INTESTINAL FLOW OF MICROBIAL PROTEIN AND RUMEN UNDEGRADABLE PROTEIN IN CATTLE FED CORN DISTILLERS GRAINS AND SOLUBLES, WITH EMPHASIS DURING LACTATION

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INTESTINAL FLOW OF MICROBIAL PROTEIN AND RUMEN UNDEGRADABLE PROTEIN IN CATTLE FED CORN DISTILLERS GRAINS AND SOLUBLES, WITH EMPHASIS DURING LACTATION

by

Ezequías Castillo López

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INTESTINAL FLOW OF MICROBIAL PROTEIN AND RUMEN UNDEGRADABLE PROTEIN IN CATTLE FED CORN DISTILLERS GRAINS AND SOLUBLES, WITH EMPHASIS DURING LACTATION

Ezequías Castillo López, Ph.D.

University of Nebraska, 2012

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Metabolizable protein (MP) is the true protein absorbed by the intestine, supplied by rumen undegradable protein (RUP) and microbial crude protein (MCP). This research was focused on evaluating the effect of dried distillers grains with solubles (DDGS) on the nature and supply of MP, with emphasis during lactation.

Experiment 1 evaluated the use of DNA markers and the effects of fermentation time and DDGS on rumen microbial growth in vitro. Treatments were 1) CONTROL, 0% DDGS; 2) 20% DDGS replacing corn; 3) 20% DDGS replacing soybean meal and 4) 20% DDGS replacing corn and soybean meal. Results indicate that microbial growth reached peak at 32 h. In addition, DDGS improves or maintains rumen microbial growth.

Experiment 2 determined the RUP of DDGS and its effect on duodenal flow of MCP. Three steers fitted with ruminal and duodenal cannulae were used in a 3 treatment, 6 period cross-over design. Diets were 1) CONTROL, 0% DDGS; 2) 10% DDGS and 3) 20% DDGS. The value of DDGS RUP as a percent of CP was determined to be 63.0 ± 0.64%. Results indicate that DDGS is a good source of RUP, but contrasting our expectations tended to decrease duodenal MCP flow.

Experiment 3 evaluated the effect of increasing levels of reduced-fat DDGS (RFDG) on lactation and duodenal microbial N flow. Sixteen Holstein cows, 4 of which fitted with ruminal and duodenal cannulae were used in a 4×4 Latin square design. Treatments were 1) CONTROL, a diet containing 0% RFDG; 2) 10% RFDG 3) 20% RFDG; and 4) 30% RFDG. Milk yield was not affected; percent of milk protein tended to increase; but percent of milk fat was not affected by treatment. In addition, duodenal microbial N flow was not affected. Overall, rations of dairy cattle can include up to 30% RFDG without negatively affecting lactation performance and rumen microbial growth.
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>CHAPTER</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Introduction</td>
<td>1</td>
</tr>
<tr>
<td>II. Review of literature</td>
<td>9</td>
</tr>
<tr>
<td>Milk and milk components</td>
<td>9</td>
</tr>
<tr>
<td>Feed ingredients commonly used in dairy rations</td>
<td>10</td>
</tr>
<tr>
<td>Corn milling byproducts</td>
<td>11</td>
</tr>
<tr>
<td>Production of DDGS</td>
<td>12</td>
</tr>
<tr>
<td>Reduced-fat DDGS</td>
<td>13</td>
</tr>
<tr>
<td>Nutritional characteristics of DDGS and RFDG</td>
<td>13</td>
</tr>
<tr>
<td>The rumen microbial community and their role in ruminant nutrition</td>
<td>14</td>
</tr>
<tr>
<td>The role of bacteria</td>
<td>14</td>
</tr>
<tr>
<td>The role of protozoa</td>
<td>15</td>
</tr>
<tr>
<td>The role of fungi/yeast</td>
<td>16</td>
</tr>
<tr>
<td>Effect of DDGS on lactational performance</td>
<td>18</td>
</tr>
<tr>
<td>Effect on milk yield</td>
<td>19</td>
</tr>
<tr>
<td>Effect on milk composition</td>
<td>20</td>
</tr>
<tr>
<td>Effect of DDGS on rumen microorganisms</td>
<td>20</td>
</tr>
<tr>
<td>Effect on total MCP</td>
<td>21</td>
</tr>
<tr>
<td>Effect on microbial dynamics</td>
<td>22</td>
</tr>
<tr>
<td>Estimation of rumen MCP</td>
<td>22</td>
</tr>
<tr>
<td>Methods and markers used to estimate rumen MCP</td>
<td>24</td>
</tr>
<tr>
<td>Application of molecular techniques in the rumen microbiome</td>
<td>28</td>
</tr>
<tr>
<td>Quantitative real-time PCR analysis</td>
<td>29</td>
</tr>
<tr>
<td>The use of sequenced bacterial DNA</td>
<td>30</td>
</tr>
<tr>
<td>Conclusions</td>
<td>33</td>
</tr>
<tr>
<td>Problem and approach</td>
<td>33</td>
</tr>
<tr>
<td>III. The effect of dried distillers grains with solubles when replacing corn or soybean meal on rumen microbial growth in vitro as measured using DNA markers</td>
<td>48</td>
</tr>
<tr>
<td>Abstract</td>
<td>48</td>
</tr>
<tr>
<td>Introduction</td>
<td>49</td>
</tr>
<tr>
<td>Materials and methods</td>
<td>50</td>
</tr>
<tr>
<td>Results and discussion</td>
<td>55</td>
</tr>
</tbody>
</table>
Conclusions ........................................................................................................................................... 60

IV. In vivo determination of rumen undegradable protein of dried distillers grains with solubles and evaluation of duodenal microbial crude protein flow .......... 68
Abstract.................................................................................................................................................... 68
Introduction ............................................................................................................................................. 69
Materials and methods ............................................................................................................................. 71
Results and discussion .............................................................................................................................. 82
Conclusions ............................................................................................................................................. 90

V. Effect of reduced fat dried distillers grains with solubles on lactation performance, rumen fermentation and duodenal flow of microbial nitrogen in Holstein cows .................................................. 102
Abstract.................................................................................................................................................... 102
Introduction ............................................................................................................................................. 104
Materials and methods ............................................................................................................................. 105
Results ...................................................................................................................................................... 119
Discussion .............................................................................................................................................. 124
Conclusions ............................................................................................................................................. 130

VI. Summary and conclusions.................................................................................................................. 148

VII. Appendices ....................................................................................................................................... 151
Abbreviations .......................................................................................................................................... 151
The purine analysis assay for the estimation of MCP ............................................................................... 153
The diaminopumelic acid assay for the quantification of BCP ................................................................. 155
Description of quantitative real-time PCR ............................................................................................. 156
Ruminal cannulation ................................................................................................................................. 157
Duodenal cannulation ............................................................................................................................... 158
Procedure for ruminal cannulation ........................................................................................................ 159
Procedure for duodenal cannulation ....................................................................................................... 160
LIST OF TABLES

TABLE                        PAGE

Chapter II

Table 2.1. Ingredients typically used and chemical composition of a ration formulated for lactating dairy cattle................................................................. 41
Table 2.2. Characteristics of rumen microorganisms and major division groups........ 42
Table 2.3. Chemical composition of different types of dried distillers grains (DDG) and dried distillers grains with solubles (DDGS)................................................................. 43
Table 2.4. Dry matter intake and lactation performance of cows when consuming diets containing corn distillers grain with solubles (DGS)................................................................. 44

Chapter III

Table 3.1. Ingredient and average chemical composition (standard deviation) of experimental treatments used in in vitro fermentation. ......................................................... 65

Chapter IV

Table 4.1. Ingredients and analyzed chemical composition of CONTROL, LOW dried distillers grains with solubles (DDGS) and HIGH DDGS treatments. ............................. 97
Table 4.2. Analyzed chemical composition (mean and standard deviation) of feedstuffs used in the formulation of CONTROL, LOW dried distillers grains with solubles (DDGS) and HIGH DDGS treatments................................................................. 98
Table 4.3. Dry matter intake, crude protein (CP) intake and ruminal pH for steers fed CONTROL, LOW dried distillers grains with solubles (DDGS) and HIGH DDGS treatments................................................................. 99
Table 4.4. Duodenal flow measurements of DM, organic matter (OM), ash, total crude protein (CP), microbial crude protein (MCP) and residual CP for steers fed CONTROL, LOW dried distillers grains with solubles (DDGS) and HIGH DDGS treatments........ 100

Chapter V

Table 5.1. Ingredient composition of formulated diets containing increasing levels of reduced fat dried distillers grains with solubles (RFDG) fed in experiment 1 and 2. .... 138
Table 5.2. Chemical composition of formulated diets containing increasing levels of reduced fat dried distillers grains with solubles (RFDG) fed in experiment 1 and 2. .... 139
Table 5.3. Analyzed chemical composition (mean and standard deviation) of feedstuffs used in the formulation of diets containing increasing levels of reduced fat dried distillers grains with solubles (RFDG) fed in experiments 1 and 2................................. 140
Table 5.4. Particle size distribution of formulated diets containing increasing levels of
reduced fat dried distillers grains with solubles (RFDG) fed in experiments 1 and 2.

Table 5.5. Effects of feeding increasing levels of reduced fat dried distillers grains with solubles (RFDG) on DMI, milk production and milk composition (Experiment 1).

Table 5.6. Effects of feeding increasing levels of reduced fat dried distillers grains with solubles (RFDG) on DMI, milk production and milk composition (Experiment 2).

Table 5.7. Effect of feeding increasing levels of reduced fat dried distillers grains with solubles (RFDG) on ruminal pH, concentration of ruminal VFA and ammonia.

Table 5.8. Fecal output, nutrient intake and nutrient total tract apparent digestibility by cows fed increasing levels of reduced fat dried distillers grains with solubles (RFDG).

Table 5.9. Duodenal digesta and N flows in cows fed increasing levels of reduced fat dried distillers grains with solubles (RFDG).
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>FIGURE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chapter I</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Figure 1.1.</strong> The supply and fates of metabolizable protein in the dairy cow.</td>
<td>8</td>
</tr>
<tr>
<td><strong>Chapter II</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Figure 2.1.</strong> Schematic representation of the dry milling process with the feed products obtained</td>
<td>45</td>
</tr>
<tr>
<td><strong>Figure 2.2.</strong> Schematic representation of the wet milling industry resulting in wet or dry corn gluten feed</td>
<td>46</td>
</tr>
<tr>
<td><strong>Figure 2.4.</strong> Example of a real-time PCR amplification plot of bacterial DNA</td>
<td>47</td>
</tr>
<tr>
<td><strong>Chapter III</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Figure 3.1.</strong> Measurements of pH from 0 to 96 h of <em>in vitro</em> fermentation from four dietary treatments</td>
<td>66</td>
</tr>
<tr>
<td><strong>Figure 3.2.</strong> Effect of dried distillers grains with solubles on synthesis of A) Bacterial crude protein (BCP), 2B) protozoal crude protein (PCP), yeast crude protein (YCP) and 2D) total microbial crude protein (MCP: BCP, PCP and YCP)</td>
<td>67</td>
</tr>
<tr>
<td><strong>Chapter IV</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Figure 4.1.</strong> Estimates of duodenal bacterial crude protein (BCP) flow averaged across treatments using DAPA or DNA as bacterial markers for steers fed CONTROL, LOW dried distillers grains with solubles (DDGS) and HIGH DDGS treatments</td>
<td>101</td>
</tr>
<tr>
<td><strong>Chapter V</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Figure 5.1.</strong> Effect of feeding increasing levels of reduced fat dried distillers grains with solubles (RFDG) on diurnal variation of ruminal pH. Time after feeding 0 represents 0930, when TMR were offered. Mean pH, $P = 0.02$.</td>
<td>147</td>
</tr>
</tbody>
</table>
CHAPTER I

Introduction

Microbial crude protein (MCP) is one of the major contributors to the supply of amino acids that are used for milk protein synthesis, therefore accurate evaluation of the effect of diet on rumen microbial growth is important. In addition, dried distillers grains with solubles (DDGS) (Stock et al., 2000) are effective feeds for dairy (Anderson et al., 2006; Kleindschmith et al., 2006) and beef (Klopfenstein et al., 2008) cattle. Ranathunga et al. (2008) reported that compared to a zero control diet, the inclusion of 21% DDGS in the rations of lactating dairy cows had no negative effect on milk production or composition and tended to improve feed efficiency. Furthermore, Anderson et al. (2006) demonstrated that DDGS can replace a portion of ground corn and soybean meal and maintain or even enhance lactational performance. However, most studies conducted on this field have been focused on evaluating the effect of DDGS on lactation performance. The effect of DDGS on the dynamics of rumen fermentation and microbial growth has not been completely elucidated. Therefore, a better understanding of the nutritional characteristics of DDGS and how it is metabolized by the ruminant microbiota is needed. Understanding the effect the effect of these by-products on the rumen microbial growth is important because MCP may contribute the majority of the metabolizable protein reaching the small intestine. In addition, protein of microbial origin has an impact on milk protein composition and quality. Therefore, every time we can increase or maintain microbial protein production in the rumen, we are making the cow more efficient in her use of feeds and supplying a more ideal protein source (Block, 2006). Given that ruminal MCP is a major contributor to MP in ruminants (Ørskov, 1982; NRC, 2000), improving
our understanding on how ruminal microbial growth is affected when including DDGS in diets is needed. The beef NRC (2000) suggests that microbial protein can supply from 50% to essentially all the MP required by beef cattle. Clark et al. (1992) found that microbial N supplies an average of 59% of the non-ammonia N that passes to the small intestine of dairy cows with a range of 34 to 89%. In addition, nutrient metabolism by rumen microorganisms plays an important role in the efficiency of feed and energy utilization by the dairy cow (Van Maanen et al., 1978), which may have an impact on production costs.

Models for balancing rations for cows balance for the protein that the cow truly needs, which is MP. The MP provides the supply of amino acids to the cow for her true needs in biological functions, with her focus being on milk production. Figure 1 illustrates the supply and fates of metabolizable protein in the dairy cow. The main fates are maintenance and production. Maintenance requirements include urinary endogenous nitrogen, scurf nitrogen and metabolic fecal nitrogen. The requirements for production include conception, growth and lactation (NRC, 2001).

The concentration of CP in DDGS is approximately 30% and it has been suggested that more than half of this CP is rumen undegradable protein (RUP) (Schingoethe et al., 2009; Kelzer et al., 2010). However, rumen degradability characteristics of protein contained in DDGS are variable (Aines et al., 1987) and may be influenced by production plant (Spiehs et al., 2002), protein originating from yeast that may also resist rumen degradation (Tagari et al., 1986; Stein and Shurson, 2008; Castillo-Lopez et al., 2010), and small particle size which may interfere in the estimation of ruminal degradation (Dewhirst et al., 1994). Accurate determination of RUP is important
because it contributes to metabolizable protein (MP). In addition to RUP of a feedstuff, sources of MP include microbial crude protein (MCP) and endogenous protein loss (Ørskov, 1982; NRC, 2000), which can represent as much as 20% of duodenal MP flow (Lapierre et al., 2006). When fed to cattle, DDGS may contribute to the supply of MP in the form of RUP. In addition, DDGS supply energy and N that rumen microbes may use to synthesize MCP.

Chibisa et al. (2012) demonstrated that wheat-based DDGS can substitute for canola meal as the major protein source in diets of dairy cows without negatively affecting ruminal fermentation, MCP production, and omasal nutrient flow. In addition, Janicek et al. (2008) reported that cows consuming 30% DDGS synthesized similar amount of MCP compared to a diet containing no DDGS. However, the phyla diversity of the rumen microbial population has been reported to differ when animals are fed DDGS compared to a control diet with no DDGS (Callaway et al., 2010). Furthermore, when feeding DDGS, total MCP has been reported to increase (Leupp et al., 2009a; Leupp et al., 2009b).

Estimation of rumen MCP relies on the use of microbial markers, such as purines, DNA, diaminopumelic acid (DAPA) or labeled isotopes (Stern, 1979; Obispo and Dehory, 1999; Sylvester et al., 2005; Castillo-Lopez et al., in press). Each of these markers has advantages and disadvantages. For example, the presence of undegraded purines or DAPA from feed may cause an overestimation of MCP (Zinn and Owens, 1986; Obispo and Dehory, 1999; Broderick and Merchen, 1992). The presence of residual yeast used in the ethanol production process may also represent a challenge in the estimation of MCP when using purines as a microbial marker due to their contribution
to total purines. The differentiation of microbial nitrogen into bacterial and protozoal nitrogen has proven to be possible (Sylvester et al., 2005), but this is difficult due to the use of conventional microbial markers sometimes found in both bacteria and protozoa (Punia et al., 1992; Robinson et al., 1996). Utilizing more specific microbial markers such as DAPA (Work and Dewey, 1953) or microbial DNA (Belanche et al., 2011a; Belanche et al., 2011b) may be useful to prevent overestimation. In addition to bacteria and protozoa, with the inclusion of DDGS in diets, there may also be a small contribution of yeast cells to total MCP (Castillo-Lopez et al., 2010). Currently, real-time PCR has been used for quantitative DNA detection and analysis of microorganisms (Tajima et al., 2001; Wellinghausen et al., 2001; Nadkarni et al., 2002; Moya et al., 2009; Fernando et al., 2010; Castillo-Lopez et. al., 2010). Using universal probes and primers as microbial markers should allow us to estimate bacteria, protozoa and yeast separately. These estimates may then provide a valuable way to evaluate the effect of DDGS not only on total ruminal MCP synthesis but also on bacterial crude protein (BCP), protozoal crude protein (PCP) and yeast crude protein (YCP) separately.

