. (2011) observed much lower starch digestibility (average of 88.5%), they also found no effect of amylase on starch digestibility.

There was an increase in apparent total tract NDF digestibility for cows fed the reduced starch diets compared with cows fed the normal starch diet. By-product feeds have highly digestible NDF (Firkins, 1997), and the increased inclusion of by-product feeds in the reduced starch rations may have contributed to increased NDF digestibility. Increased NDF digestibility in reduced starch diets with amylase as compared to reduced starch without amylase has been previously observed (Gencoglu et al., 2010; Weiss et al., 2011). The increased NDF digestibility in the presence of enzyme may be attributed to the cross feeding mechanism where amylase produces hydrolysis products that provide substrate to non-amylolytic organisms (Tricarico et al., 2008).

In the current trial cows fed RSE had increased DM and CP digestibility compared to those fed RS. There was also a tendency for OM digestibility to be greater for cows fed RSE in comparison with those fed RS. Gencoglu et al. (2010) similarly observed an increase in DM, OM and CP total tract digestibility for cows fed the reduced starch diet with exogenous amylase compared to the reduced starch diet without amylase. In contrast Weiss et al. (2011) reported no effect of enzyme on DM, OM or CP digestibility, but found that reduced dietary starch resulted in decreased DM and OM digestibility. In contrast, the current trial and that of Gencoglu et al. (2010) observed increased DM and OM digestibility in cows fed the reduced starch

rations compared to the normal starch rations. In the 3 trials, the addition of exogenous amylase to a reduced starch ration most often increased NDF, DM, OM and CP digestibility, but did not consistently improve animal production performance. In the current trial cows were mid to late lactation animals and were not in a situation of negative energy balance which may have impeded any amylase performance response, as animals on all treatments were gaining weight (Table 6). While not statistically significant, cows on the RSE treatment gained 5.6 kg more BW than cows on the RS treatment. Because of the increased DM and CP digestibility in response to amylase treatment, we suspect that this slight increase in weight change may have been due to amylase treatment.

The rate and extent of starch digestion in the rumen influences the concentration and composition of ruminal VFA, along with the amount of starch available for post-ruminal digestion (Kotarski et al., 1992). Because fecal VFA profiles reflect rumen VFA (Hoover, 1978; Siciliano-Jones and Murphy, 1989), it was hypothesized that there would be an increase in total fecal VFA concentration and fecal propionate for cows fed the NS ration compared to cows fed the reduced starch diets because of the higher dietary starch content. It has been suggested that by-product NDF has much greater post-ruminal NDF digestion (Firkins, 1997). It was hypothesized that the higher inclusion of by-products in the reduced starch rations would increase post-ruminal NDF digestion resulting in increased proportions of fecal acetate and butyrate. We also expected the inclusion of amylase to further increase acetate and butyrate compared to the reduced starch diet without amylase due to amylase stimulating NDF digestion via the cross feeding mechanism. For example,

previous studies have shown that feeding of amylase increased ruminal butyrate concentration (Hristov et al., 2000; Tricarico et al., 2002). Contrary to our hypotheses, fecal butyrate tended to be greater for NS than for the reduced starch diets, and no other treatment effects on fecal VFA measures were observed.

Total fecal VFA concentration was plotted against fecal NDF and fecal starch percentages to evaluate whether residual carbohydrate concentration affected VFA concentrations (Figures 5 and 6). We suspected that samples with greater carbohydrate would have reflected increased carbohydrate available to hindgut fermentation and hence increased hindgut VFA concentrations. However, no relationships were observed between fecal VFA and fecal NDF percentage or between fecal VFA and fecal starch percentage.

The effect of amylase on in vitro starch digestibility was measured following the lactation trial due to the absence of production response and the uniformity in total tract apparent starch digestibility across treatments. We hypothesized that in vitro, amylase would increase starch digestibility in the NS grain mix, RS grain mix and CS substrate at increasing amylase levels, and that the 18 h overnight pre-incubation of samples with amylase would increase starch digestibility compared to those that were co-incubated. Co-incubated samples had the greatest starch digestibility at amylase levels of 382 and 1274 KNU/kg substrate DM (Table 8; Figure 8). It appears that the amylase level that was used in the lactation trial (323 KNU/kg TMR DM) was sufficient to increase ruminal starch digestibility. However, the highest amylase dose (3822 KNU/kg substrate DM) was not different from the control. Quadratic effects of dosage have been previously observed with fibrolytic

enzymes (Beauchemin et al., 1995; Kung et al., 2000) and may also explain the lack of effect of 3822 KNU/kg DM amylase. It is possible that overtreatment with enzymes could negate any positive effects incurred from the enzyme at a lower dosage. Overtreatment with fibrolytic enzymes may interfere with the attachment of rumen bacteria to the feed particles, which in turn may decrease rumen digestibility (Treacher and Hunt, 1996). A key step in bacterial digestion of both fiber and starch is bacterial attachment (Huntington, 1997; McAllister et al., 1994), and some amylolytic bacteria adhere to grain particles in the rumen and hydrolyze amylose and amylopectin (Kotarski et al., 1992).

Because of the importance of adsorption and binding of enzyme to substrate (Forwood et al., 1990; Beauchemin et al., 2003), we hypothesized that pre-incubating amylase with substrate would also increase starch digestibility. Pre-incubation without amylase increased starch digestibility compared to the co-incubated samples without amylase. This suggests that overnight hydration alone enhanced starch digestibility. When amylase was included at 1274 KNU/kg substrate DM, the pre-incubated samples had increased starch digestibility compared to co-incubated samples at the same level of amylase (Figure 10). When fibrolytic enzymes were pre-incubated with forage, Forwood et al. (1990) reported increased in vitro DM digestibility. In vivo, Forwood et al. (1990) and Beauchemin et al. (1999) reported that liquid exogenous enzymes should be applied to TMR before feeding to allow the enzyme to bind to the feed particles. Because the amylase that was fed during the lactation trial was in a dry form, the lack of hydration time may have impeded enzyme attachment to the feed particles.

CONCLUSIONS

Feeding a reduced-starch diet formulated by partially replacing corn grain with soyhulls and citrus pulp resulted in decreased milk, FCM, milk protein yield, and milk lactose yield, and increased MUN and NDF digestibility compared with a normal starch diet. The addition of exogenous amylase to the reduced-starch diet resulted in increased milk protein percentage, decreased milk lactose yield and increased DM, and CP digestibility, and tended to decrease milk yield and 3.5% FCM yield. While exogenous amylase increased nutrient digestibility in the reduced starch diet, this was not accompanied by improvements in animal performance.

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Appendix A

TABLES

Table 1. Ration composition

	Treatments[1]		
Ingredient, %	NS	RS	RSE
Corn silage	43.30	43.30	43.30
Alfalfa silage	10.50	10.50	10.50
Alfalfa hay	5.20	5.20	5.20
Corn grain, ground	12.81	6.06	6.06
Soybean hulls	2.88	6.95	6.95
Citrus pulp	2.85	6.92	6.92
Sucrose	1.03	0.00	0.00
Protected soybean meal[2]	6.94	6.94	6.94
Canola meal	3.46	3.46	3.46
Dried corn distillers grains	3.46	3.46	3.46
Soybean meal	2.93	2.57	2.57
Blood meal	0.97	0.97	0.97
Rumen bypass fat[3]	0.75	0.75	0.75
$NaHCO_3$	0.63	0.63	0.63
$CaCO_3$	0.56	0.56	0.56
NaCl	0.43	0.43	0.43
Trace mineral and vitamin mix[4]	0.33	0.33	0.33
$CaSO_4 \bullet 2H_2O$	0.27	0.27	0.27
Monensin[5]	0.27	0.27	0.27
Urea	0.21	0.21	0.21
MnO	0.16	0.16	0.16
Protected methionine[6]	0.06	0.06	0.06
Amylase[7]	0.00	0.00	0.10

[1]Treatments: NS = Normal Starch; RS = Reduced Starch; RSE = Reduced Starch Enzyme.
[2]Extruded and expelled soybean meal, J. L. Moyer & Sons, Inc., Turbotville, PA.
[3]Bergafat F-100, Berg & Schmidt, Hamburg, Germany.
[4]Contained 33.0% Mg, 8.0% S, 4.5% K, 12,028 ppm Zn, 6017 ppm Mn, 2252 ppm Cu, 1918 ppm Fe, 218 ppm I, 164 ppm Co, 84 PPM Se, 1,411 IU/g Vitamin A, 353 IU/g Vitamin D, 7 IU/g Vitamin E.
[5]Rumensin 90®, Elanco, Greenfield, IN.
[6]Smartamine M, Adisseo, Alpharetta, GA.
[7]RONOZYME® RumiStar DSM Nutritional Products, Ltd., Kaiseraugst, Switzerland.

Table 2. Analyzed ration nutrient composition[1]

	Treatments[2]		
Item	NS	RS	RSE
Nutrient composition, % DM			
DM	51.9	52.1	52.0
CP	16.2	16.2	16.3
RDP	10.9	10.9	11.0
RUP	5.3	5.3	5.3
NDF	29.9	33.1	32.9
ADF	20.0	22.8	22.7
NFC	43.8	40.7	40.7
Starch	27.5	23.2	22.4
Ash	6.6	6.8	7.0
Ca	0.89	0.94	0.98
P	0.36	0.34	0.34
Mg	0.39	0.37	0.40
K	0.42	0.41	0.43
NE_L, Mcal/kg	1.72	1.67	1.67

[1]Cumberland Valley Analytical Services, Hagerstown, MD.
[2]Treatments: NS = Normal Starch; RS = Reduced Starch; RSE = Reduced Starch Enzyme.

Table 3. Amylase inclusion levels and activity

Period	Amylase inclusion rate[1]	Expected amylase activity[2]	Measured amylase activity[3]	Measured activity as % of expected[4]	Calculated amylase activity in TMR[5]
1	2.29	892	996	112	408
2	2.29	892	998	112	409
3	2.14	835	576	69	236
Average		873	857	98	351

[1]Amylase inclusion rate in g amylase/ kg grain mix (as fed basis).

[2]Expected amylase activity of the RSE grain mix, KNU/kg grain mix DM. Calculated using the measured KNU/g of the amylase product.

[3]Measured amylase activity in the RSE grain mix, KNU/kg grain mix DM.

[4]Measured activity as a percentage of expected activity = Measured activity/Expected activity × 100.

[5]Amylase activity in the TMR was calculated as the measured amylase activity × 0.41 kg grain per kg TMR DM.