The overall objectives of this research were 1) to explore the ability of using DNA markers through real-time PCR to estimate MCP synthesis, 2) to determine the RUP of DDGS and its effect on rumen MCP synthesis and 3) to evaluate the impact of DDGS on lactation performance and rumen fermentation in lactating dairy cows.
Literature cited


Figure 1.1. The supply and fates of metabolizable protein in the dairy cow.

Adapted from the dairy NRC (2001).
CHAPTER II

Review of literature

Milk and milk components

In cattle, the chemical composition of milk varies across breeds. The gross composition of cow’s milk is 87% water, 3.9% fat, 2.6% casein, 0.6% whey, 4.6% lactose and 0.7% ash. More specifically, the sources of milk fat are dietary fat and de novo fat synthesis, lactose is synthesized from glucose and galactose, and proteins are synthesized from absorbed amino acids of RUP and MCP origin (Jenness, 1985).

Milk is a complex fluid with hundreds of individual soluble minor components. However, the major nutritive value of milk is accounted for by its gross composition of fat, protein and carbohydrates. Milk is synthesized and stored within the lumen of the alveoli and ductular system until it is removed (Oftendal, 1984).

Most protein in milk are synthesized from free amino acids or peptides absorbed from the bloodstream. The basolateral cell membrane serves to regulate the uptake of these molecules. The major proteins in milk are caseins and whey proteins. These proteins are synthesized by membrane-associated ribosomes and the newly made proteins have short signal sequences of amino acids that allow binding of the nascent protein into the cisternal space of the rough endoplasmic reticulum. The signal peptide is ultimately cleaved as the protein moves to the Golgi apparatus, where it is stored in secretory vesicle (Akers, 2002).

Most of the glucose that dairy cows need to meet their nutritive requirements is originated from hepatic gluconeogenesis of propionate (Nafikov and Beitz, 2007).
Lactose synthase acts to combine the monosaccharides of glucose and galactose to produce lactose. Lactose is packaged into secretory vesicles along with other specific protein. Because lactose cannot pass across the secretory vesicle membrane, it has a dramatic osmotic effect such that water enters the secretory vesicles.

The three sources of the fatty acids in milk triglycerides are (1) metabolism of glucose which is converted to pyruvate, citrate, and ultimately acetyl CoA, (2) diet via hydrolysis of chylomicra, and (3) de novo synthesis within the mammary cell from non-glucose sources. The malonyl CoA pathway, which sequentially adds two carbon units to the growing fatty acid chain, is the major synthesis pathway. In ruminants, acetate and beta-hydroxibutyric acid from the blood provide most of the carbon needed for fatty acid synthesis. Lipid droplets form as micro-droplets near the rough endoplasmic reticulum; the droplets progressively enlarge and make their way to the apical plasma membrane. (Mather and Keenan, 1998). These enlarged droplets protrude from the cell, pushing a portion of the plasma membrane into the alveolar lumen, where they are finally excreted (Akers, 2002).

Feed ingredients commonly used in dairy rations

Table 1.1 lists the ingredient and chemical composition of a typical ration formulated for lactating dairy cattle, excluding by-products from the corn ethanol industry. These rations may include corn silage, alfalfa hay, alfalfa silage, ground corn, soybean hulls, cottonseed, soypass and soy bean meal. Rations are normally formulated to contain approximately 18% CP, 22% starch, 34% neutral detergent fiber (NDF), 5% ether extract (EE) and 1.5 Mcal/kg of net energy for lactation (NE\textsubscript{L}) (Janicek et al., 2008). The rational of formulating diets with this ingredient and chemical composition is
to provide enough metabolizable protein for production and maintenance and avoid excess loss of nitrogen though urine. Neutral detergent fiber, specifically effective NDF plays a major role in promoting chewing activity and saliva production to buffer the rumen. Ether extract provides energy to the animal; however this should not be provided in excessive amount because it may interfere with feed degradation by rumen microorganisms.

**Corn milling byproducts**

With the expansion of the corn ethanol industry, new products are being included in rations formulated for dairy cattle. Two primary types of corn milling processes currently exist, resulting in quite different feed products. The dry milling process, illustrated in Figure 2.1, produces distillers grains (DG) or distillers grains with solubles (DGS). Byproducts from the dry milling process can be marketed as wet distillers grains (WDG), wet distillers grains with solubles (WDGS), dried distillers grains (DDG) or dried distillers gains with solubles (DDGS). The wet milling process, illustrated in Figure 2.2, produces corn gluten feed (CGF). Byproducts from the wet milling process can also be marketed as wet corn gluten feed (WCGF) or dry corn gluten feed (DCGF). The majority of corn ethanol plants in Nebraska are dry milling plants and the current production rates will likely be maintained. Therefore, these feeds are very attractive for cattle producers to use as feed sources (Erickson et al., 2007).

Byproducts of the dry and wet milling processes, or a combination of both byproducts, offer many feeding options when included in cattle rations. These byproduct feeds may effectively improve cattle performance and operation profitability (Smith et al., 2009; Buckner et al., 2008).
Schingoethe et al. (2009) reported that most of the ethanol byproducts are currently available as DDGS. He also reported that it is only during recent years that large quantities of DDGS have become widely available and at competitive prices. In addition, the products available today usually contain more protein and energy (Birkelo et al., 2004). This reflects the improved fermentation efficiency of the new generation corn ethanol plants (Spiehs et al., 2002).

Production of DDGS

Stock et al. (1999) described the dry milling process, which is illustrated in Figure 2.1. During this process, the grain is ground and the starch is fermented by yeast to produce alcohol. Then, the fermented mash is processed by various techniques to remove the alcohol and the large volume of water associated with the residual DM. After distillation, the remaining feed slurry contains 5 to 10% DM and is called whole or spent stillage. The whole stillage then is either screened and pressed or is centrifuged to remove the coarser grain particles. Another approach is to remove the coarser grain particles before processing through the distillation column. With this method, only the liquid fraction is distilled. The coarser grain particles removed from the whole stillage may be sold as WDG, or they may be dried and sold as DDG. The liquid fraction (5 to 10% DM) remaining after separation of the grains is called thin stillage. Thin stillage contains fine grain particles and yeast cells. Thin stillage is evaporated to produce a syrup-like by-product containing 20 to 35% DM and is called condensed distillers solubles (CDS). The CDS may be dried with DDG to produce DDGS.
**Reduced-fat DDGS**

The production of DDGS by the corn dry milling industries increased from approximately 2.7 million metric tons in 2000 to approximately 23 million metric tons in 2008 (Renewable Fuel Association, 2009). Because of this sustained growth new products are emerging. Reduced-fat DDGS (RFDG) is an example of this growth. The use of a solvent extraction process that removes corn oil from DG results in a product that is lower in fat compared with conventional DDGS (Mjoun et al., 2010a). Mjoun et al. (2010a, 2010b) reported that with the improved production processes of DDGS, heat damage is less likely to occur. In addition, these authors suggested that the removal of a portion of the fat in RFDG may reduce the potential for reduced milk fat percentage. Consequently, this may allow for a greater inclusion of this byproduct in dairy rations.

**Nutritional characteristics of DDGS and RFDG**

Two-thirds of the grain DM of corn consists of starch. After fermentation, approximately one-third of the original grain DM is recovered in the whole stillage. Because only the starch is removed during the fermentative process, the other nutrients associated with the grain become more concentrated (Kleinschmit et al., 2006). Table 2.1 lists the chemical composition of various types of DDG and DDGS. Kelzer et al. (2010) analyzed the chemical composition of three types of DG: 1) DDGS1, DDGS with no heating or cooking before fermentation; 2) DDGS2, DDGS that had heating and cooking before fermentation and 3) HPDDG, high protein DDG with no solubles included. They observed that the chemical composition of DDG1 was 26.9% CP, 6.7% starch, 30.2% NDF, 13.0% crude fat and 7.6% ash. For DDG2, the observed chemical composition was 25.9% CP, 6.85% starch, 33.9% NDF, 11.8% crude fat and 6.3% ash. The chemical
composition of HPDG was 45.4% CP, 9.5% starch, 22.5% NDF, 4.0% crude fat and 4.21% ash. In addition to DDGS and HPDG, RFDG is now being produced and marketed. The reported chemical composition of RFDG is 34.0 CP, 5.6% starch, 42.8% NDF, 3.5% crude fat and 5.6% ash (Mjoun et al., 2010).

The rumen microbial community and their role in ruminant nutrition

Rumen microorganisms play an important role in ruminant nutrition, specifically in feed degradation and contribution to MP. The national research council (NRC, 2001) defines MP as the true protein absorbed by the intestine, and that it is mostly supplied by MCP and RUP. The two major contributors of MCP are rumen bacteria and protozoa. For the purpose of this work, a brief description of the role of rumen bacteria, protozoa and yeast in ruminant nutrition is given. Table 2.2 lists the main characteristic of bacteria, protozoa and fungi found in the rumen. In the rumen, RUP supplies nitrogen that microorganisms utilize to grow. When these microorganisms reach the small intestine they provide MP to the animal in the form of MCP.

The role of bacteria. Russell (2002) reported that bacterial density in the rumen can be as high as $10^{10}$ cells per g of ruminal content. Although bacterial mass has not been precisely determined, values are typically in the range of 14 to 18 mg bacterial dry weight per mL; this means that there is 7 to 9 mg bacterial protein per mL. Bacteria are approximately 10% N (62.5% crude protein). However only 80% of the crude protein is true protein, the remainder is contributed by nucleic acid nitrogen. Approximately 75% of the rumen bacteria are bound to feed particles and only 25% are free-floating and their size may range from 1-5 µm.

Spicer et al. (1986) suggested that BCP alone can supply from 50% to essentially
all the MP required by beef cattle, depending on the RUP content of the diet. Furthermore, these authors mentioned that the efficiency of synthesis of BCP is critical to meeting the protein requirements of ruminants. This fact demonstrates the vital importance of the rumen microbial population for ruminant nutrition and that the prediction of BCP synthesis and RUP of diets and diet ingredients is important for being critical components of the MP system. The major bacterial phyla found in the rumen are listed in Table 1.1 (Russell, 2002).

The role of protozoa. Protozoa are eukaryotic organisms and their numbers in the rumen do not normally exceed $10^7$ cells per mL, but they can account for as much as half of the total mass of MCP. Sylvester et al. (2005) determined protozoal nitrogen to be 4.8 and 12.7% of the rumen microbial nitrogen pool and 5.9 and 11.9% of the duodenal flow of microbial nitrogen on diets containing low (16%) or high (21%) forage neutral detergent fiber (cellulose, hemicellulose and lignin), respectively.

It has been reported that when protozoa numbers increase, bacterial mass declines and vice versa. Protozoa associate with the feed particles and this association prolongs their residence time in the rumen. For example, if the diet has little nitrogen, bacterial predation and protozoal lysis leads to a recycling of microbial protein in the rumen, and there is a direct relationship between the presence of protozoa and ruminal ammonia (Koenig et al., 2000). Ruminal protozoa appear to be both advantageous and detrimental to the host. For example, if the ration is based on grain, protozoal engulfment of starch grains can modulate pH and protect the animal from acidosis (Russell, 2002). Conversely, Koenig et al. (2000) reported that the defaunation of sheep improved microbial nitrogen flow by 60% and organic matter flow by 68% to the duodenum, the
synthesis of MCP. These authors also reported that the total tract nitrogen digestibility lowered in defaunated animals.

Rumen protozoa can be divided in Holotrichs (isotricha and dasytricha), which have cilia over their entire body and entodinomorphs, which only have cilia over discreet regions. Holotrichs utilize sugars and are prevalent in animals consuming fresh forage. Entodinomorphs are found in close proximity to feed particles, and use starch as well as a variety of plant materials (Russell, 2002). Studies have demonstrated that the total numbers of protozoa are not affected when including 25% DDGS in the diets of finishing steers and that the proportions of *Entodiniummium*, *Isotricha*, and *Dasytricha* are not affected either (Li et al., 2011).

**The role of fungi/yeast.** It has been shown that ruminal fungi digest cellulose and other fibrous materials, and fungi appear to have better enzymes than the bacteria for feed degradation. Because the mycelium penetrates deep into feed particles and breaks the fibers apart, there is an increase in surface area available for fungi as well as bacteria (Orpin and Joblin, 1997). Because fungi are usually attached to feed particles, it has been difficult to estimate their biomass. It has been suggested, however, that fungi zoospores may be as high as $10^6$ per mL. Another report (Akin, 1987) has suggested that fungi can account for as much as 8% of the rumen microbial biomass. However, their contribution to MCP is not well documented.

Over the last three decades, significant research has been conducted on ruminal fermentation when supplementing yeast cells of *Saccharomyces cerevisiae* to ruminants. Moya et al. (2009) studied the effect of supplementation with yeast culture on ruminal microbial fermentation and found that yeast culture reduced the foam strength, suggesting
potential benefits to the animal for reducing the risk of developing bloat. However, the yeast culture addition had no significant impact on rumen fermentation. Furthermore, Williams et al. (1991) studied the effects of the inclusion of yeast culture of *S. cerevisiae* in the diet of dairy cows on milk yield and forage degradation and fermentation patterns in the rumen. These authors concluded that the yeast culture increased DM intake of the cows by a mean of 1.2 kg/d and it also increased milk yield by 1.4 liters/d (corrected to 4% butter fat) and that the effect was greatest in diets containing 60:40 (concentrate:forage) ratio. The concentrate represented by rolled barley and the forage represented by straw and hay. In addition, they reported that in steers the yeast culture increased ruminal pH for 4 h after the meal. The elevation in pH was suggested to be due to a reduction in the concentration of lactate in the ruminal liquor of steers given the yeast culture. Overall, an improvement in rumen fermentation has been reported by yeast supplementation (Desnoyers et al., 2009).

Yeast cells of *S. cerevisiae* may also be incorporated in the rumen through the addition of DG in the rations, but their effect on rumen fermentation is not fully understood. Indledew (1999) suggested that 3.9% of the dry weight of DDGS may be contributed by yeast cell biomass and that the greater value of distillers byproducts (averaged daily gain, feed efficiency) compared to corn may be at least partially explained by the contribution of yeast components that influence ruminal fermentation. Castillo-Lopez et al. (2010) determined that the concentration of yeast protein contained in DDGS was 1.4% (DM-basis). In addition, they found that a small amount of *S. cerevisiae* DNA is reaching the omasum of dairy cows. However they reported that yeast DNA escaping degradation in the rumen does not significantly alter the amount of total
purines in digesta; therefore the estimation of MCP when using purines as a microbial marker may not be grossly overestimated due to the presence of yeast cells contained in DDGS included in dairy rations.

Effect of DDGS on lactational performance

Schingoethe et al. (2009) reported that diets can contain DDGS as partial replacement for both concentrates and forages. Concentrates normally supplied by ground corn and soybean meal, and forages commonly supplied by corn silage, alfalfa haylage, alfalfa hay and brome hay. However, DDGS usually replaces concentrates because of the need to maintain the level of effective fiber in the diet to avoid milk fat depression. He also suggested that when fed to dairy cattle, adequate effective fiber is needed to avoid milk fat depression when DDGS replaces forages. In addition, he reported that nutritionally balanced diets can be formulated that contain 20% or more of the diet dry matter as DDGS and obtain similar or higher milk production compared with feeding diets containing no DDGS. These authors suggested that there is usually no nutritional advantage of feeding more than 20% DDGS because such diets may contain excess protein and P, although production performance is high even with more than 30% dried DDGS in the diet. Excessive excretion of nitrogen and P may have negative environmental consequences, N and P contamination of surface water may cause algae blooms, which shade aquatic vegetation, reducing photosynthetic activity (Burkholder et al., 2004). Table 2.2 lists the effect on feeding DGS on lactational performance of Holstein dairy cows. The data indicate that milk production and composition are maintained when including up to 30% DM basis of DGS in the diet (Kalscheur, 2005).
Effect on milk yield. Schingoethe et al. (2009) conducted a review on the use of DGS on dairy rations and reported that the amounts of DGS usually fed ranges from 4.2 (Broderick et al., 1990) to 41.6% of total DM (Van Horn et al., 1985). Data presented by Schingoethe et al. (2009) indicate that milk production is similar or higher when feeding DGS compared with feeding control diets. Mjoun et al. (2010a) fed either a control diet, a diet containing 22% regular DDGS or a diet containing 20% RFDG to cows in early lactation. They found that milk yield was not different among treatments and averaged 39.3 ± 0.46 kg/d during a 12-wk experiment. In addition, Mjoun et al. (2010b) found that milk production (35.0 kg/d) was similar for all treatments when including from 0 to 30% RFDG in the diets of dairy cows in mid lactation. Conversely, Janicek et al. (2008) reported a linear increase in milk production with increasing levels of DDGS. Specifically, they reported 27.4, 28.5, 29.3 and 30.6 kg/d of milk yield for cows consuming a 0, 10, 20 and 30% DDGS diets, respectively. This different response in lactation performance can be attributed to the increase in DMI observed by Janicek et al (2008) when feeding DDGS. A meta-analysis conducted by Kalscheur (2005) indicated that milk production was the same as or higher when feeding DG compared with feeding control diets except when feeding 30% or more of diet DM as WDGS. However, in dairy cattle only a few studies have been conducted to include up to 30% DDGS in the diets. Kleinschmit et al. (2006) fed either a control diet with no DDGS or a diet containing 20% DDGS and found that cows fed diets containing DDGS had greater yields of milk (31.2 vs. 34.6 kg/d for control and DDGS containing diet, respectively). In addition, Nichols et al. (1998) reported an increase in milk production, 34.3 and 35.3 kg/d for cows consuming a control diet with no DDGS and cows consuming a diet containing 20%
DDGS. These findings demonstrate that corn ethanol byproducts, namely DDGS are an affective feed ingredient in rations of lactating dairy cattle.

**Effect on milk composition.** Mjoun et al. (2010a) reported that the composition of milk is usually not negatively affected by feeding distillers byproducts unless routinely recommended ration formulation guidelines are not followed, such as feeding insufficient amounts of functional fiber. In addition, they reported that yields of milk fat were similar for cows fed either a control diet, a diet containing 22% regular DDGS or a diet containing 20% RFDG and averaged 1.36 kg/d. However, these authors found that yield of protein increased 0.075 kg/d for cows fed DDGS diets compared with the control diet. Mjoun et al. (2010b) reported that milk fat percentage increased linearly from 3.18 to 3.72% as RFDG increased from 0 to 30% of the diet. Similarly, milk fat yield tended to increase linearly from 1.08 to 1.32 kg/d. They also found that milk protein percentage (2.99, 3.06, 3.13, and 2.99% for diets with RFDG from 0 to 30%) increased when RFDG increased from 0 to 20%, but it was similar to control when 30% RFDG was included in diet. Janicek et al. (2010) found that milk fat and protein percent were not affected by DDGS inclusion, averaging 3.64 and 3.16% of milk fat and milk protein when DDGS increased from 0 to 30% in the diet. These data clearly indicate that milk composition is not negatively affected when lactating dairy cows consume large amounts (up to 30% DM basis) of DDGS. Besides DMI, other factors that may affect lactational response include total energy intake, chemical composition and nutrient balance of diets.

**Effect of DDGS on rumen microorganisms**

The use of DDGS for ruminant nutrition has increased in the last 30 years because
of their availability and lower price compared to some of the feed ingredients that they replace (Ham et al., 1994). This situation may play an important role in rumen microbial growth and activity. Besides contributing to MP, rumen microorganisms play a crucial role in ruminal parameters and nutrient metabolism. For example, biohydrogenation influences the profile of fatty acids reaching the duodenum and ultimately the mammary gland (Beam et al., 2000; Abdelqader et al., 2009). In addition, fatty acid metabolism in the rumen has an influence on the fatty acid composition of ruminant meats (Jenkins et al., 2008); and may affect the nutritional properties and susceptibility to oxidation of milk fat (Pottier et al., 2006; Palmquist et al., 1993). Therefore, the study of the effect of DDGS on the rumen microbiome is needed.

**Effect on total MCP.** Chibisa et al. (2012) demonstrated that wheat-based DDGS can substitute for canola meal as the major protein source in dairy cow diets without negatively affecting ruminal fermentation. These investigators reported mean ruminal pH were similar across treatments, total VFA, ruminal ammonia and MCP production, which was measured through the purine derivative method, were unaffected. In addition, Janicek et al. (2008) reported that cows consuming 30% DDGS synthesized similar amount of MCP compared to a diet containing no DDGS using the purine derivative approach, with an average of 1,642 g of MCP/d. In another trial (Kelzer et al., 2009) MCP synthesis, estimated through purine derivatives, was similar for dairy cows fed a control diet with no DDGS, a diet containing 15% DDGS or a diet containing 15% HPDDG (DM basis). The average estimate of MCP was 1,161 g/d. In contrast to these studies, Leupp et al. (2009b) fed beef steers 0, 15, 30, 45, or 60% of DDGS (DM basis) replacing a combination of dry rolled corn, sunflower meal, and urea and observed an
increase in total MCP from 0 to 45% DDGS inclusion. Estimates were 545, 576, 563, 593 and 515 g of MCP/d when DDGS was included from 0 to 60% in the diets. Overall, these results demonstrate that DDGS maintains or enhances MCP synthesis. The dairy NRC (1989) calculated MCP based on the net energy for lactation and the NRC (2001) assumes that the yield of MCP is 130 g/kg of total digestible nutrient intake and that the requirement for RDP is 1.18 x MCP. Therefore, the changes in the net energy intake or in total digestible nutrient intake when feeding DDGS would be expected to directly impact the yield of MCP.

**Effect on microbial dynamics.** Several studies have been conducted focusing on the dynamics of rumen microbes (Tajima et al., 2000; Moya et al., 2009; Fernando et al., 2010). Although the effect of DDGS on changes in microbial species has not been fully elucidated, it has been reported that the diversity of the rumen microbial population changes when animals are fed DDGS (Callaway et al., 2010). Shifts in the rumen bacterial populations have been observed at the levels of phyla, order, class and genera. These shifts in the composition of the rumen microbiome may influence animal performance and partially explain the greater feeding value of DDGS compared to feed ingredients typically used in ruminant rations like ground corn or high moisture corn (Buckner et al., 2008).

**Estimation of rumen MCP**

Accurate measurement of MCP supply in ruminants is important because of the fact that it is a major component of the MP reaching the small intestine. For instance, if MCP is affected, this will be reflected on the supply of MP to the animal. The microbial
population within the rumen provides a substantial proportion of the amino acids that are available for absorption from the small intestine of ruminants. In view of the importance of MCP to ruminant nutrition, the understanding of MCP synthesis in the rumen is essential for optimizing the production performance of livestock (Dijkstra et al., 1998).

An accurate estimation of rumen MCP is dependent upon a reliable microbial marker, which can be an internal or external marker. Examples of internal markers include total purines, diaminopumelic acid (DAPA), RNA, DNA; external markers include labeled isotopes infused exogenously such as $^{35}\text{S}$, $^{15}\text{N}$ and $^{32}\text{P}$ (Obispo and Dehority, 1999). These maker are naturally or become part of the microbial cells, therefore they can be utilized to detect and quantify rumen microbial growth and measure MCP.

Dehority (1995) suggested that the ideal microbial marker should possess the following criteria; 1) not be present in the feed; 2) not be absorbed; 3) be biologically stable; 4) have a relatively simple assay procedure; 5) occur in a similar percentage between the various types of microbes like bacteria and protozoa; 6) be a constant percentage of the microbial cell in all stages of growth; and 7) all forms should flow at a similar rate out the rumen.

Once the microbial marker has been selected, the ratio of marker to nitrogen from a ruminal microbial pellet is established, and then the concentration of the marker in a given sample of digesta is also measured. From these values, the amount of MCP in the sample is estimated (Hristov et al., 2005).

Digesta flow makers that are typically used for the measurement of digesta flow rate to the intestine may be internal or external markers. Examples of internal markers are
lignin and silica. External markers are dosed ruminally and include chromic oxide, inert metals, Cr EDTA, etc. In order to be able to collect samples from a postruminal compartment, placement of cannulae is necessary. To ensure that the sample collected is representative of the total digesta passing a given site, several sample collections throughout the day and composites of those collected samples should be made (Titgemeyer, 1997).

The synthesis of rumen MCP is usually expressed in g of MCP per d. In addition, the supply of rumen MCP to the animal per unit of feed ingested can be expressed as g microbial N or MCP/kg of digestible organic matter fermented in the rumen (Wattiaux et al., 1995), which gives a better indication of the efficiency of MCP synthesis.

**Methods and markers used to estimate rumen MCP**

During the last 4 decades, researchers have developed multiple methods for the accurate estimation of rumen MCP supply. Most of those techniques have proven to be useful, but each has advantages and disadvantages. Following is a brief description of the two most widely used methods to measure rumen MCP.

**Purine analysis.** Purine bases (Adenine and Guanine) are part of nucleic acids of microbial cells. Purine analysis is a technique that was developed by Zinn et al. (1986) and utilized a rapid procedure for the separation and quantification of purines. Briefly, this is a procedure that combines standard methods for the hydrolysis of nucleotides by perchloric acid. The first step is followed by precipitation of free purines with silver nitrate to separate the purines from interfering compounds. In this method, acid resolubilized purines are quantitated with a spectrophotometer at 260 nm. Then, microbial protein is estimated by the ratio of purines to nitrogen of isolated bacteria. The
authors applied this technique to ruminal and intestinal digesta in order to estimate net MCP synthesis in the rumen using animals that were equipped with ruminal and duodenal cannulas.

Obispo and Dehority (1999), who also utilized the technique, suggested that is a simple procedure and they indicate that purines may be useful as a microbial marker for quantifying rumen MCP synthesis. However, the use of purines for estimation of MCP is considered to have some inherent challenges. Zinn et al., (1986) suggested that purines from feed, which escape destruction in the rumen may cause MCP flow to the lower gut to be overestimated. Sloughed epithelial gut cells may also contribute purines to the digesta and therefore cause an overestimation of MCP flow. Belanche et al. (2011) reported greater purine bases concentration in duodenum than in abomasum in lambs, which was attributed to sloughed cells and bile secretion. Other major challenges encountered when using total purines as a bacterial marker seems to be whether the purines are present 1) in a similar percentage in the different species and 2) in all stages of microbial growth. It has been reported that values found for purines in mixed ruminal bacteria vary widely. For example, Clark et al. (1992) carried out a meta-analysis and found a mean purine concentration of 7.28% with values ranging from 2.40 to 13.02%. Calsamiglia et al. (1996) have also reported the purine concentrations of mixed ruminal bacteria grown in continuous culture using eight different protein sources. This variation in the concentration of purines has also been suggested by Obispo et al. (1999), who reported a considerable variation among 10 pure cultures of ruminal bacteria cultured in the laboratory. Concentrations as a percentage of DM ranged from 0.69 to 5.57%, with a mean value of 2.98%. These investigators also suggested that samples of mixed bacteria
isolated from ruminal contents and representing the majority of ruminal bacteria may be contaminated with plant material resulting in lower purine to protein ratio. This situation means that if the ratio purine to protein is used to estimate MCP at the duodenal level, MCP would be overestimated. Among the biological factors that may be responsible for these variations are the difference in chemical composition among liquid and particle associated bacteria and the stage of bacterial growth (Martin et al., 1994). The procedure used for the isolation of the rumen bacterial pellet can also represent an analytical factor contributing to these observed differences. The bacterial isolation procedure should yield a bacterial pellet that represents not only different locations of the rumen, but also liquid and particle associated bacteria (Hristov et al., 2005). In general, the concentrations of purines when measured in mixed ruminal bacterial samples tend to be higher than those in pure cultures; however, some studies have reported values for mixed ruminal bacteria to be considerably lower. For example, Obispo et al. (1999) found that the concentration of purines were 1.44 and 2.98% for mixed bacteria and pure cultures, respectively.

**Diaminopumelic acid (DAPA) analysis.** This is an assay that measure DAPA, which is a bacterial chemical component that was first used by Weller et al. (1958) to estimate the rate of synthesis of ruminal BCP. DAPA is an amino acid derivative closely related to lysine. Hogan and Weston (1970) reported that the DAPA method involves estimating the ratio of DAPA to nitrogen in rumen bacteria and the amount of DAPA in digesta. From these values the amount of bacterial nitrogen in digesta may be calculated. Webster et al. (1990) described the use of HPLC for the determination of DAPA from ruminal digesta samples. Briefly, lyophilized samples are hydrolyzed with methasulfonic acid then centrifuge. Then, 20 µL of derivatized sample are injected into the column and
subjected to HPLC analysis. A refinement of this method is described by Csapó et al., (2008). The method is based on oxidation with performic acid preceding the hydrolysis of proteins. During the oxidation process, methionine is converted into methionine-sulfone. During the ion-exchange column chromatographic separation, methionine-sulfone elutes between aspartic acid and threonine, making available the space between valine and isoleucine for the elution of DAPA.

Dufva et al., (1982) proposed that DAPA is a good microbial marker based on the fact that it is found in the cell membrane of ruminal bacteria and it is absent in feedstuffs commonly fed to ruminants. The accuracy of the technique is dependent on a constant DAPA:Nitrogen ratio among various microbial species, or the maintenance of a constant ratio of microbial species in the rumen. The latter assumption is not consistent with the sequential nature of rumen fermentation. It also has been demonstrated that the DAPA:Nitrogen ratio may vary among ruminal bacterial species (Stern and Hoover, 1970). The different bacteria have different peptidoglycans in the cell wall, therefore different DAPA concentration. For example, gram-positive bacteria contain 30-70% peptidoglycan in the cell wall; the gram-negative bacteria contain only 10%. For that reason, the use of DAPA as a bacterial maker may also have challenges (Csapo et al., 2008). For instance, if cattle are fed with only forage diets, the gram-negative bacteria such as *Ruminococcus albus, Succinomonas amylovorans, Veillonella alcalescens, Megasphaera elsdenii, Selenomonas lactilytica, Succinivibrio dextrinosolvens, Anaerorhabdus lipolytica, S. ruminantium, Bacteroides amylophilus, B. succinigenes,* and *Butyrivibrio fibrisolvens* will be predominant in the rumen. However if cattle consume more concentrate, the proportion of gram-positive bacteria, such as
Eubacterium cellulosolvens, Lactobacillus vitulinus, L. ruminus, R. flavefaciens, Streptococcus bovis will increase (Arambel et al., 1982; Van Baale et al., 2004). Therefore, variations in the relative presence of gram-positive and gram-negative bacteria may affect the estimation of total bacterial nitrogen synthesis. For example, if gram-positive bacteria predominates in the rumen, the ratio DAPA:Nitrogen will be greater, which would lead to an underestimation of total bacterial nitrogen synthesis.

Application of molecular techniques in the rumen microbiome

The microbiome is defined as the ecological community of commensal, symbiotic, and pathogenic microorganisms that share the body space and can be determinants of health and disease (Dewhirst et al., 2010). Rumen microbiology has a rich history of discovery. Although molecular approaches have expanded the boundaries of the study of microbial diversity in the rumen, key aspects of the microbiome have not been well linked to efficiency of nutrient usage (Krause, 2009). Currently emerging technology is expanding our ability to analyze archived samples from nutrition experiments to demonstrate the importance of microbial populations, among animals, among particulate versus fluid fractions, or their interactions (Firkins, 2009). Molecular techniques have evolved rapidly over the past 20 years. This expansion of knowledge provided by genome sequencing projects and improvements in computing resources represent new research tools and approaches for ruminant nutritionists and microbiologists. Polymerase chain reaction and DNA sequencing are the basis of many molecular techniques applied in studies related to the rumen microbial community (Knapp, 2009).
Quantitative real-time PCR analysis

Quantitative real-time (QPCR) is a technique that has been extensively used for the study of microbial communities (Moya et al., 2009; Tajima et al., 2001; Castillo-Lopez et. al., 2010). Moya et al. (2009) utilized the technique to describe the changes occurring in the rumen during a digestive upset induced by rapidly changing from a high forage diet to a high concentrate diet. These authors utilized specific primers to quantify the 16s rRNA gene copies of *Megasphaera elsdenii* and *Streptococcus bovis* during the transition from the forage based diet to the high concentrate diet. Castillo-Lopez et al. (2010) used the technique for the detection of yeast DNA escaping ruminal degradation in dairy cows consuming DDGS.

The genes targeted by primer and probes when using QPCR for the quantification of rumen microbial protein are the 16S rRNA gene (Yu et al., 2005), 18S rRNA gene (Sylvester et al., 2005) and the II chromosome (Castillo-Lopez et al., 2010) for bacteria, protozoa and yeast, respectively.

The use of QPCR has the advantage of being highly specific in targeting a particular DNA segment of interest. When utilizing markers designed for bacteria, protozoa and yeast, we can calculate the relative contribution of each of these microbial types to total MCP.