Table 4. Milk production, composition and intake[1]

	Treatments[2]				*P*- values					
Item	NS	RS	RSE	SEM	Treatment	Starch	Enzyme	Period	Parity	Treat × Period
DMI, kg/d	25.4	25.4	24.8	0.71	0.48	0.50	0.32	0.65	<0.001	0.11
Milk, kg/d	47.8[a]	46.7[ab]	45.1[b]	2.04	0.008	0.01	0.06	<0.001	0.02	0.30
3.5FCM, kg/d[3]	42.4[a]	41.6[ab]	40.2[b]	1.87	0.03	0.04	0.09	<0.001	0.004	0.07
Milk/DMI	1.89	1.86	1.83	0.07	0.42	0.24	0.56	0.04	0.98	0.16
Milk fat										
%	2.82	2.83	2.84	0.17	0.98	0.88	0.89	0.09	0.30	0.16
kg/d	1.34	1.32	1.28	0.09	0.28	0.23	0.29	0.003	0.01	0.06
Milk protein										
%	2.96[a]	2.91[b]	2.98[a]	0.08	0.02	0.51	0.006	<0.001	0.37	0.78
kg/d	1.40[a]	1.35[b]	1.33[b]	0.04	0.03	0.01	0.44	0.002	0.01	0.45
Milk lactose										
%	4.78	4.77	4.73	0.07	0.19	0.18	0.21	0.03	0.17	0.41
kg/d	2.28[a]	2.22[a]	2.13[b]	0.10	0.003	0.006	0.03	<0.001	0.06	0.28
MUN, mg/dL	10.32[b]	11.23[a]	10.89[ab]	0.49	0.04	0.02	0.33	<0.001	0.47	0.008
SCS[4]	3.86	2.61	3.78	0.56	0.14	0.27	0.10	0.64	0.50	0.09

[1]Treatment by parity interactions were not significant for any parameters.
[2]Treatments: NS = Normal Starch; RS = Reduced Starch; RSE = Reduced Starch Enzyme.
[3]3.5% Fat corrected milk, calculated as [0.4324 × milk (kg/d)] + [16.216 × fat (kg/d]/[100 ×milk (kg/d)].
[4]Somatic cell score, calculated as the $\log_2$(SCC/100,000) + 3.

Table 5. Apparent total tract nutrient digestibility

	Treatments[1]				*P*-values				
Item	NS	RS	RSE	SEM	Treatment	Starch	Enzyme	Period	Treat × Period
DM, %	71.0[ab]	70.1[b]	72.1[a]	0.6	0.08	0.98	0.02	0.06	0.005
OM, %	73.2[ab]	72.0[b]	73.4[a]	0.7	0.19	0.45	0.10	0.001	0.01
Starch, %	99.0	99.2	99.1	0.10	0.26	0.14	0.44	0.04	0.21
NDF, %	41.4[b]	46.0[a]	48.0[a]	1.5	0.01	0.005	0.30	0.04	0.09
CP, %	72.8[ab]	71.1[b]	73.4[a]	0.8	0.12	0.59	0.05	0.53	0.006

[1]Treatments: NS = Normal Starch; RS = Reduced Starch; RSE = Reduced Starch Enzyme.

Table 6. Body weight and body weight change

	Treatments[1]				*P*- values					
Item	NS	RS	RSE	SEM	Treatment	Starch	Enzyme	Period	Parity	Treat × Period
Body weight, kg	671.9	694.7	675.7	13.6	0.28	0.16	0.19	0.03	0.03	0.28
Body weight change, kg	13.6	15.1	20.7	6.7	0.68	0.60	0.53	0.31	0.29	0.81

[1]Treatments: NS = Normal Starch; RS = Reduced Starch; RSE = Reduced Starch Enzyme

Table 7. Fecal VFA concentration[1]

	Treatments[2]				*P*- values				
	NS	RS	RSE	SEM	Treatment	Starch	Enzyme	Period	Hour
VFA, m*M*									
Acetate	57.07	54.83	56.73	3.14	0.80	0.68	0.60	0.14	0.04
Propionate	11.12	10.45	10.87	0.68	0.71	0.52	0.60	0.04	0.03
Butyrate	8.76[a]	7.43[b]	7.72[b]	0.53	0.15	0.06	0.67	0.03	0.03
Isobutyrate	0.60	0.53	0.52	0.58	0.49	0.24	0.97	0.50	<0.001
Valerate	0.12	0.09	0.09	0.04	0.68	0.40	0.87	0.05	0.02
Isovalerate	0.12	0.10	0.09	0.26	0.70	0.41	0.92	0.12	0.45
Lactate	0.06	0.06	0.05	0.01	0.90	0.84	0.69	<0.001	0.005
Total VFA, mM^2	77.79	73.42	76.06	4.36	0.69	0.50	0.61	0.09	0.04

[1]Treatment by period and treatment by hour interactions were not significant for any parameters.
[2]Treatments: NS = Normal Starch; RS = Reduced Starch; RSE = Reduced Starch Enzyme.
[3]Total VFA was calculated as the sum of acetate, propionate, butyrate, isobutyrate, valerate and isovalerate.

Table 8. Effect of the co-incubation of different levels of amylase on in vitro starch digestibility[1]

	Amylase level, KNU/kg DM					*P*-values		
Day	0	382	1274	3822	SEM	Sub.[2]	Amy.[3]	Amy. × Sub.
1	51.1[d]	57.2[b]	58.5[a]	53.9[c]	0.8	<0.001	<0.001	<0.001
2	70.1[b]	73.3[ab]	75.8[a]	70.2[b]	1.2	<0.001	0.02	0.02
Combined	60.6[b]	65.2[a]	67.2[a]	62.1[b]	1.4	<0.001	<0.001	<0.001

[1]Data reported as a percentage.
[2]Sub. = substrate, substrates were normal starch grain mix, reduced starch grain mix and corn starch.
[3]Amy. = amylase.

Table 9. Effect of pre-incubation and amylase on in vitro starch digestibility[1]

	0 KNU/kg Amylase		1274 KNU/kg Amylase			*P*-values		
Day	Coinc.	Preinc.	Coinc.	Preinc.	SEM	Preinc.	Amylase	Preinc. × Amylase
1	51.1[d]	56.4[c]	58.5[b]	63.5[a]	1.1	<0.001	<0.001	0.89
2	70.1[c]	71.2[c]	75.8[b]	79.5[a]	1.2	0.05	<0.001	0.28
Combined	60.6[d]	63.8[c]	67.2[b]	71.5[a]	0.8	<0.001	<0.001	0.48

[1]Data reported as a percentage.