There are a number of studies using real-time PCR for the study of ruminal microorganisms. For example, Tajima et al. (2001) used the technique to study the diet driven shifts in the bacterial population of the rumen. These researchers developed sets of primers for quantification and identification of bacterial species isolated from the rumen. In addition, Sylvester et al. (2005) used the technique for the estimation of protozoal flow.
to the omasum and duodenum of dairy cows. Primers used by these researchers targeted part of the 18S rRNA gene. More recently, Belanche et al. (2011) used real-time PCR to target 16S rRNA and 18S rRNA genes to measure the flow of ruminal microbial nitrogen to the omasum, abomasum and duodenum of sheep. The authors concluded that this procedure gave similar results of microbial synthesis as the method based on measuring purine bases. They also indicated that rDNA markers showed a greater specificity for the estimation of microbial flow. One of the limitations of the technique is the higher costs compared to conventional methods like the DAPA procedure or the purine analysis assay. Higher variability has also been reported when using DNA as microbial markers (Belanche et al., 2011). The use of real-time PCR may also be combined with sequenced bacterial DNA for a more detailed description of the rumen microbiome, which seems a promising field of study in ruminant nutrition (Fernando et al., 2010). In another study (Lee et al. 2012) real-time PCR and bacterial DNA pyrosequencing technique (described below) were utilized for the description of the microbial community in different bovine and caprine species. Bioinformatic analysis conducted by these researchers revealed that the bacterial sequences were predominantly affiliated with four phyla, which are *Bacteroidetes, Firmicutes, Fibrobacteres*, and *Proteobacteria* in both caprine and bovine species.

**The use of sequenced bacterial DNA**

Modern microbial genetics and molecular phylogenetic techniques for identifying and classifying microorganisms by DNA pyrosequencing (Benson et al., 2010; Davis et al., 2011) may help advance knowledge of the role and contribution of specific microbial species in ruminal metabolism (Jenkins et al., 2008; Krause, 2009). Sequencing
technology has since advanced even further, and new platforms based on pyrosequencing are available (Benson et al., 2010).

Pyrosequencing is a four-enzyme DNA sequencing technology monitoring the DNA synthesis detected by bioluminescence. The technique is based on sequential addition of nucleotides to a primed template and the sequence of the template is deduced from the order different nucleotides are incorporated into the growing DNA chain, which is complementary to the target template (Ronaghi et al., 1998). The four enzymes included in the pyrosequencing system are the Klenow fragment of DNA polymerase I, ATP sulfurylase, Luciferase and Apyrase. The reaction mixture also contains the enzyme substrates adenosine phosphosulfate, d-luciferin and the sequencing template with an annealed primer to be used as starting material for the DNA polymerase. The four nucleotides are added one at a time in a cyclic manner and a charge-coupled device, also known as CCD camera, detects the light produced (Ahmadian et al., 2006).

Ahmadian et al. (2006) described the basic steps in pyrosequencing. The first reaction, the DNA polymerization, occurs if the added nucleotide forms a base pair with the sequencing template and thereby is incorporated into the growing DNA strand. Then, the inorganic pyrophosphate (PPi) released by the Klenow DNA polymerase serves as substrate for ATP Sulfurylase, which produces ATP. Through the third and fourth reactions, the ATP is converted to light by Luciferase and the light signal is detected. Hence, only if the correct nucleotide is added to the reaction mixture, light is produced.

The use of sequenced microbial DNA and bioinformatic programs to study the rumen microbiome is still an emerging field. Callaway et al. (2010) subjected bacterial DNA to pyrosequencing and described the bacterial diversity in the rumen and feces of
cattle fed different levels of DDGS. These authors described the bacterial diversity at various levels including phylum, order, class, family and genus. In another study, Hristov et al. (2012) used pyro sequenced DNA to rumen bacterial, archaeal, and fungal diversity of dairy cows. They reported that in dairy cows consuming a diet based on alfalfa silage and ground peas, the most abundant bacterial genera in the rumen are prevotella and clostridium. Methanobrevibacter and Methanosphaera were the most abundant archeal genera. In addition, they indicated that Eupenicillium was the most abundant fungi genus in the rumen. More recently, Ramirez-Ramirez et al. (2012) studied the effect of brown midrib corn silage and dried distillers' grains with solubles on the microbial community structure in dairy cows and reported changes in the rumen bacterial phyla level. Specifically, these authors observed a decrease in the ratio Firmicutes:Bacteriodetes in the rumen when cattle consumed DDGS. In addition, they reported that when cows were fed brown midrib corn silage, the population of *Fibrobacter sp.* tended to represent a larger proportion of the total bacterial population. The utilization of sequenced microbial DNA may provide the ability to better understand the relationship between the host and the gastrointestinal microbiota because of their role in nutrient degradation and energy utilization. Describing the effect of diet composition on rumen microbial shifts may provide the tools to enhance animal performance (Bergen and Bates, 1984). The intensive research applying pyrosequencing DNA in the human has contributed to lower the costs to characterize the microbial communities in other species like ruminants which used to be a limitation due to high costs associated with the utilization of the technique (Gootembergh and Turnbaugh, 2011). Luke and Frenzel (2012) indicated that the pyrosequencing technology still has its limitations. For example, problems may occur in
particular during sequencing of homopolymeric stretches, which may introduce errors in the process.

**Conclusions**

Corn ethanol byproducts are effective feed ingredients for ruminants. Rations fed to dairy cattle can contain DDGS as replacements for portions of both concentrates and forages. Nutritionally balanced diets can be formulated to contain up to 30% of DDGS DM basis with no negative effect on lactation performance. When DDGS is included in rations fed to ruminants, some research results have reported a small increase in the growth of rumen microorganisms. Other results suggest no difference in the synthesis of rumen total MCP. The estimation of rumen MCP has been conducted through several techniques. Even though those methods were developed a few decades ago, some of them are still effective in the present. With the generation of new feed ingredients included in ruminant rations and with the advancement in technology and laboratory molecular techniques, new approaches to estimate rumen microbial dynamics and total MCP are being explored. Some of these techniques like real-time PCR and pyrosequencing have shown to be more accurate and more efficient for the description of the rumen microbiome. Overall, given that MCP represents a significant contribution to MP, the application of molecular techniques for a better understanding of the effect of ration composition on lactational performance and rumen MCP synthesis is a promising field of research.

**Problem and approach**

The impact of feeding DDGS on rumen microflora and the supply of MCP is not
clearly understood. Few studies have been conducted evaluating the effect of DDGS on the presence of rumen bacteria, protozoa and yeast separately. Quantifying the presence of these microorganisms is important given that each have a unique role in nutrient contribution and feed degradation, which may impact the efficiency of energy utilization by the animal. In addition, rumen MCP may supply the majority of amino acids reaching the small intestine that are incorporated into milk protein. Therefore, understanding the effect of diet composition on rumen microbial dynamics is beneficial to the dairy industry. The most commonly employed analytical method is to measure purines or the excretion of purine derivatives in the urine. The measurement of urinary purine derivatives is an indirect approach and is fundamentally flawed because it does not directly measure microbial material and may be susceptible to contamination by undegraded feed particles. To address this problem we will explore the use of DNA markers through real-time PCR to estimate not only total MCP synthesis, but also separate estimates of bacterial protozoa and yeast in vitro and in vivo. We will also compare the use of DNA markers with other microbial makers such as purines or DAPA. Then, once this approach is established, when feeding DDGS to lactating cattle we will determine the impacts on milk production and on the proportion of MCP in the supply of MP of a lactating dairy cow.
Literature cited


Li, Y. L., T. A. McAllister, K. A. Beauchemin, M. L. He, J. J. McKinnon and W. Z. 2011. Yang Substitution of wheat dried distillers grains with solubles for barley grain or barley silage in feedlot cattle diets: Intake, digestibility, and ruminal


**Table 2.1.** Ingredients typically used and chemical composition of a ration formulated for lactating dairy cattle.

<table>
<thead>
<tr>
<th>Ingredient, % DM</th>
<th>Treatment Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn silage</td>
<td>30.1</td>
</tr>
<tr>
<td>Ground corn</td>
<td>16.3</td>
</tr>
<tr>
<td>Alfalfa hay</td>
<td>10.9</td>
</tr>
<tr>
<td>Alfalfa haylage</td>
<td>10.9</td>
</tr>
<tr>
<td>Cottonseed</td>
<td>6.61</td>
</tr>
<tr>
<td>Soybean meal</td>
<td>4.77</td>
</tr>
<tr>
<td>Soybean hulls</td>
<td>10.4</td>
</tr>
<tr>
<td>Soy-Pass</td>
<td>5.6</td>
</tr>
<tr>
<td>Magnesium Oxide</td>
<td>0.17</td>
</tr>
<tr>
<td>Trace mineral(^1)</td>
<td>0.04</td>
</tr>
<tr>
<td>Limestone</td>
<td>0.73</td>
</tr>
<tr>
<td>Salt</td>
<td>0.52</td>
</tr>
<tr>
<td>Vitamin ADE(^2)</td>
<td>0.12</td>
</tr>
<tr>
<td>Sodium bicarbonate</td>
<td>0.52</td>
</tr>
<tr>
<td>Dicalcium phosphate</td>
<td>0.41</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chemical composition, %</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>CP</td>
<td>19.4</td>
</tr>
<tr>
<td>NDF</td>
<td>34.6</td>
</tr>
<tr>
<td>Starch</td>
<td>22.4</td>
</tr>
<tr>
<td>Ether extract</td>
<td>5.14</td>
</tr>
<tr>
<td>NFC(^3)</td>
<td>37.2</td>
</tr>
</tbody>
</table>

\(^1\)Formulated to contain 1.0% Ca, 0.50% P, 0.36% Mg, 1.3% K.
\(^2\)Formulated to supply approximately 120,000 IU/d vitamin A, 24,000 IU/d of vitamin D, and 800 IU/d Vitamin E in total ration.
\(^3\)NFC = Nonfiber carbohydrate calculated by difference 100 – (%NDF + %CP + %Fat + %Ash).

Adapted from Janicek et al. (2008).
<table>
<thead>
<tr>
<th>Name</th>
<th>Domain</th>
<th>Density cell/mL</th>
<th>Size µm</th>
<th>Contribution to MP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteria</td>
<td>Prokaryote</td>
<td>$10^{10}$</td>
<td>1-5</td>
<td>40-80%</td>
</tr>
<tr>
<td>Bacteroidetes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Firmicutes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TMZ</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tenericutes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actinobacteria</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chloroflexi</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spirochaetes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proteobacteria</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protozoa</td>
<td>Eukaryote</td>
<td>$10^7$</td>
<td>60-135</td>
<td>As high as 50%</td>
</tr>
<tr>
<td>Holotrichs</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Entodinomorphs</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fungi zoospores</td>
<td>Eukaryote</td>
<td>$10^6$</td>
<td>6-10</td>
<td>---</td>
</tr>
</tbody>
</table>

Adapted from Russell (2002).
Table 2.3. Chemical composition of different types of dried distillers grains (DDG) and dried distillers grains with solubles (DDGS).

<table>
<thead>
<tr>
<th>Item</th>
<th>TYPE OF BYPRODUCT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DDGS1&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>DM</td>
<td>86.7</td>
</tr>
<tr>
<td>CP, %DM</td>
<td>26.9</td>
</tr>
<tr>
<td>ADF, %DM</td>
<td>13.1</td>
</tr>
<tr>
<td>NDF, %DM</td>
<td>30.2</td>
</tr>
<tr>
<td>Starch, %DM</td>
<td>7.65</td>
</tr>
<tr>
<td>Crude fat, %DM</td>
<td>13.3</td>
</tr>
<tr>
<td>NFC, %DM</td>
<td>18.8</td>
</tr>
<tr>
<td>Lignin, %DM</td>
<td>4.4</td>
</tr>
<tr>
<td>Ash, %DM</td>
<td>7.62</td>
</tr>
</tbody>
</table>

<sup>1</sup>DDGS1: dried distillers grains plus solubles (no heating or cooking before fermentation). Kelzer et al. (2010).
<sup>2</sup>DDGS2: dried distillers grains plus solubles (had heating and cooking before fermentation). Kelzer et al. (2010).
<sup>3</sup>HPDDG: high protein dried distillers grains (no solubles included). Kelzer et al. (2010).
<sup>4</sup>HPDDG: high protein dried distillers grains (no solubles included). Schingoethe et al. (2009).
<sup>5</sup>RFDG: reduced-fat dried distillers grains with solubles. Mjoun et al. (2010).
Table 2.4. Dry matter intake and lactation performance of cows when consuming diets containing corn distillers grain with solubles (DGS)\(^1\).

<table>
<thead>
<tr>
<th>Inclusion level, % of DM</th>
<th>DMI, kg/d</th>
<th>Milk, kg/d</th>
<th>Fat, %</th>
<th>Protein, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>22.1(^b)</td>
<td>33.0(^{ab})</td>
<td>3.39</td>
<td>2.95(^a)</td>
</tr>
<tr>
<td>4–10</td>
<td>23.7(^a)</td>
<td>33.4(^a)</td>
<td>3.43</td>
<td>2.96(^a)</td>
</tr>
<tr>
<td>10–20</td>
<td>23.4(^{ab})</td>
<td>33.2(^{ab})</td>
<td>3.41</td>
<td>2.94(^a)</td>
</tr>
<tr>
<td>20–30</td>
<td>22.8(^{ab})</td>
<td>33.5(^a)</td>
<td>3.33</td>
<td>2.97(^a)</td>
</tr>
<tr>
<td>&gt;30</td>
<td>20.9(^c)</td>
<td>32.2(^b)</td>
<td>3.47</td>
<td>2.82(^b)</td>
</tr>
<tr>
<td>SEM</td>
<td>0.8</td>
<td>1.4</td>
<td>0.08</td>
<td>0.06</td>
</tr>
</tbody>
</table>

\(^{a-c}\) Values within a column followed by a different superscript differ (\(P < 0.05\)).
\(^1\) Adapted from Kalscheur (2005).
Figure 2.1. Schematic representation of the dry milling process with the feed products obtained.

Adapted from Erickson et al. (2007)
Figure 2.2. Schematic representation of the wet milling industry resulting in wet or dry corn gluten feed.

Adapted from Erickson et al. (2007)
Figure 2.3. Example of a real-time PCR amplification plot\(^1\) of bacterial DNA.

\(^1\)On the Y axis is the magnitude of reporter’s signal and on the X axis are number of cycles. During the first cycles, the amount of signal produced is not enough to be detected. However, after a few cycles, the plot exhibits an exponential amplification, then linear amplification and finally it reaches a plateau is reached because one or several of the reagents utilized run out. The sooner the amplification plot reaches the threshold line, the more DNA template is originally present in the sample.
CHAPTER III

The effect of dried distillers grains with solubles when replacing corn or soybean meal on rumen microbial growth in vitro as measured using DNA markers

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Department of Animal Science, University of Nebraska-Lincoln, Lincoln, Nebraska, 68583-0908

ABSTRACT

The objectives were to evaluate the use of DNA markers and the effects of dried distillers grains with solubles (DDGS) and in vitro fermentation time on the growth of rumen bacteria and protozoa, and to measure the contribution of yeast originating from DDGS to total microbial crude protein (MCP). Treatments were: 1) CONT, control with no DDGS, but with alfalfa hay, corn silage (CS), ground corn (GC) and soybean meal (SBM) included at 25% (DM basis); 2) RC, 20% (DM Basis) DDGS replacing GC; 3) RS, 20% (DM basis) DDGS replacing SBM; 4) RCS, 20% DDGS replacing 10% GC and 10% SBM (DM basis). For each treatment, 0.5 g of substrate was incubated in vitro in 50 mL of inoculum in duplicate. At 0, 4, 16, 32, 48 and 96 h of fermentation DNA was extracted from each treatment and microbial protein was measured by real-time PCR. Microbial markers used are from the 16S rRNA gene, 18S rRNA gene and the II chromosome; for bacteria, protozoa and yeast, respectively. Data were analyzed as a completely
randomized design with repeated measures to test the effects of treatments and fermentation time. Treatment did not affect \( P = 0.23 \) mean bacterial crude protein (BCP) which was observed to be \( 30.98 \pm 3.19 \) mg/g of substrate (DM basis) across treatments. However, a treatment by time interaction was observed \( P < 0.05 \).

Specifically, at 16 h the RCS diet yielded higher \( P < 0.01 \) BCP than CONT \( (58.20 \) and \( 26.86 \pm 6.79 \) mg/g of substrate DM for RCS and CONT, respectively). However, at 32 h only the RS yielded higher \( P < 0.05 \) BCP than the CONT \( (70.75 \) and \( 45.20 \pm 6.79 \) mg/g of substrate DM for RS and CONT, respectively). In addition, compared to the CONT, BCP of RCS at 32 h tended \( P = 0.09 \) to be higher \( (61.86 \) and \( 45.20 \pm 6.79 \) mg/g of substrate DM for RCS and CONT, respectively). At 32 h, the RCS diet yielded higher \( P < 0.01 \) protozoal crude protein (PCP) when compared to the CONT \( (29.76 \) and \( 21.17 \pm 1.92 \) mg/g of substrate DM for RCS and CONT, respectively). Treatment did not affect \( P = 0.21 \) yeast crude protein (YCP) and averaged \( 0.04 \pm 0.01 \) mg/g of substrate DM.

Overall, results suggest that rumen microbial growth was improved when DDGS replaced SBM and it was maintained when DDGS replaced GC. In addition, yeast contribution to total MCP was minimal.

**Key words:** bacteria, DNA markers, dried distillers grains with solubles, protozoa, yeast

**INTRODUCTION**

Ethanol byproducts are a good source of energy and protein in ruminant diets (Ham et al., 1994). Dried distillers grains with solubles (DDGS) has been successfully included in rations of beef (Klopfenstein et al., 2008) and dairy cattle (Janicek et al., 2008; Anderson et al., 2006). Given that ruminal microbial crude protein (MCP) is an
important contributor to metabolizable protein in ruminants (Ørskov, 1982; NRC, 2000), improving our understanding of how ruminal microbial growth is affected when including DDGS in diets is important. The differentiation of microbial nitrogen into bacterial and protozoal nitrogen has proven to be possible (Sylvester et al., 2005), but this is difficult due to the use of conventional microbial markers sometimes found in both bacteria and protozoa (Punia et al., 1992; Robinson et al., 1996). In addition to bacteria and protozoa, with the inclusion of DDGS in diets, there may also be a small contribution of yeast cells to total MCP (Castillo-Lopez et al., 2010). Currently, real-time PCR has been used for quantitative DNA detection and analysis of microorganisms (Wellinghausen et al., 2001; Nadkarni et al., 2002; Moya et al., 2009). Fernando et al. (2010) used the technique to evaluate the dynamics of the rumen population of rumen microbes during adaptation to a high grain diet. Using universal probes and primers should allow us to estimate bacteria, protozoa and yeast. These estimates may then provide a valuable way to evaluate the impact of different feed ingredients on ruminal MCP production. The objectives of this experiment were to evaluate the use of DNA markers and the effects of fermentation time and DDGS when replacing corn or SBM on the growth of rumen bacteria and protozoa, secondly to measure the contribution of yeast originating from DDGS to total MCP.

MATERIALS AND METHODS

Dietary treatments

Dietary ingredients typically used in dairy cattle rations were utilized in this experiment.
Table 3.1 lists the ingredients and analyzed chemical composition of the four dietary treatments used for *in vitro* fermentation. Experimental treatment fermentation substrates were as follows: 1) **CONT**, control with no DDGS, containing an equal mixture (25% DM basis) of alfalfa hay, corn silage (CS), ground corn (GC) and SBM; 2) **RC**, 20% (DM Basis) DDGS replacing GC; 3) **RS**, 20% (DM basis) DDGS replacing SBM; 4) **RCS**, 20% DDGS replacing 10% GC and 10% SBM (DM basis). Treatment ingredients were sampled and analyzed for chemical components. Feed samples were dried for 48 h at 55°C in a forced air oven, ground to pass through a 1-mm screen (Wiley Mill, Arthur A. Thomas Co., Philadelphia, PA). Samples were analyzed for DM (AOAC, 2000); N (Leco FP-528 Nitrogen Combustion Analyzer, Leco corp. St. Joseph, MI); NDF (Van Soest et al., 1991); starch (Hall, 2009); ether extract (AOAC, 2006) and ash (AOAC, 2000), ash analysis included 1.5 g of sample weight and 4 h of ash time.

*In vitro* fermentation

Two liters of ruminal fluid were collected and pooled from two rumen fistulated crossbred Angus steers being fed a diet of 70% and 30% forage and concentrate, respectively. Rumen content collected was squeezed through four layers of cheese cloth into a pre-warmed thermos. Then fluid was transferred into separatory funnels, placed in 39°C water bath and mixed with one volume of warmed McDougall’s Buffer (McDougall, 1948). The basic *in vitro* incubation procedure was carried out according to Tilley and Terry (1963); briefly, 0.5 g of dietary substrate (CONT, RC, RS, RCS) was weighed in duplicate and placed in a 100-mL fermentation tube. Tubes were flushed with CO₂ and 50 mL of inoculum was added to tubes and were placed in water bath at 39°C. Fermentation tubes were swirled gently immediately after inoculation (50% rumen fluid
and 50% McDougal’s buffer) and at 6-h intervals for 24 h and every 12 h thereafter as described by Grant (1994). Fermentation times were terminated at 0, 4, 16, 32, 48 and 96 h. At each time point as tubes were removed from the water bath the pH of the contents of each substrate was measured to monitor pH variation. Therefore, there were 6 independent measurements per treatment and a total of 24 measurements among the 4 treatments (n = 24).

**DNA extraction**

Collecting DNA at different time points required multiple replicates from each treatment thus two replicates of each treatment were made for each time point resulting in a total of 12 fermentation tubes for each of the dietary treatments. At 0, 4, 16, 32, 48 and 96 h a crude pellet was isolated from each fermentation tube by a high speed centrifugation (30,000 x g) according to Shabi et al. (2000). Then DNA was extracted by the repeat bead beating plus column method according to the improved extraction method for PCR-quality DNA from digesta and fecal samples described by Yu and Morrison (2004). Briefly, a sample of isolated pellet was combined with lysis buffer and zirconia beads then shaken for physical disruption of cells and exposure of cellular contents. Then DNA and RNA were precipitated; and finally DNA was purified by applying a series of centrifugation steps and to eliminate the RNA and proteins. The concentration of DNA in each sample was measured by spectrophotometry (NanoDrop ND-1000 Spectrophotometer, NanoDrop Technologies, Inc. Wilmington, DE) and stored at -20°C in aliquots of 25 µL for later analysis.

**Microbial markers**

For bacteria, marker (composed of forward primer, reverse primer and probe) was
designed from the gene encoding part of the 16S rRNA, which has been shown to be highly preserved in bacteria (Ogier et al., 2002; Yu et al., 2005; Zimmermann et al., 2010). The National Center of Biotechnology Information accession number (NCBI) of the targeted DNA amplicon is FJ715623. Forward primer: 5’-act cct acg gga ggc agc agc-3’. TaqMan probe: 5’-FAM/tgc cag cag ccg cgg taa tac/TAMRA-3’. Reverse primer: 5’-gac tac cag ggt atc taa tcc-3’.

For protozoa, marker (composed of forward primer, reverse primer and probe) was designed from DNA encoding part of the 18S rRNA gene (Sylvester et al., 2005). The NCBI accession number of the targeted DNA is EU796177. Forward primer: 5’-gct ttc gat ggt agt gta tt-3’. TaqMan Probe: 5’-FAM/cgg aag gca gca ggc gc/TAMRA-3’. Reverse primer: 5’-act tgc cct cta atc gta ct-3’.

For yeast, marker (composed of forward primer, reverse primer and probe) was designed from part of the II chromosome of the yeast (Saccharomyces cerevisiae) genome. To design these oligonucleotides, the NCBI blast software was used to verify that this DNA sequence belongs only to S. cerevisiae so that it will not be cofounded by other related fungi species. The NCBI accession number of the targeted DNA amplicon is Y08934. The oligonucleotides composing the yeast marker were analyzed with the oligo analyzer of the Integrated DNA Technology (IDT) program; this was to verify whether those oligonucleotides are adequate for being used in real-time PCR assays. Forward primer: 5’-cct gct aaa ctg cag ctt gac-3’. TaqMan probe: 5’-FAM/ctg cgg acc ctg cag tcc agc/TAMRA-3’. Reverse primer: 5’-cag cgt ttg cgt tcc atg ac-3’. Bacterial and protozoal primers and probes were not analyzed because they were already tested.
Reactions and real-time PCR conditions

The concentration of DNA of each sample was measured. Real-time PCR reactions were as follows, 8 µL of DNA sample were combined with 2.4 µL of 10 µM forward primer, 2.4 µL of 10 µM reverse primer, 0.72 µL of 10 µM TaqMan probe, 24 µL of TaqMan Master Mix (Applied Biosystems, Foster City, CA, USA) and 10.48 µL of PCR water. Two samples with no DNA were included as non-template controls. Each sample was run in duplicate in separate wells of the 384-well real-time PCR plate. DNA samples were subjected to real-time PCR using a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). Temperature cycling was as described by Moya et al. (2009) with some variations, specific conditions were as follows: stage 1: 50ºC for 2 minutes; stage 2: 95ºC for 10 minutes; stage 3: 45 cycles alternating denaturation at 95ºC for 15 seconds, then annealing and polymerization at 60ºC for 1 minute.

Calculation of bacterial crude protein (BCP), protozoal crude protein (PCP) and yeast crude protein (YCP)

Once real-time PCR was performed, BCP, PCP and YCP were measured according to the procedure described by Castillo-Lopez et al. (2010), and expressed in mg of CP/g of DM. Briefly; real-time PCR results and concentration reactions (amount of DNA placed in each well, and amount of total DNA recovered from each sample) were used to calculate the abundance of DNA marker per g of DM from each dietary treatment. In addition, we estimated the abundance of DNA marker per g of CP from a pure sample of each microbial type (bacteria, protozoa and yeast) this was to obtain the ratio of DNA marker to CP. To do so, a pure sample of yeast (S. cerevisiae) was used and pure bacterial and protozoal samples were isolated from a portion of the ruminal fluid by
differential centrifugation as described by Shabi et al. (2000). From those values, the amount of BCP, PCP and YCP were calculated and reported in mg of CP/g of DM. Calculation were conducted as described by Castillo-Lopez et al. (2010). DDGS was also analyzed for YCP and yeast mass by the same procedure.

**Statistical analysis**

The data collected were analyzed in the MIXED procedure of SAS (Version 9.1; SAS Institute, Inc., Cary, NC) as a completely randomized design with repeated measures to test the effects of treatments and fermentation time on BCP, PCP and YCP. Treatment means are presented as least square means, and the largest standard error of the mean (SEM) is reported.

**RESULTS AND DISCUSSION**

**pH measurements**

The pH measurements taken at each time point (0, 4, 16, 32, 48 and 96 h in vitro fermentation) are illustrated in Figure 3.1. Mean pH among the four dietary treatments from 0 to 96 h of in vitro fermentation was not observed to be different ($P = 0.14$). Time however, had a significant effect ($P < 0.01$) on pH. All treatments started with a mean pH value of 6.73 ± 0.04. Treatment RC (replacing 20% corn) and treatment RS (replacing 20% SBM) reached the lowest pH at 32 h, 6.43 and 6.33 for the RC and RS, respectively; whereas, control and treatment RCS (replacing 10% corn and 10% SBM) reached the lowest pH value at 48 h, 6.37 and 6.39 for control and RCS, respectively. From 48 to 96 h of fermentation, pH was either maintained (control and RC treatment) or slightly increased (RS and RCS treatments). These data on pH are similar to the observations of
Callaway et al. (2010) who found that, compared to 0% DDGS inclusion, ruminal pH in cattle did not change when animals consumed a 25% DDGS ration and that ruminal pH only decreased when they consumed a 50% DDGS ration.

**Effect of DDGS and fermentation time on BCP**

Changes in BCP from 0 to 96 h of *in vitro* fermentation are illustrated in Figure 3.2 A. For all treatments, bacterial growth reached a peak at 32 h and was not affected by treatment \((P = 0.23)\) by treatment and averaged 30.98 ± 3.19 mg/g of DM across treatments. However, a treatment by time interaction was observed \((P < 0.05)\).

Specifically, at 16 h the RCS treatment yielded higher \((P < 0.01)\) BCP than CONT (58.20 and 26.86 ± 6.79 mg/g of substrate DM for RCS and CONT respectively). Compared to CONT, at 32 h the RS treatment yielded higher \((P < 0.05)\) BCP (70.75 and 45.20 ± 6.79 mg/g of substrate DM for RS and CONT, respectively). In addition, compared to CONT, BCP of RCS at 32 h tended \((P = 0.09)\) to be higher (61.86 and 45.20 ± 6.79 mg/g of substrate DM for RCS and CONT, respectively). After 32 h of fermentation, BCP started to decline in all treatments. Overall, BCP was maintained when replacing corn and it was increased when SBM was replaced. This may be due to higher content of starch available for microbial growth in the RS compared to the RC treatment. The numerical increase of BCP observed when DDGS was included was similar to that of Luepp et al. (2009), who observed that microbial efficiency expressed as g of microbial N/kg of organic matter truly fermented increased linearly when including 15, 30, 45 or 60% of DDGS diet DM replacing a combination of dry rolled corn, sunflower and urea. Bacterial growth may also be a result of stimulation produced by yeast cells contained in DDGS as suggested by Newbold et al. (1995) who reported that some strains of *S. cerevisiae* may enhance
bacterial growth. In addition, this increment may reflect the fact that the inclusion of corn milling byproducts in diets produces changes in some particular species of the bacterial population. In addition, Fron et al. (1996) reported that including corn milling co-products in a ruminant diet enhanced the capacity of microbial population to utilize lactic acid by increasing lactic utilizing bacteria. Furthermore, Callaway et al. (2010) reported that, compared to feeding 0% DDGS, ruminal and fecal bacteria populations were changed when animals were fed DDGS. Specifically the Firmicutes:Bacteroidetes ratio was smaller in 25 or 50% DDGS diets.

**Effect of DDGS and fermentation time on PCP**

Maintaining protozoa *in vitro* has been proven to be possible by other researchers (Yoshida and Katsuki, 1980; Takenaka et al., 2004). During the 96 h of fermentation we monitored changes in PCP as illustrated in Figure 3.2 B. Similar to results obtained for bacteria, PCP reached a peak at 32 h. The RCS treatment yielded higher ($P < 0.05$) PCP compared to CONT (13.42 and 10.60 ± 0.84 mg/g of substrate DM for RCS and CONT respectively). Similar to BCP, a treatment by time interaction was observed ($P < 0.05$). Specifically, at 32 h the RCS treatment yielded higher ($P < 0.01$) PCP compared to the CONT (29.76 and 21.17 ± 1.92 mg/g of substrate DM for RCS and CONT, respectively). After 32 h of fermentation, abundance of protozoa started to decline and by 96 h PCP was virtually undetectable (0.41 ± 0.96 mg/g of substrate DM, averaged across treatments). This overall growth pattern was similar to that reported by Yoshida and Katsuki (1980) who incubated rumen protozoa with corn starch, wheat starch, rice powder or barley powder. They indicated that protozoa reached a peak after 24 h of incubation, after 48 h protozoal populations decreased markedly and by 96 h protozoal
numbers were undetectable. These authors also indicated that only *Entodinium* species remained until 72 h of incubation. Protozoal generation time has been reported to vary by other authors *in vitro* (Dehority, 2008) and *in vivo* in sheep (Williams and Withers, 1993). Furthermore, protozoal cell division have been reported to be discrete not continuous. Sylvester et al. (2009) reported that protozoa decrease their generation time and that cell division can adjust and synchronize with substrate availability in batch cultures. The results obtained in this *in vitro* study may vary from *in vivo* experimental results because of different environmental and nutritional conditions.

**Effect of DDGS and fermentation time on YCP**

Figure 3.2 C illustrates the amount of YCP measured during *in vitro* fermentation over 96 h. Treatment did not affect \((P = 0.21)\) YCP, which averaged 0.04 ± 0.01 mg/g of substrate DM. The addition of DDGS to treatments only produced a numerical increase in the levels of YCP detected at 0 h fermentation compared to control. The level of YCP measured from 4 to 96 h fermentation was near 0 mg/g of substrate DM. On average, the highest amount of YCP was measured from the RS treatment and it was 0.048 mg/g of substrate DM. No treatment by time interaction was observed \((P = 0.48)\). Stein and Shurson (2008) suggested that 3.9% of the dry weight of DDGS may be contributed by yeast cell biomass. However, the level of yeast protein mass measured from DDGS samples utilized on this experiment was only 1.42% (DM basis). Our findings indicate when DDGS is included at 20% of the diet DM, YCP represented only 0.06% of total MCP. Additionally, only a small portion of yeast originating from DDGS is not being degraded, this is similar to results obtained by Castillo-Lopez et al. (2010), who observed that only a small proportion of yeast cells escape ruminal degradation and reach the
omasum when DDGS is included in the diet of dairy cows. However, Bruning and Yokoyama (1988) suggested that, when treated with heat, yeast cells of *S. cerevisiae* are morphologically affected and may be more resistant to degradation because the cell wall and soluble proteins contained in the yeast cytoplasm are denatured, rendering them more resistant to lysis and microbial degradation.