Appendix B

FIGURES

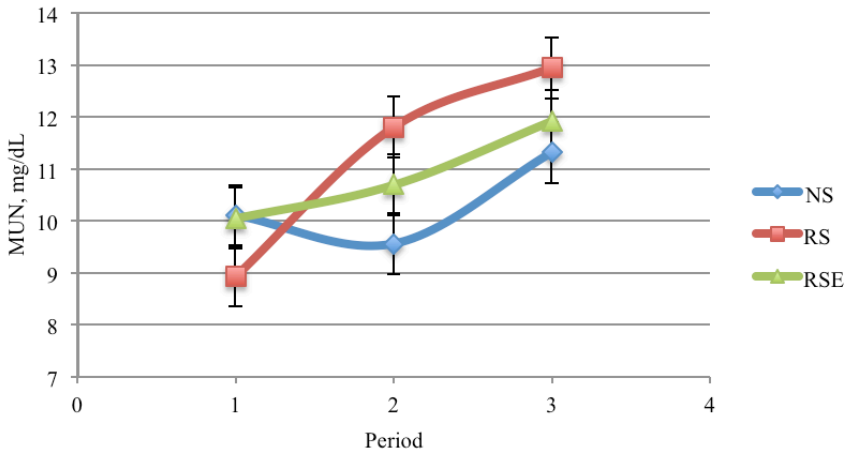

Figure 1. Milk urea nitrogen treatment by period interaction.

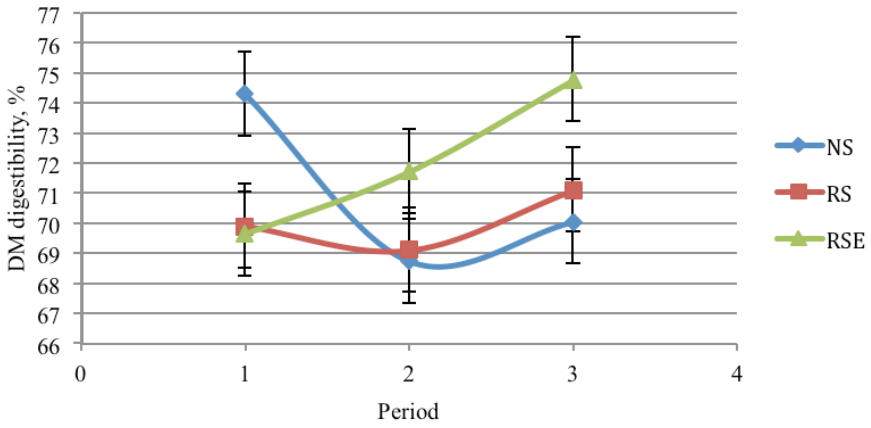

Figure 2. Apparent total tract dry mater digestibility.

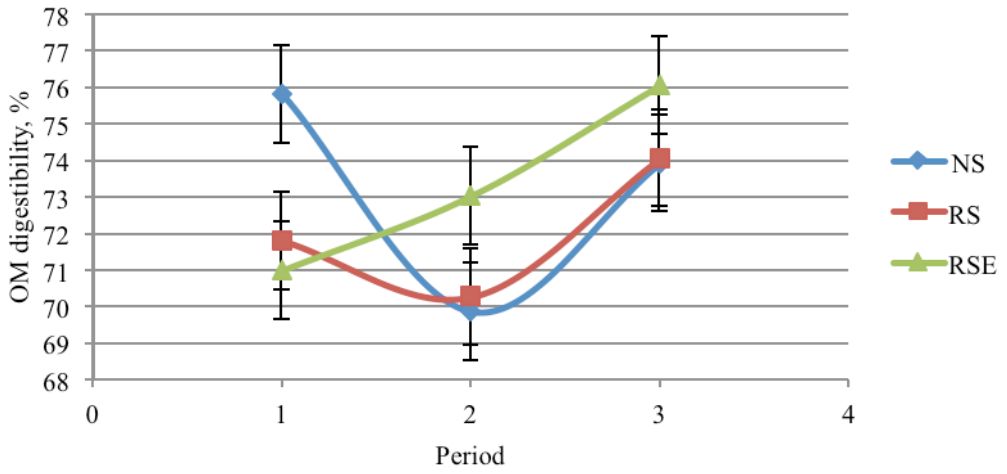

Figure 3. Apparent total tract organic matter digestibility.

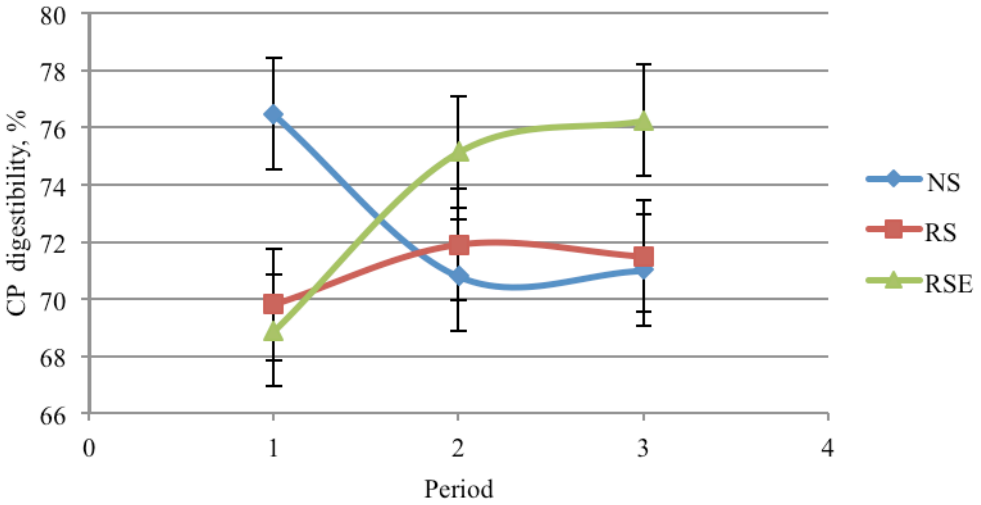

Figure 4. Apparent total tract crude protein digestibility.

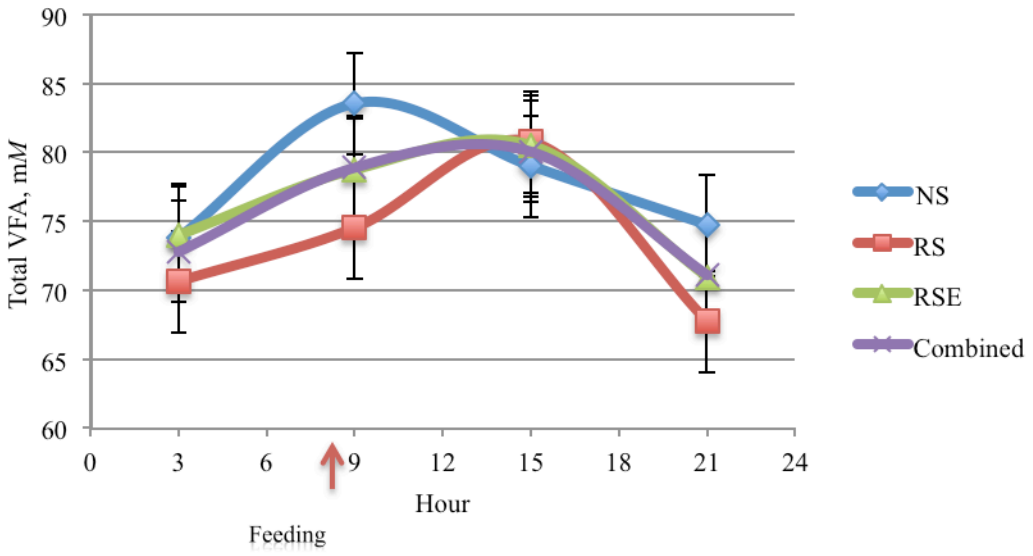

Figure 5. Total fecal VFA concentration.

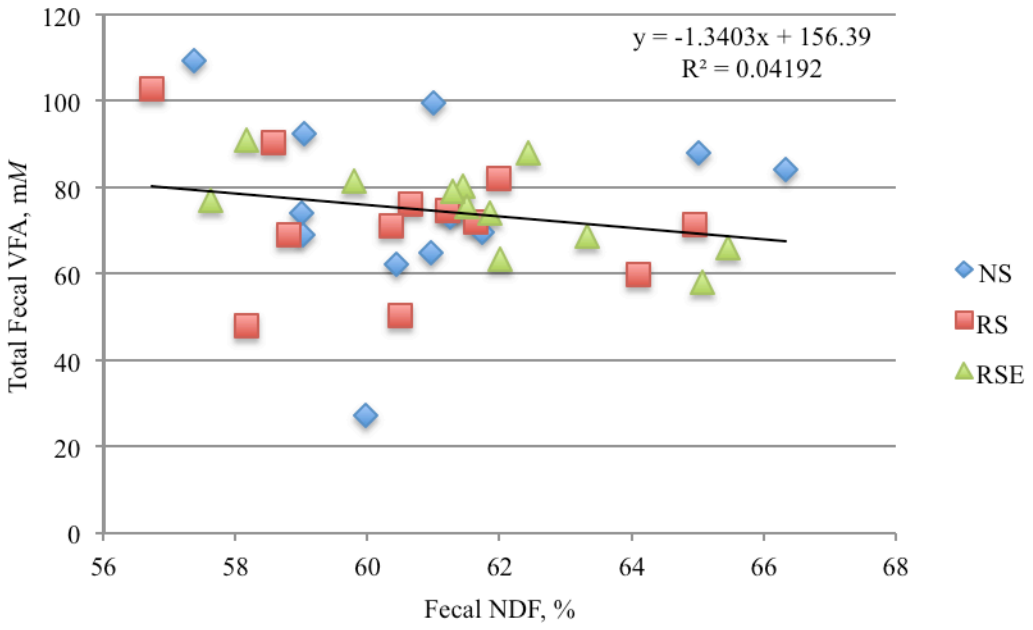

Figure 6. Total fecal VFA vs. fecal neutral detergent fiber.