**Effect of DDGS and fermentation time on total MCP**

Figure 3.2 D illustrates the total amount MCP (the sum of BCP, PCP and YCP) in mg/g of substrate DM. Compared to CONT, replacing corn resulted in a small increase in total MCP (39.35 and 37.63 ± 3.64 mg/g of substrate DM for RC and CONT, respectively). However, when SBM or a portion of corn and SBM were replaced, the increase in MCP was greater (41.75 and 47.44 ± 4.45 mg/g of substrate DM for RS, RCS, respectively). This represents a practical biological benefit of DDGS when included in ruminant diets, which may partially explain the fact that DDGS can replace a portion of corn and SBM while maintaining or enhancing milk protein yield and lactational performance in dairy cows (Anderson et al., 2006). Microbial efficiency has been reported to increase by other authors (Luepp et al., 2009) when feeding DDGS. This may be explained in part by the existence of yeast and yeast derived components contained in those byproducts (Newbold et al., 1995). In addition, results may represent a practical economic benefit of feeding DDGS because of its lower price compared to corn and SBM. When averaged across treatments, total MCP yielded 41.54 mg/g of substrate DM and PCP yielded 10.87 mg/g of substrate DM. This means that on average, the amount of PCP accounted for 20.74% of total MCP, which is slightly lower compared to results obtained by Punia and Leibholz (1994) who found that protozoal nitrogen contributes 23-
29% of total microbial nitrogen flow to the omasum. Sylvester et al. (2005) suggested that protozoal nitrogen accounts for 12.7% of the rumen microbial nitrogen pool on diets containing 16% forage NDF. The dramatic decrease in protozoa at 48 and 96 h may be due the increase of bacteria during the first hours of fermentation with a subsequent lowering of pH affecting protozoa (Dehority, 2008) or due to a decrease in fermentative substrate by the end of in vitro fermentation. Additionally, data suggests that YCP had only minor effects on increasing total MCP, although as discussed previously, small amounts of yeast was detected particularly in DDGS-containing treatments. Compared to total MCP, the amount of YCP in fermentation treatments represented only 0.06%.

**CONCLUSIONS**

Dietary changes may produce variations in ruminal microorganisms. Specific variations in ruminal microbiota when feeding DDGS remain largely unknown. However, a good understanding on how ruminal microbial growth is affected by feeding distillers byproducts, like DDGS, may help us explain their high feeding value when fed to ruminants. This study was designed to help us understand its effect on ruminal microorganisms when included in dairy rations replacing a portion of corn, SBM or both from a diet based on corn silage plus alfalfa hay. By applying real-time PCR and utilizing DNA markers we evaluated the effect of DDGS not only on BCP and PCP in vitro, but also on YCP that presumably originated from DDGS included in treatments. Overall, results suggest that rumen microbial growth was improved when DDGS replaced SBM and it was maintained when DDGS replaced GC. This represents a practical biological benefit of DDGS when included in dairy ration, besides its relatively lower price.
compared to corn and SMB. In addition, data suggest that YCP contribution to total MCP was minimal when including 20% DDGS in treatments.
Literature cited


Leupp, J. L., G. P. Lardy, K. K. Karges, M. L. Gibson, and J. S. Caton. 2009. Effects of increasing levels of corn distillers dried grains with solubles to steers offered


<table>
<thead>
<tr>
<th>Ingredient, % DM</th>
<th>CONT</th>
<th>RC</th>
<th>RS</th>
<th>RCS</th>
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<td>25.0</td>
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Chemical composition, %

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<td>18.7 ± 0.16</td>
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<td>NDF</td>
<td>23.4 ± 0.60</td>
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<td>Starch</td>
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<td>29.5 ± 0.82</td>
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<td>5.6 ± 0.03</td>
<td>6.1 ± 0.02</td>
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1**CONT**: Control with no DDGS, but with alfalfa hay, corn silage (CS), ground corn (GC) and soybean meal (SBM) included at 25% (DM basis). **RC**: 20% (DM Basis) DDGS replacing GC. **RS**: 20% (DM basis) DDGS replacing SBM. **RCS**: 20% DDGS replacing 10% GC and 10% SBM (DM basis).
Figure 3.1. Measurements of pH from 0 to 96 h of *in vitro* fermentation from four dietary treatments.

1) CONT: Control with no DDGS, but with alfalfa hay, corn silage (CS), ground corn (GC) and soybean meal (SBM) included at 25% (DM basis); 2) RC: 20% (DM Basis) DDGS replacing GC; 3) RS: 20% (DM basis) DDGS replacing SBM and 4) RCS: 20% DDGS replacing 10% GC and 10% SBM (DM basis). Treatment, \( P = 0.14 \); time, \( P < 0.01 \). All treatments started with a mean pH value of 6.73 ± 0.04. Treatment RC and treatment RS reached the lowest pH at 32 h, 6.43 and 6.33 for the RC and RS, respectively; whereas, control and treatment RCS reached the lowest pH value at 48 h, 6.37 and 6.39 for control and RCS, respectively.

There were 6 independent measurements per treatment and a total of 24 measurements among the 4 treatments. \( n = 24 \).
Figure 3. Effect of dried distillers grains with solubles on synthesis of
(A) Bacterial crude protein (BCP), (B) protozoal crude protein (PCP), (C) yeast crude protein (YCP) and (D) total microbial crude protein (MCP: BCP, PCP and YCP).

There were 6 independent measurements per treatment and a total of 24 measurements among the 4 treatments. N = 24.

For BCP; treatment, $P = 0.23$; time, $P < 0.01$; treatment x time, $P < 0.05$. For PCP; treatment, $P < 0.05$; time, $P < 0.01$; treatment x time, $P < 0.05$. For YCP; treatment, $P = 0.21$; time, $P < 0.05$; treatment x time, $P = 0.48$. For MCP; treatment $P = 0.31$; time, $P < 0.01$; treatment x time, $P = 0.07$. Values are expressed in mg/g of substrate (DM basis) and measured from 0 to 96 h of in vitro fermentation from four dietary treatments.

Treatments are 1) CONT: Control with no DDGS, but with alfalfa hay, corn silage (CS), ground corn (GC) and soybean meal (SBM) included at 25% (DM basis); 2) RC: 20% (DM Basis) DDGS replacing GC; 3) RS: 20% (DM basis) DDGS replacing SBM and 4) RCS: 20% DDGS replacing 10% GC and 10% SBM (DM basis).
CHAPTER IV

In vivo determination of rumen undegradable protein of dried distillers grains with solubles and evaluation of duodenal microbial crude protein flow*

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ABSTRACT

The objectives of this trial were to determine the rumen undegradable protein (RUP) of dried distillers grains with solubles (DDGS), to compare the estimates of duodenal bacterial crude protein (BCP) flow using diaminopumelic acid (DAPA) or DNA as bacterial markers, and to estimate duodenal protozoal crude protein (PCP) and yeast crude protein (YCP) flow when DDGS are fed. Three crossbred steers fitted with ruminal and double L-shaped duodenal cannulae (average BW 780 ± 137 kg) were used in a 3 treatment, 6 period cross-over design. Animals were housed in individual free stalls and fed twice daily at 0700 and 1900. Diets (DM basis) were 1) CONTROL, 0% DDGS, but with 19.5% corn bran, 20% sorghum silage, 60% brome hay, 0.5% trace minerals and 0.25% urea; 2) LOW DDGS, inclusion of 9.75% DDGS replacing equal percentage of corn bran; 3) HIGH DDGS, inclusion of 19.5% DDGS completely replacing corn bran. At the end of each period, 200 mL of duodenal digesta were collected from each steer every 4 h over the course of 4 d. Duodenal BCP flow was estimated using DAPA and DNA as bacterial markers. In addition, duodenal PCP and YCP flow were estimated
using DNA markers. The value of DDGS RUP as a percent of CP was determined to be 63.0 ± 0.64%. Estimates of duodenal BCP flow using DAPA were 473, 393, 357 ± 78 g/d ($P = 0.09$) for CONTROL, LOW DDGS and HIGH DDGS, respectively. Estimates of duodenal BCP flow using DNA were 479, 397 and 368 ± 74 g/d ($P = 0.14$) for CONTROL, LOW DDGS and HIGH DDGS, respectively. Average BCP flow across treatments was unaffected ($P = 0.71$) by marker type and were 404 and 417 ± 83 g/d for DAPA and DNA markers, respectively. Estimates of duodenal PCP flow were 82, 80 and 78 ± 12 g/d ($P = 0.64$) for CONTROL, LOW DDGS and HIGH DDGS, respectively. Estimates of duodenal YCP flow were 0.15, 1.94 and 4.80 ± 0.66 g/d ($P < 0.01$) for CONTROL, LOW DDGS and HIGH DDGS, respectively. Duodenal BCP flow tended to decrease with DDGS inclusion, but estimates were not affected by marker type. In addition, DDGS did not affect duodenal PCP supply and provided small amounts of duodenal YCP. Overall, the value of DDGS RUP determined in this study will contribute to better understand the effect of this byproduct in ruminant nutrition.

**Key words:** dried distillers grains with solubles, rumen undegradable protein, duodenal microbial crude protein flow

**INTRODUCTION**

The rumen undegradable protein (RUP) of dried distillers grains with solubles (DDGS) has been suggested to range from 56% (Kelzer et al., 2010a) to 72% of CP (Archibeque et al., 2008; NRC, 2000). More specifically rumen degradability characteristics of protein contained in DDGS are variable (Aines et al., 1987) and may be influenced by production plant (Spiehs et al., 2002), protein originating from yeast that
may also resist rumen degradation (Tagari et al., 1986; Castillo-Lopez et al., 2010), and small particle size (Dewhirst et al., 1994). Accurate determination of RUP is important because it contributes to metabolizable protein (MP) (NRC, 2000). When fed to cattle, DDGS may contribute to the supply of MP from RUP and supply energy and N that rumen microbes may use to synthesize MCP.

Determination of the RUP concentration of feed is conducted through in vivo (Vanzant et al., 1996), in situ (Ørskov and McDonald, 1979; Kelzer et al., 2010a) or in vitro (Krishnamoorthy et al., 1983; Poos-Floyd et al., 1985) methods. Compared to in situ, in vivo estimations of RUP are more expensive and time consuming (Vanzant et al., 1996), but may be superior because it involves exposure of the feed to all of the biochemical processes in the digestive tract (Johnson, 1966).

The estimation of MCP relies on the use of microbial markers, such as, purines (Zinn and Owens, 1986; Obispo and Dehority, 1999), DNA (Sylvester et al., 2005; Belanche et al. 2011a), diaminopumelic acid (DAPA) (Broderick and Merchen, 1992; Webster et al., 1990) or labeled isotopes (Stern, 1979). The objectives of this experiment were to 1) determine the RUP of DDGS, and 2) to compare the estimates of duodenal bacterial crude protein (BCP) flow using DAPA or DNA as bacterial markers, and 3) to estimate duodenal protozoal crude protein (PCP) and yeast crude protein (YCP). We hypothesize that the addition of DDGS to diets may increase rumen MCP synthesis and provide duodenal protein of yeast origin.
MATERIALS AND METHODS

Animals and treatments

The experimental steers were managed according to the guidelines stipulated by the University of Nebraska Animal Care and Use Committee. Three British-bred crossbred steers fitted with ruminal and double L-shaped duodenal cannulae were used in this experiment, which was a 3 treatment, 6 period cross-over design. Duodenal cannulations were conducted as described by Streeter et al. (1991). Briefly, the cannula was constructed from cyclopolyvinyl chloride water pipe fittings. Construction materials are fairly rigid, but by connecting the split cannula pieces with elastic castration bands the cannula had some flexibility. A short cone was placed over the exposed cannula barrel to reduce mechanical damage to the intestine. Cattle were fasted for 12 h before surgery. Surgeries were conducted after local anesthesia of the surgical area with 2% lidocaine. Surgeries were conducted while animals were standing blindfolded to reduce disturbance to the animal by the movements of the surgical team. Standard aseptic surgical conditions were used. Laparotomy was performed to place the cannula in the proximal duodenum of each animal. Steers received each treatment twice on 1 of six 19 d experimental periods. Animals averaged 780 ± 137 kg of BW throughout the trial and were housed in individual free box stalls.

Treatments were formulated to include 0, 9.75 or 19.5% DDGS (DM basis), which were obtained from a Nebraska-based corn-ethanol plant (Green Plains Renewable Energy, Central City, NE). Table 4.1 lists the ingredients and chemical composition of each treatment diet. In addition, the chemical composition of feed ingredients utilized in this experiment is listed in Table 4.2. These diets were formulated so that DDGS would
only replace corn bran and urea. Total mixed rations (TMR) were mixed by hand daily and animals were fed 2 times per d at 0700 and 1900 h. The amount of feed offered was adjusted before the initiation of the trial to 95% of \textit{ad libitum} intake during a 10 d period followed by the experimental periods. Water was available for \textit{ad libitum} consumption. Chromic oxide (Cr$_2$O$_3$) was used as a marker for the estimation of duodenal flow (Hutton et al., 1971; Harvatine et al., 2002; Sylvester et al., 2005). Seven and a half grams of Cr$_2$O$_3$ (Taylor and Allen, 2005) was weighed and placed in gelatin capsules (Torpac Inc., Fairfield, NJ), then dosed into the rumen via the ruminal cannula twice daily at 0700 and 1900 during d 9 through 19 of each experimental period to provide a marker to estimate duodenal digesta flow. It is important to note that, some limitations have been suggested in the use of Cr$_2$O$_3$ as a digesta marker. For example, studies indicate that Cr$_2$O$_3$ mixes poorly with total ruminal contents especially with digesta in the dorsal blind sac (Owens and Hanson, 1991; Waller et al., 1980). In addition, great diurnal variation has been reported in the concentration of Cr$_2$O$_3$ in digesta of ruminants (Hopson and McCroskey, 1972). However, this problem can be overcome if enough samples are collected throughout the day to provide an average sample in which the marker concentration is representative of the entire day (Titgemeyer, 1997).

\textbf{Ruminal pH measurement}

Ruminal pH was measured according to the protocol described by Rolfe et al. (2009). Briefly, during d 16 through 19 of each period ruminal pH was measured once every minute continuously using a wireless pH probe (Dascor Inc., Escondido, CA). The probe was placed into the ventral sac of the rumen of each steer. Each probe contained a data logger, 9-volt battery, and an electrode cable housed in a watertight capsule.
constructed out of PVC material. Each pH electrode was enclosed in a weighted, PVC cover that maintained the electrode in the ventral sac of the rumen. Measurements of ruminal pH were averaged by steer across the 4 collection days, so that a period of 24 h was represented in each period. This would allow us a better illustration of ruminal pH variation with time after feeding. From these values, minimum, maximum and mean ruminal pH was calculated for each steer. The time and area below the mean ruminal pH (averaged across treatments) are also presented. Wireless ruminal pH probes were calibrated before inserting them into the rumen and after downloading pH data collected into calculation spread sheets at the end of each period. The use if this method and type of rumen pH probes has also been reported to be effective by other researchers (Penner et al., 2006; Silveira et al., 2007).

**Duodenal digesta and feed sampling**

Duodenal digesta contents (200 mL) were collected every 4 h and placed in 250-mL Nalgene bottles (Thermo Scientific Inc., Waltham, MA) during d 16 through 19 of each period. Collection time was advanced 1 h in subsequent collection d, so that every 60-minute interval in a 24-h period was represented (6 samples per d and a total of 24 samples per steer per period). Samples were collected on d 16 at 0700, 1100, 1500, 1900, 2300 and 0300; d 17 at 0800, 1200, 1600, 2000, 0000 and 0400; d 18 at 0900, 1300, 1700, 2100, 0100 and 0500; and d 19 at 1000, 1400, 1800, 2200, 0200 and 0600. Samples were then composited by steer, by day within period and immediately frozen at -20°C for subsequent analyses. Samples of individual feed ingredients as well as samples of the TMR for each treatment were also collected twice daily immediately after feeding on d 16 through 19 of each period and frozen at -20 °C for later analysis.
Isolation of ruminal bacteria

On d 18 and 19 of each experimental period, 1.5 L of whole ruminal digesta contents were collected from 4 different locations within the rumen at 1000 and 1600 (d 18) and 1200 and 1800 (d 19). Then ruminal bacteria were isolated according to the procedure described by Hristov et al. (2005). Briefly, whole ruminal contents were composited and squeezed through 2 layers of cheesecloth and the filtrate was retained. Solids remaining on the cheesecloth were added to a volume of cold buffer (McDougall, 1948) equal to the volume of filtrate, and shaken manually in a screw-capped jar to dislodge the ruminal microorganisms loosely associated with feed particles. This suspension was then squeezed through 2 layers of cheesecloth and the 2 filtrates were combined (1:1) and preserved with 5% (vol/vol) formalin. From this sample, bacteria were harvested via differential centrifugation (Hristov and Broderick, 1996) with an initial low-speed centrifugation at 400 × g for 5 min at 4 °C and a subsequent high-speed centrifugation at 20,000 × g for 15 min at 4°C. Samples were maintained on ice while being processed. The supernatant was then discarded and the isolated bacterial pellets were composited by steer and period and frozen at –20 °C for later analysis.