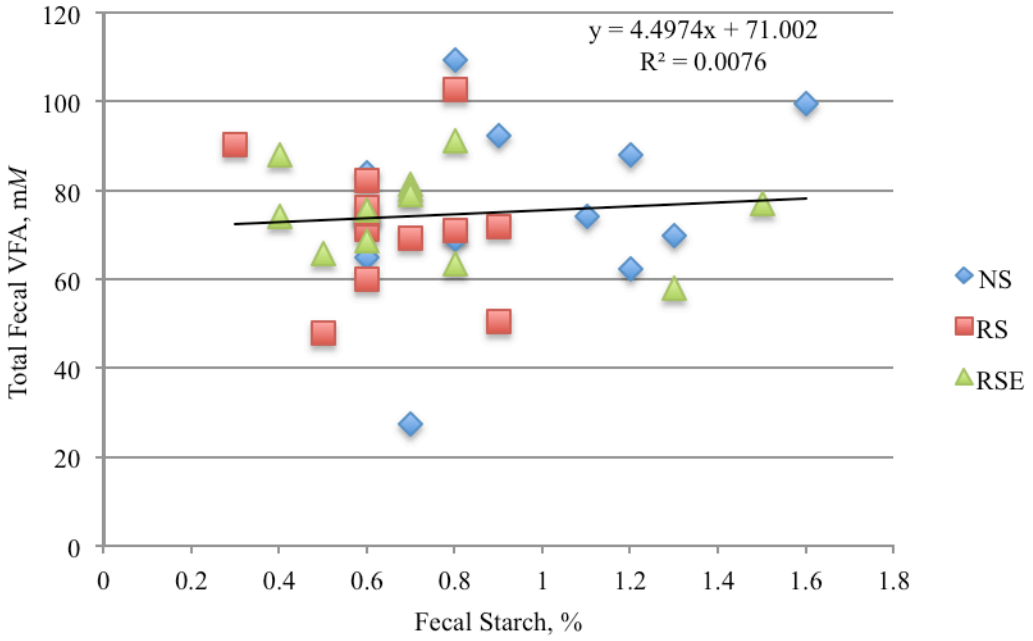

Figure 7. Total fecal VFA vs. fecal starch.

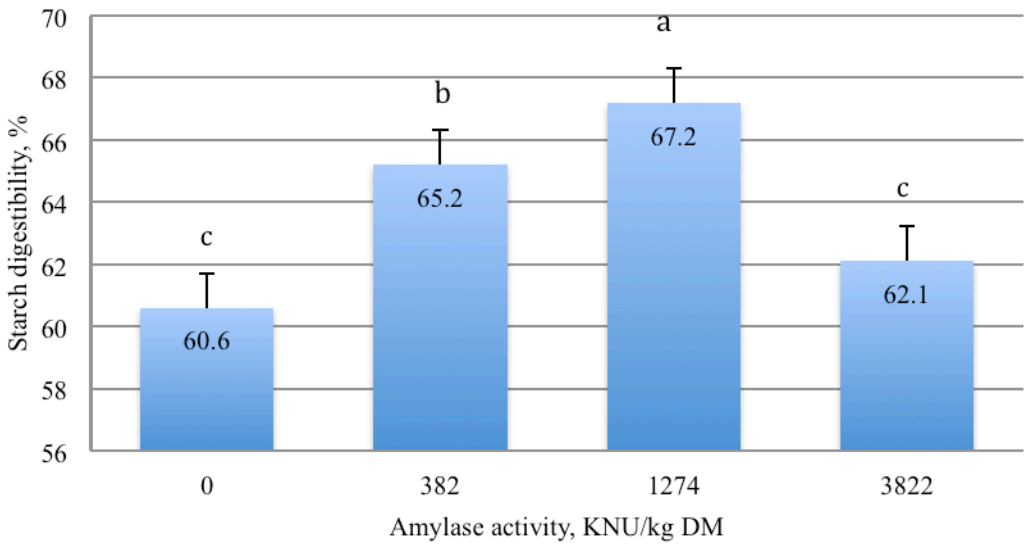

Figure 8. In vitro starch digestiblity co-incubation for all substrates.

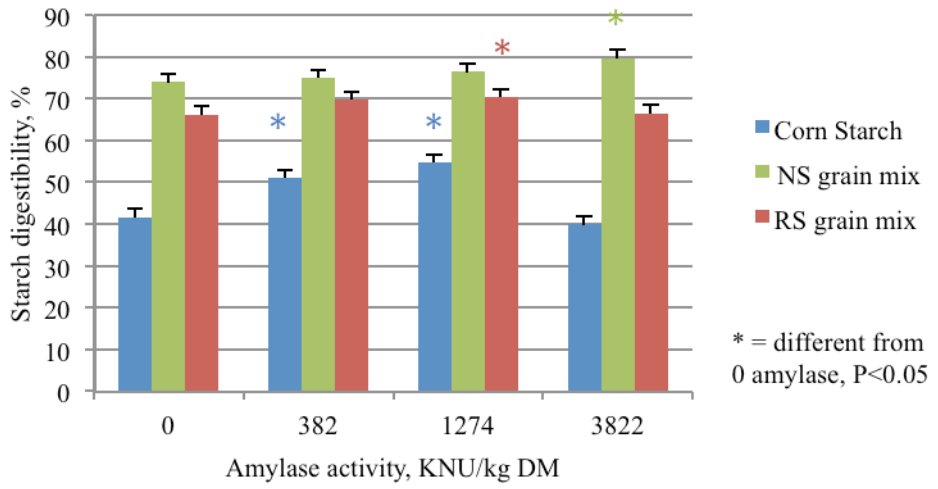

Figure 9. In vitro starch digestiblity co-incubation for each substrate.

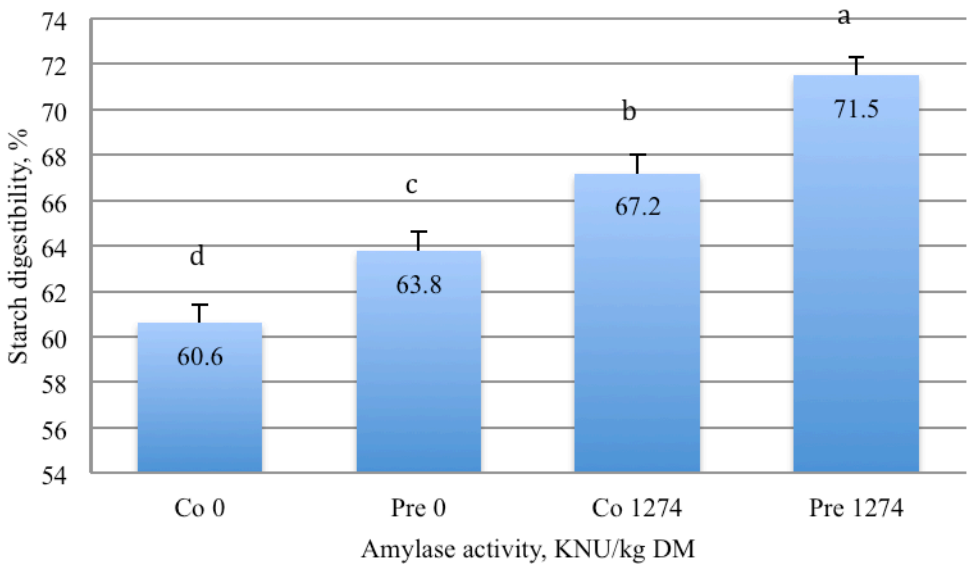

Figure 10. In vitro starch digestiblity pre-incubation (Pre) vs. co-incubation (Co) for all substrates.

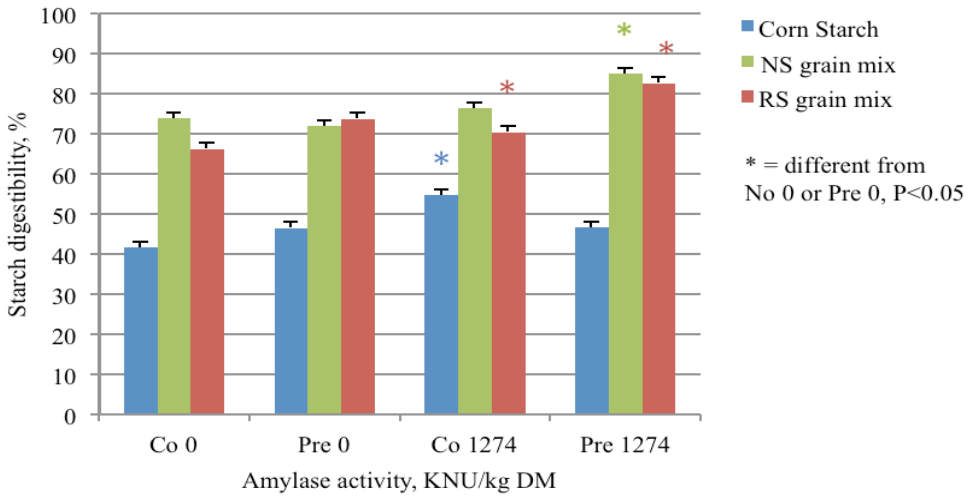

Figure 11. In vitro starch digestiblity pre-incubation (Pre) vs. co-incubation (Co) for each substrate.

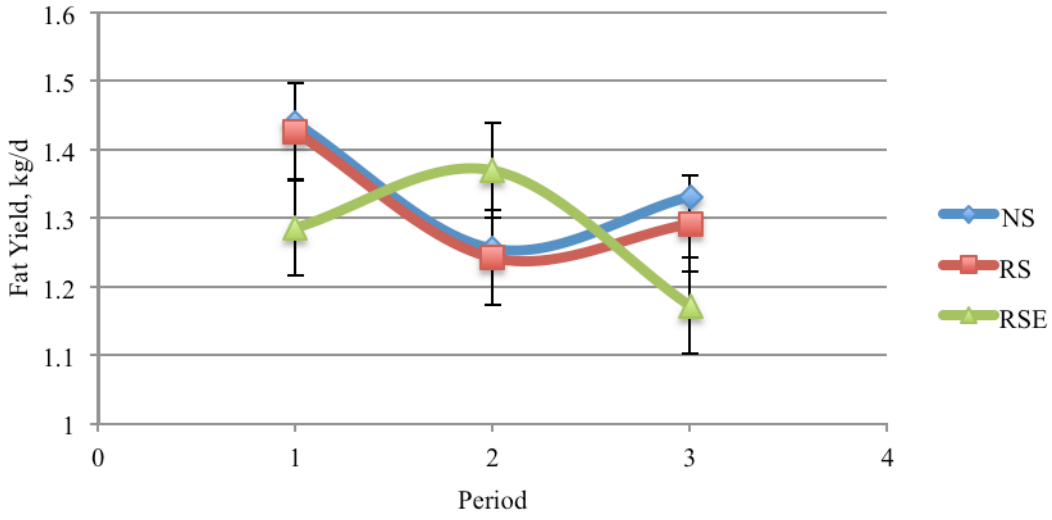

Figure 12. Fat yield treatment by period interaction.

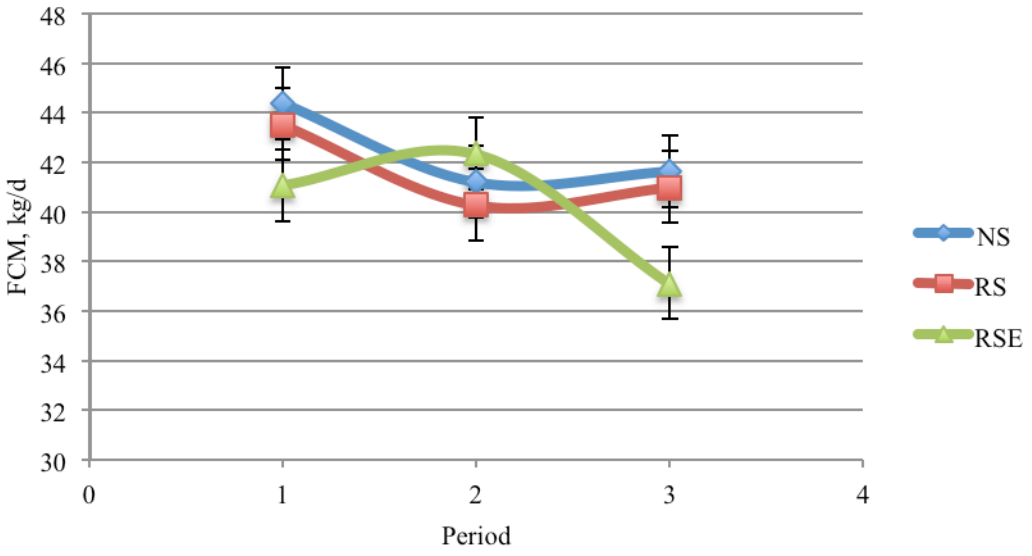

Figure 13. 3.5% fat corrected milk treatment by period interaction.