Laboratory procedures

a) Analysis of feed, duodenal digesta and bacterial pellets

Collected feed ingredient and TMR samples were dried for 48 h at 60 °C in a forced air oven, ground to pass through a 1-mm screen (Wiley Mill, Arthur A. Thomas Co., Philadelphia, PA) and analyzed for chemical composition by an external laboratory (Cumberland Valley Analytical Services, Hagerstown, MD), which included DM (AOAC, 2000), N (Leco FP-528 Nitrogen Combustion Analyzer, Leco corp. St. Joseph,
MI), NDF (Van Soest et al., 1991), starch (Hall, 2009), ether extract (AOAC, 2006) and ash (AOAC, 2000). In addition, the concentration of yeast (Saccharomyces cerevisiae) protein was measured from subsamples of DDGS collected. This was conducted by using DNA markers as described by Castillo-Lopez et al. (2010). Briefly, the ratio yeast DNA marker:protein was established from a pure sample of yeast. Then the yeast DNA marker was measured from subsamples of DDGS. From those values, the amount of yeast protein in samples was determined. Nutrient composition of the diets (Table 4.1) was calculated based on analysis of individual feed ingredient and the rate of inclusion to the diet.

Collected duodenal contents were lyophilized and ground to pass through a 1-mm screen using a Wiley Mill (Arthur H. Thomas, Philadelphia, PA). Then, ground samples were analyzed for DM (100 °C oven for 24 h) and ash (AOAC, 2000). Duodenal samples were also analyzed for Cr$_2$O$_3$ by an external laboratory (Servi-Tech Laboratories, Hastings, NE) by sample digestion in 10 mL nitric acid and 3 mL peroxide, with a hydrochloric acid addition and analyzed by inductive coupled plasma.

Isolated bacterial pellets were lyophilized and ground using a mortar and pestle, then analyzed for DM (AOAC, 2000) and N (Leco FP-528 Nitrogen Combustion Analyzer, Leco corp. St. Joseph, MI).

b) Estimation of BCP using DAPA as bacterial marker

The use of DAPA as a bacterial marker has been widely reported (Dufva et al., 1982; Webster et al., 1990; Csapo et al., 2008). In the present study, isolated ruminal bacterial pellets and collected duodenal digesta samples were analyzed for DAPA using a 717 HPLC system (Waters Corporation Inc., Milford, MA) according to the procedure
described by Webster et al. (1990). Briefly, between 150 to 180 mg of ground duodenal
digesta samples or between 28 to 30 mg of ground bacterial pellets were weighed and
placed into screw cap culture tubes (25 × 125 mm). Then, 3 mL of methasulfonic acid
was added to each tube and samples were hydrolyzed at 110°C for 22 h. Samples were
neutralized with sodium hydroxide and pH was adjusted to 7.0 to 7.5 with sulfuric acid or
sodium hydroxide. This step was followed by 2 clean up procedures. First by adding
HCl-washed charcoal (Sigma chemical Co., St. Louis, MO) and centrifuging samples at
1,000 × g for 20 minutes, and then by filtering samples through a Sep-Pak C-18 cartridge
filter (Waters Associates, Milford, MA). Then, 1 mL of o-phthalaldehyde derivatization
solution was added to 1.0 mL of sample in a small test tube; contents were mixed
thoroughly and reacted for 14 h at 4 °C. Finally, 20 µL of derivatized sample were
injected into the column.

Results from HPLC were used to calculate the concentration of DAPA in the
samples. Then, the ratios DAPA:N (N measured by N combustion from isolated ruminal
bacterial pellets) were utilized to estimate the concentration of BCP from each duodenal
sample. To do so, the ratio DAPA:N was independently established for isolated bacterial
pellets from each steer in each period and was utilized to calculate duodenal BCP flow
from samples taken from the corresponding animal and period.

c) Estimation of BCP using DNA as bacterial marker

Bacterial CP was also estimated based on the ratio bacterial DNA marker:N. To
do so, bacterial DNA was extracted by the repeat bead beating plus column method
according to the extraction method for PCR-quality DNA from digesta samples described
by Yu and Morrison (2004). Briefly, collected samples of duodenal digesta and ruminal
bacteria were combined with lysis buffer and beads, and then they were shaken for physical disruption of cells and exposure of cellular contents. Then, DNA and RNA were precipitated; and finally DNA was purified by applying a series of centrifugation steps and by eliminating the RNA and proteins. The concentration of DNA in each sample was measured by spectrophotometry (NanoDrop ND-1000 Spectrophotometer, NanoDrop Technologies, Inc. Wilmington, DE) and stored at -20°C in aliquots of 25 µL for later analysis for BCP using real-time PCR.

Bacterial DNA marker used in this study have been reported elsewhere (Yu et al., 2005) and it is part of the gene encoding the 16S rRNA, which has been shown to be highly preserved in bacteria (Ogier et al., 2002; Zimmermann et al., 2010). The national center of biotechnology information accession number (NCBI) of the targeted bacterial DNA marker is FJ715623. The marker is composed of a forward primer, a TaqMan probe and a reverse primer. Forward primer: 5’-act cct acg gga ggc agc ag-3’. TaqMan probe: 5’-FAM/tgc cag cag ccg cgg taa/TAMRA-3’. Reverse primer: 5’-gac tac cag ggt atc taa tcc-3’.

Real-time PCR reactions were as follows, 4 µL of DNA sample were combined with 1 µL of 10 µM forward primer, 1 µL of 10 µM reverse primer, 0.25 µL of 10 µM TaqMan probe, 7.5 µL of TaqMan Master Mix (Applied Biosystems, Foster City, CA, USA) and 1.25 µL of nanopure water. Two samples with no DNA were included and used as non-template controls. Each sample was run in duplicate in separate wells of the 384-well real-time PCR plate. DNA samples were subjected to real-time PCR using a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA).
Temperature cycling was as described by Moya et al. (2009) with some variations, specific conditions were as follows: stage 1: 50°C for 2 minutes; stage 2: 95°C for 10 minutes; stage 3: 45 cycles alternating denaturation at 95°C for 15 seconds, then annealing and polymerization at 60°C for 1 minute. Results from real-time PCR were used to estimate BCP according to calculations described by Castillo-Lopez et al. (2010). Briefly; the ratio of bacterial DNA marker:N (from isolated ruminal bacterial pellet) was established and used to estimate BCP from duodenal samples. Similar to the DAPA assay, the ratio bacterial marker:N was established for isolated bacterial pellets from each steer in each period.

d) Estimation of PCP and YCP

For the estimation of duodenal PCP and YCP flow, DNA extraction method, real-time PCR reactions and conditions and calculations were identical to those used for the estimation of BCP however, the ratio of protozoal DNA marker:N was established from isolated ruminal protozoal pellets (Shabi et al., 2000). The protozoal DNA marker utilized has been reported elsewhere (Sylvester et al., 2005) and it is part of the gene encoding the 18S rRNA. This marker is composed of a forward primer, a TaqMan probe and a reverse primer. Forward primer: 5’-gct ttc gwt ggt agt gta tt-3’. TaqMan Probe: 5’-FAM/cgg aag gca gca ggc gc/TAMRA- 3’. Reverse primer: 5’-act tgc cct cya atc gtw ct-3’. From the ratio protozoal DNA marker:N, the amount of duodenal PCP was calculated. The ratio of yeast DNA marker:N from a sample of yeast pellet (S. cerevisiae) was also established. Yeast marker was designed from part of the II chromosome of S. cerevisiae genome (Castillo-Lopez et al., 2010). To design these oligonucleotides, the NCBI blast software was used to verify that this DNA sequence belongs only to S.
cerevisiae, so that it will not be cofounded by other related fungi species. The NCBI accession number of the targeted yeast DNA marker is Y08934. Similar to bacterial and protozoal DNA markers, yeast DNA marker is composed of a forward primer, a TaqMan probe and a reverse primer. Forward primer: 5’-cct gct aaa ctg cag ctt gac-3’. TaqMan probe: 5’-FAM/ctg cgg acc ctg cag tcc agc/TAMRA-3’. Reverse primer: 5’-cag cgt ttg cgt tcc atg ac-3’. These oligonucleotides were analyzed with the oligo analyzer of the Integrated DNA Technology (IDT) program this was to verify whether those oligonucleotides are adequate for being used in real-time PCR assays. From the ratio yeast DNA marker:N, the amount of duodenal YCP was calculated.

**Calculation of duodenal flow**

The use of Cr₂O₃ as a marker for the estimation of digesta flow has been reported elsewhere (Christiansen and Webb, 1990; Titgemeyer, 1997; Kozloski et al., 1998). Duodenal flows were calculated as described by Erasmus et al. (1992). Briefly, the flow of DM was calculated by dividing the amount of daily Cr₂O₃ dosed by Cr₂O₃ concentration in ground duodenal samples. Then, DM flow was expressed in g per d. Duodenal flow of CP was calculated by multiplying DM flow by the concentration of CP (N × 6.25) in duodenal samples (DM basis). Flow of organic matter (OM), ash, BCP, PCP and YCP were calculated in a similar fashion.

**Estimate of RUP content of DDGS**

Equation 1 denotes the calculations conducted for the estimation of RUP as a percent of CP contained in DDGS. Given that DMI was constant across treatments (Table 4.3), DDGS RUP was determined by difference of residual duodenal CP flow (between CONTROL and HIGH DDGS treatment) divided by DDGS CP intake. Briefly, estimate
of residual duodenal CP (non-BCP) flow of CONTROL diet was subtracted from estimate of residual duodenal CP flow of HIGH DDGS treatment, then result was divided by CP originating from DDGS intake and the final result was expressed as a percent of DDGS CP.

In addition, we corrected this value for corn bran RUP. To do so; duodenal RUP flow originating from corn bran was calculated and subtracted from residual duodenal CP of CONTROL diet, this calculation was based on the assumption that the value of RUP in corn bran is 13% (Herold, 1999; Mass et al., 1999). The amount of RUP originating from corn bran was determined to be 31 g/d and the intake of CP originating from DDGS was determined to be 570 g/d.

DDGS RUP

\[
\frac{\left[ (\text{CP flow} \frac{g}{d})^1 - (\text{BCP flow} \frac{g}{d})^2 \right] - \left[ (\text{CP flow} \frac{g}{d})^3 - (\text{BCP flow} \frac{g}{d})^4 \right] - (\text{CB RUP} \frac{g}{d})^5}{(\text{Intake of DDGS CP} \frac{g}{d})^6} \quad \text{.. [1]}
\]

Where;

1: Total duodenal CP flow in g/d for HIGH DDGS diet

2: Duodenal BCP flow in g/d for HIGH DDGS diet

3: Total duodenal CP flow in g/d for CONTROL diet

4: Duodenal BCP flow in g/d for CONTROL diet

5: Duodenal RUP flow in g/d originating from corn bran (contained in CONTROL diet), which was calculated by multiplying daily DMI by percent of corn bran in diet by percent RUP of corn bran
Intake of CP originating from DDGS, which was calculated by multiplying daily DMI by percent DDGS in diet by percent CP of DDGS.

The calculation of DDGS RUP was conducted first by utilizing results of duodenal BCP flow obtained when using DAPA as bacterial marker, and then by utilizing duodenal BCP flow obtained when using DNA as bacterial marker (data presented in Table 4.4).

Calculation of DDGS RUP when using DAPA as bacterial marker:

\[ DDGS \text{ RUP} = \frac{\left[ (1168 \frac{g}{d})^1 - (357 \frac{g}{d})^2 \right] - \left[ \left( \left(953 \frac{g}{d}\right)^3 - (473 \frac{g}{d})^4 \right) - (31 \frac{g}{d})^5 \right]}{(570 \frac{g}{d})^6} \]

\[ DDGS \text{ RUP} = \frac{811 \frac{g}{d} - 449 \frac{g}{d}}{(570 \frac{g}{d})^6} \]

\[ DDGS \text{ RUP} = \frac{362}{570} = 0.635 = 63.5\% \]

Calculation of DDGS RUP when using DNA as bacterial marker:

\[ DDGS \text{ RUP} = \frac{\left[ (1168 \frac{g}{d})^1 - (368 \frac{g}{d})^2 \right] - \left[ \left( \left(953 \frac{g}{d}\right)^3 - (479 \frac{g}{d})^4 \right) - (31 \frac{g}{d})^5 \right]}{(570 \frac{g}{d})^6} \]

\[ DDGS \text{ RUP} = \frac{800 \frac{g}{d} - 443 \frac{g}{d}}{(570 \frac{g}{d})^6} \]

\[ DDGS \text{ RUP} = \frac{357}{570} = 0.626 = 62.6\% \]
Statistical analysis

Data collected on ruminal pH and duodenal flow were analyzed using the MIXED procedure of SAS (Version 9.1; SAS Institute, Inc., Cary, NC) as a 3 treatment, 6 period cross-over design assuming treatment and period as fixed effects and steer as a random effect. Linear and quadratic effects of treatments were tested. In this design all animals received each treatment twice. To compare the estimates of duodenal BCP flow using DAPA or DNA as bacterial markers, data were analyzed with a model that included treatment, period and method as fixed effects and steer as a random effect. Treatment means are presented as least square means and the largest standard error of the mean (SEM) is reported. Significance was declared at $P \leq 0.05$ and tendency was declared if $P > 0.05$ and $\leq 0.15$.

RESULTS AND DISCUSSION

Animals, DMI and CP intake

During the last period, 1 animal was removed from the experiment because of a dramatic decrease in DMI and a suspected health problem. Table 4.3 lists DMI and CP intake from steers fed CONTROL, LOW DDGS and HIGH DDGS. As designed, averaged DMI was similar for all treatments. Intake of CP increased ($P < 0.01$) with increasing levels of DDGS.

Ruminal pH measurements

Table 4.3 lists ruminal pH measurements collected averaged across d 16 through 19 of experimental periods. Mean ruminal pH was not affected ($P = 0.88$) by level of DDGS and averaged $6.4 \pm 0.09$ across treatments. In addition, the time ($P = 0.68$) and
area ($P = 0.98$) below pH 6.4 were not affected and averaged $567 \pm 362$ min/d and $80 \pm 61$ pH × min/d, respectively. Measurements indicate that ruminal pH was highest just before feeding and declined for approximately 4 to 5 h thereafter before gradually increasing and return to near its previous value before feeding.

Mean ruminal pH observed in this study was higher compared to values reported in other studies for animal consuming DDGS. For example, Leupp et al. (2009b) found mean ruminal pH to be 6.35 for steers consuming a diet containing 15% DDGS. In addition, Peter et al. (2000) reported mean ruminal pH to be 6.23 for steers consuming a diet based on alfalfa hay and cracked corn with 20% inclusion of DDGS. Overall, ruminal pH observed in this study was in normal range and was not expected to have a detrimental effect on rumen fermentation and ruminal microbial protein synthesis (Russell et al., 1979; Russell and Rychlik, 2001).

**Duodenal flow of DM, OM, ash and CP**

Table 4.4 lists the duodenal flow of DM, OM, ash and total CP for steers fed CONTROL, LOW DDGS and HIGH DDGS treatments. High variability in duodenal DM flow was observed in this study. The great variability in DM flow when using Cr$_2$O$_3$ as a digesta marker has been reported elsewhere (Waller et al., 1980; Drennan et al., 1970; Faichney, 1975) and it has been suggested that this may be caused by diurnal variations in the concentration of Cr$_2$O$_3$ in digesta. The effect of diurnal variation, however, was expected to be overcome by compositing samples collected throughout the same day (Titgemeyer, 1997). Studies have also suggested that Cr$_2$O$_3$ may not consistently flow with the digesta as a result of poor mixing (Drennan et al., 1970; Faichney, 1975) when supplied in gelatin boluses (Titgemeyer, 1997), which have contributed to the high
variability in our observations.