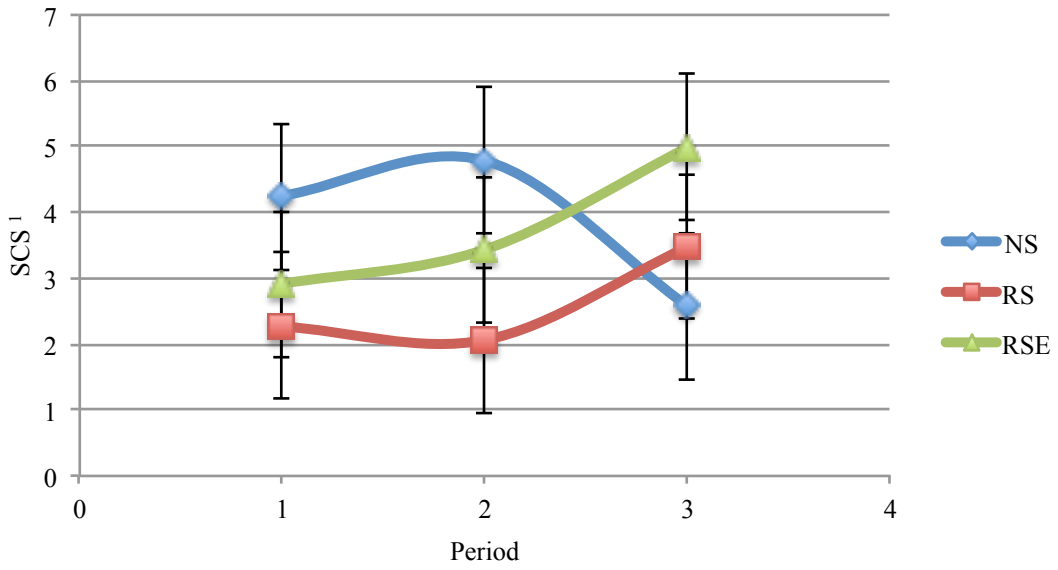

Figure 14. Somatic cell score treatment by period interaction.
[1]Somatic cell score, calculated as the $\log_2(SCC/100,000) + 3$.

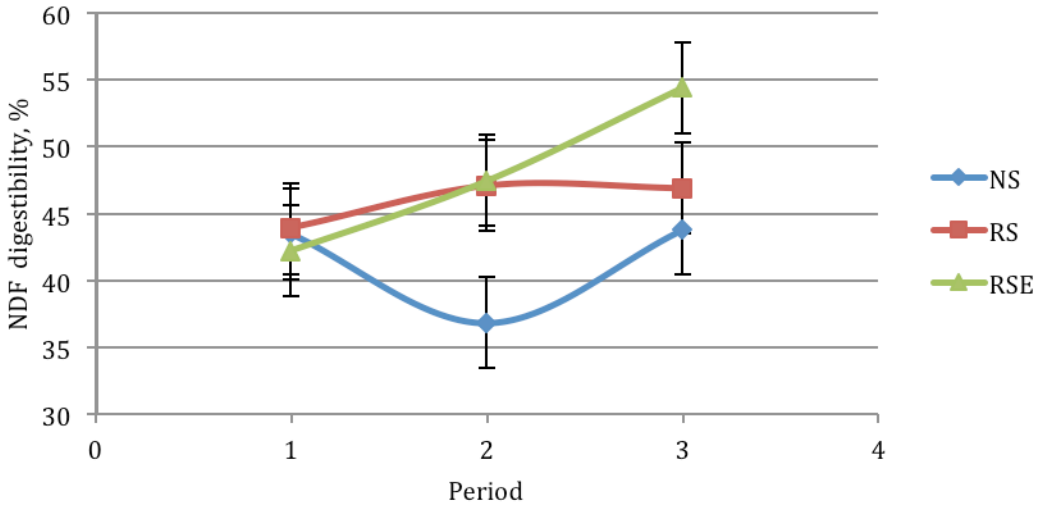

Figure 15. Apparent total tract neutral detergent fiber digestibility treatment by period interaction.

Appendix C

PERMISSION LETTER

Animal care and use committee approval form.

Fwd: Protocol (10) 03-05-10R Has Been Approved Inbox x

Tanya Gressley to me show details Jul 18 (2 days ago) Reply

AACUC info.

---------- Forwarded message ----------
From: **Alphin, Robert** <ralphin@udel.edu>
Date: Thu, Mar 11, 2010 at 2:52 PM
Subject: Protocol (10) 03-05-10R Has Been Approved
To: "Severson, Dan" <severson@udel.edu>, Daniel Bautista <02973@udel.edu>, "Frank Warren (E-mail)" <fwarren@udel.edu>, "Hopkins, Scott" <hopkins@udel.edu>, "klmurray@udel.edu" <klmurray@udel.edu>, "Hall, Meg" <meghall@udel.edu>, "Robert Dyer (E-mail)" <94713@udel.edu>, "Thea (Thea@IrishTulip.com)" <Thea@irishtulip.com>
Cc: Tanya Gressley <gressley@udel.edu>

Dear Dr. Gressley,

Your protocol has been approved by the full AACUC.

Best regards,

Robert L Alphin, Jr., M.S.
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