The inclusion of DDGS in diets had no effect \((P = 0.87)\) on duodenal DM flow, which was observed to be 5,406, 5,305 and 5,541 ± 1,025 g/d for CONTROL, LOW DDGS and HIGH DDGS, respectively. In addition, duodenal OM flow was unaffected \((P = 0.83)\) with estimates of 4,681, 4,588 and 4,805 ± 871 g/d for CONTROL, LOW DDGS and HIGH DDGS treatments, respectively. Furthermore, duodenal ash flow was not affected \((P = 0.91)\) by DDGS inclusion; estimates were 725, 717 and 736 ± 157 g/d for CONTROL, LOW DDGS and HIGH DDGS treatments, respectively. These observations were expected because DMI was similar across treatments. Also, DDGS replaced corn bran which likely has similar digestibility. In contrast, total duodenal CP flow tended \((P = 0.12)\) to increase with increasing levels of DDGS. Estimates were 953, 1,030 and 1,168 ± 201 g/d for CONTROL, LOW DDGS and HIGH DDGS treatments, respectively. Although, duodenal flow of OM was not affected in this study, when steers are fed \textit{ad libitum}, an increase in duodenal OM flow with increasing DDGS has been reported (Leupp et al., 2009a). These authors observed that duodenal OM flow was 3.51 and 4.10 kg/d when animals were fed 0 or 1.2% of BW daily of DDGS. However, the increase in OM flow observed by these researchers was associated with an increase in DMI. It is important to note that in that study DDGS replaced smooth brome hay, whereas in the current study DDGS replaced corn bran. Relative differences in ruminal digestibility and retention time may also affect flow (Bach et al., 2005). An increase in duodenal CP flow with increasing DDGS levels in the current study could be explained by higher CP content in DDGS added treatments compared to CONTROL (10.6, 11.9 and 13.0 ± 1.2% CP for CONTROL, LOW DDGS and HIGH DDGS, respectively) and due to higher RUP
supplied by DDGS, which provided more protein resistant to ruminal degradation compared to corn bran that has lower levels of CP mostly degradable in the rumen (Herold, 1999).

**RUP content of DDGS**

The estimate of DDGS RUP determined in this experiment averaged 63.0 ± 0.64%. This value is higher than that reported by Schingoethe et al. (2009), who suggested that the RUP contained in DDGS is 55% when fed to dairy cows. Dairy cattle would have a higher passage rate than the steers utilized in the present experiment and this should result in a higher RUP (Bateman et al., 2005). In addition, our estimate is higher than that reported by Kelzer et al. (2010a), who used a 16 h nylon bag rumen incubation and reported that the RUP contained in DDGS is 56.3%. However, the observed value of RUP in DDGS in the current study is lower compared to DDGS RUP (70.3%) reported by the NRC (2001). Our results are similar to dried distillers grains RUP reported by Cao et al. (2009). These authors incubated samples in nylon bags for 3, 6, 12, 18, 24 and 48 hours and used an exponential model to determine *in situ* degradation curves of CP (Ørskov and McDonald, 1979; McDonald, 1981) and observed that 64% of dried distillers grains CP escapes ruminal degradation. Interestingly, the estimate of RUP contained in DDGS determined in the current study is higher compared to dried corn grain RUP (58.8% CP) or cracked corn grain RUP (55.3% CP) (NRC, 2001). This observation may support the suggestion of Kleinschmit et al. (2007), that the heat applied during the drying process may reduce the availability of protein to ruminal degradation. Heat treated yeast would likely have a minor effect on DDGS RUP, because only small amounts of yeast (described below) were found to escape ruminal degradation.
in this study. However, Stein and Shurson (2008) suggested higher levels of yeast cell biomass in DDGS, which may become resistant to lysis and microbial degradation when treated with heat (Bruning and Yokoyama, 1988). The RUP content of DDGS has been reported to be influenced by the processing method during ethanol production. For example, Kelzer et al. (2010a) reported that RUP content of DDGS that had no heat exposure before fermentation is 33.2%, whereas the RUP content of DDGS that had heat exposure before fermentation is 56.3%.

**Duodenal BCP flow using DAPA and DNA as markers**

Table 4.4 lists estimates of duodenal BCP flow obtained by the DAPA procedure. Duodenal estimates of BCP flow tended ($P = 0.09$) to decrease with the inclusion of DDGS and were 473, 393 and 357 ± 78 g/d for CONTROL, LOW DDGS and HIGH DDGS treatments, respectively. In addition, BCP as a percent of total duodenal CP flow was lower ($P < 0.01$) with increasing levels of DDGS. Estimates were 49, 38 and 30 ± 3.55%, for CONTROL, LOW DDGS and HIGH DDGS, respectively. Table 4.4 also lists duodenal BCP flow estimated by using DNA marker through the real-time PCR assay. As observed when using DAPA as a marker, the inclusion of DDGS also tended to decrease duodenal BCP flow ($P = 0.14$). Estimates were 479, 397 and 368 ± 84 g/d for CONTROL, LOW DDGS and HIGH DDGS treatments, respectively. In addition, BCP estimated with DNA marker as a percent of total duodenal CP flow decreased ($P < 0.01$) with increasing levels of DDGS. Estimates were 50, 38 and 31 ± 1.65%, for CONTROL, LOW DDGS and HIGH DDGS, respectively. In contrast to our results, the inclusion of DDGS in ruminant diets has been reported to maintain or increase ruminal MCP synthesis. For example, Leupp et al. (2009a) fed beef steers 0, 15, 30, 45, or 60% of
DDGS (DM basis) replacing a combination of dry rolled corn, sunflower meal, and urea and observed no change in total duodenal BCP supply using purines as a microbial marker. In addition, Kelzer et al. (2009b) reported that MCP synthesis estimated with purine derivatives was similar for cows fed a control diet with no DDGS, a diet containing 15% DDGS or a diet containing 15% high protein DDGS (DM basis). These authors observed that MCP averaged 1,161 g/d. Furthermore, Chibisa et al. (2012) demonstrated that wheat-DDGS can substitute for canola meal as the major protein source in dairy cow diets without negatively affecting MCP production using purine derivatives as a microbial marker. Another study (Leupp et al., 2009b) demonstrated that duodenal BCP flow increase linearly from 380 to 551 g/d when steers were supplemented from 0 to 1.2% of BW daily of DDGS. In addition, Janicek et al. (2008) reported that cows consuming 30% DDGS synthesized similar amount of MCP compared to a diet containing no DDGS using the purine derivative method, with an average of 1,642 g of MCP/d.

When microbial protein reached the small intestine it represents an important supply of peptides and amino acids to the host animal (Lapierre et al., 2006). Some of the factors that may influence MCP synthesis are the availability of N, energy availability for rumen fermentation and ruminal passage rate (Purser, 1970; Stern and Hoover, 1979). Readily available N in the form of urea (0.25 versus 0% for CONTROL and HIGH DDGS diets, respectively) and higher starch content (5.7 versus 3.5% for CONTROL and HIGH DDGS diets, respectively) may have supported more microbial growth leading to more BCP synthesis when CONTROL diet was fed in the present experiment. The lower duodenal flow of MCP observed in this study compared to that observed by Leupp et al.
(2009b) may reflect differences in the chemical composition of diets fed in these 2
scenarios when feeding DDGS. Specifically, the 15% DDGS diet fed by these researchers
contained 31.2% nonfiber carbohydrate (NFC); whereas in this study, the 20% DDGS
diet contained only 15.9% NFC. Another difference between these 2 diets is the level of
CP and urea supplementation. In this study, the 20% DDGS diet was not supplemented
with urea and contained only 13% CP; whereas the 15% DDGS diet fed by Leupp et al.
(2009b) contained 16.2% CP supplemented with 0.67% urea.

No difference was observed ($P = 0.71$) in duodenal BCP flow averaged across
treatments when using either DAPA or DNA as bacterial markers (Figure 4.1). The
overall estimates of duodenal BCP flow averaged across treatments were 404 and 417 ±
83 g/d for DAPA and DNA markers, respectively. The use of DNA as a bacterial marker
through real-time PCR to estimate BCP has also been reported by other researchers. For
example, Belanche et al. (2011a) tested and demonstrated that bacterial 16S rDNA
sequences may persist through the gastric digestive tract and their utilization as a highly
specific bacterial marker should not be neglected. Several studies have reported the use of
DAPA as a bacterial marker (Work and Dewey, 1953; Dennehy, 2001; Webster et al.,
1990). However, limitations have been suggested for DAPA. For example, its presence in
feed sources (Theurer, 1982; Rahnema and Theurer, 1986; Broderick and Merchen, 1992)
would represent a source of contamination leading to an overestimation of BCP. In
addition, the ratio DAPA:N may vary substantially within the same animals on the same
diet with time after feeding (Broderick and Merchen, 1992). The utilization of DAPA and
DNA for the estimation of BCP in this experiment was based on our hypothesis that the
inclusion of DDGS in diets may also provide residual yeast cells, which would contribute
to duodenal purines and therefore confound our estimates of microbial protein flow and DDGS RUP if purines are used as a microbial marker. One advantage of using DNA as a marker is the fact that it involves a faster and simpler procedure compared to the DAPA protocol performed in this study. In addition, the utilization of DNA as a microbial marker permits an independent quantification of the bacterial and protozoal N contribution (Belanche et al., 2011b).

**Duodenal PCP and YCP flow**

As listed in Table 4.4, no difference ($P = 0.64$) was observed in the estimates of duodenal PCP flow and averaged 80 ± 2 g/d. On average, the duodenal PCP flow represented 16% of total duodenal MCP flow. These results are higher than that of Sylvester et al. (2005), who reported that protozoal N accounts for 11.9% of the duodenal flow of microbial N on diets containing 21% forage neutral detergent fiber. On the other hand, our result is lower compared to data reported by Punia and Leibholz (1994), who observed that protozoal N contributes 26-29% of total microbial N flow in steers being fed kikuyu (*Pennisetum clandestinum*) hay containing 6.2, 34.5 and 3.8% of CP, acid detergent fiber and acid detergent lignin, respectively.

Table 4.4 lists the estimates of duodenal YCP flow, which increased ($P < 0.01$) with increasing levels of DDGS. Estimates were 0.15, 1.94 and 4.80 ± 0.66 g/d for CONTROL, LOW DDGS and HIGH DDGS, respectively. This observation agrees with our hypothesis that the inclusion of DDGS to diets would also provide yeast reaching the intestine. When HIGH DDGS treatment was fed, duodenal YCP flow represented only 1.06% of total MCP. The amount of yeast protein biomass estimated from DDGS in this study was 1.42% (DM basis). Consequently on average, animals were consuming 29 g/d
(or 0.27% of total DMI) of protein from yeast origin when HIGH DDGS treatment was fed and only 17% of those yeast cells resisted ruminal degradation and reached the duodenum. Bruning and Yokoyama (1988) suggested that, when treated with heat, yeast cells of *S. cerevisiae* are morphologically affected and may be more resistant to degradation. In addition, DNA from yeast has been reported to resist ruminal degradation by Castillo-Lopez et al. (2010). However, observations from the present trial suggest that when feeding DDGS the amount of yeast based protein resisting ruminal degradation is small and supplied only minimal amounts of RUP to the animal.

**CONCLUSIONS**

Byproducts of the ethanol industry, namely DDGS are important sources of energy and protein in ruminant diets. Thus, accurate estimates of RUP and MCP supply are important in the successful application of metabolizable protein systems. This study was designed to estimate DDGS RUP value and to evaluate the effect of DDGS on duodenal MCP flow when included in ruminant forage-based diets. The observed value of RUP contained in DDGS was higher compared to that of ground corn grain or dried rolled corn. This suggests that the heat applied to DDGS during ethanol production may decrease the degradability of CP of this byproduct by ruminal microorganisms increasing intestinal RUP supply. Duodenal flow of protozoa was not affected by DDGS inclusion. However, a small increase flow of duodenal yeast was observed, suggesting that a portion of yeast utilized in the fermentation process of ethanol production resists ruminal degradation. Furthermore, the inclusion of 19.5% of DDGS had no effect on total duodenal in flow of DM, OM and ruminal pH. Results from this experiment should
contribute to a more complete understanding of the effect of DDGS on rumen microflora, and ruminant nutrition.
Literature cited

Dennehv, J. J. 2001. Influence of social dominance rank on diet quality on pronghorn


Ørskov, E. R., and I. McDonald. 1979. The estimation of protein degradability in the


Table 4.1. Ingredients and analyzed chemical composition of CONTROL, LOW dried distillers grains with solubles (DDGS) and HIGH DDGS treatments.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>CONTROL</th>
<th>LOW DDGS</th>
<th>HIGH DDGS</th>
</tr>
</thead>
<tbody>
<tr>
<td>DDGS</td>
<td>0.0</td>
<td>9.75</td>
<td>19.5</td>
</tr>
<tr>
<td>Corn bran</td>
<td>19.5</td>
<td>9.75</td>
<td>0.0</td>
</tr>
<tr>
<td>Sorghum silage</td>
<td>20.0</td>
<td>20.0</td>
<td>20.0</td>
</tr>
<tr>
<td>Brome hay</td>
<td>60.0</td>
<td>60.0</td>
<td>60.0</td>
</tr>
<tr>
<td>Trace minerals</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
</tr>
<tr>
<td>Urea</td>
<td>0.25</td>
<td>0.125</td>
<td>0.0</td>
</tr>
<tr>
<td>DM</td>
<td>78.9</td>
<td>79.0</td>
<td>79.2</td>
</tr>
<tr>
<td>CP, % DM</td>
<td>10.6</td>
<td>11.8</td>
<td>13.0</td>
</tr>
<tr>
<td>NDF, % DM</td>
<td>66.2</td>
<td>62.9</td>
<td>59.6</td>
</tr>
<tr>
<td>Starch, % DM</td>
<td>5.7</td>
<td>4.6</td>
<td>3.5</td>
</tr>
<tr>
<td>Ether extract, % DM</td>
<td>1.7</td>
<td>2.6</td>
<td>3.6</td>
</tr>
<tr>
<td>Ash, % DM</td>
<td>7.2</td>
<td>7.6</td>
<td>7.9</td>
</tr>
</tbody>
</table>

1CONTROL: 0% (DM basis) DDGS, but with 19.5% corn bran, 20% sorghum silage, 60% brome hay, 0.5% trace minerals and 0.25% urea. LOW DDGS: 9.75% (DM basis) DDGS was included replacing equal percentage of corn bran. HIGH DDGS: 19.5% (DM basis) DDGS was included completely replacing corn bran.

2DDGS obtained from a Nebraska-based ethanol plant (Green Plains Renewable Energy, Central City, NE).
Table 4.2. Analyzed chemical composition (mean and standard deviation) of feedstuffs used in the formulation of CONTROL, LOW dried distillers grains with solubles (DDGS) and HIGH DDGS treatments.

<table>
<thead>
<tr>
<th>FEEDSTUFF</th>
<th>Component</th>
<th>DDGS ¹</th>
<th>Corn bran</th>
<th>Sorghum Silage</th>
<th>Brome hay</th>
</tr>
</thead>
<tbody>
<tr>
<td>DM</td>
<td></td>
<td>90.7 ± 1.93</td>
<td>89.0 ± 0.70</td>
<td>35.9 ± 0.52</td>
<td>90.5 ± 1.22</td>
</tr>
<tr>
<td>CP, % DM</td>
<td></td>
<td>27.8 ± 0.76</td>
<td>11.8 ± 0.30</td>
<td>7.2 ± 0.30</td>
<td>10.2 ± 0.73</td>
</tr>
<tr>
<td>NDF, % DM</td>
<td></td>
<td>31.5 ± 0.70</td>
<td>65.4 ± 0.99</td>
<td>54.9 ± 0.47</td>
<td>70.8 ± 1.74</td>
</tr>
<tr>
<td>Starch, % DM</td>
<td></td>
<td>5.2 ± 0.22</td>
<td>16.5 ± 0.91</td>
<td>4.2 ± 0.24</td>
<td>2.7 ± 0.57</td>
</tr>
<tr>
<td>Ether extract, % DM</td>
<td></td>
<td>11.7 ± 0.51</td>
<td>2.0 ± 0.15</td>
<td>1.5 ± 0.27</td>
<td>1.7 ± 0.18</td>
</tr>
<tr>
<td>Ash, % DM</td>
<td></td>
<td>4.3 ± 0.12</td>
<td>1.0 ± 0.09</td>
<td>8.2 ± 0.25</td>
<td>8.2 ± 0.50</td>
</tr>
<tr>
<td>YCP, % DM ²</td>
<td></td>
<td>1.4 ± 0.02</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

¹DDGS obtained from a Nebraska-based ethanol plant (Green Plains Renewable Energy, Central City, NE).

²YCP, yeast crude protein measured using DNA markers through real-time PCR.
Table 4.3. Dry matter intake, crude protein (CP) intake and ruminal pH for steers fed CONTROL, LOW dried distillers grains with solubles (DDGS) and HIGH DDGS treatments.

<table>
<thead>
<tr>
<th>Item</th>
<th>CONTROL</th>
<th>LOW DDGS</th>
<th>HIGH DDGS</th>
<th>SEM</th>
<th>Linear</th>
<th>Quadratic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intake, kg/d</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total DM</td>
<td>10.50</td>
<td>10.50</td>
<td>10.50</td>
<td>0.32</td>
<td>&lt; 0.01</td>
<td>0.82</td>
</tr>
<tr>
<td>CP</td>
<td>1.12</td>
<td>1.24</td>
<td>1.37</td>
<td>0.32</td>
<td>&lt; 0.01</td>
<td>0.82</td>
</tr>
<tr>
<td>Ruminal pH</td>
<td></td>
<td></td>
<td></td>
<td>0.12</td>
<td>0.86</td>
<td>0.78</td>
</tr>
<tr>
<td>Minimum</td>
<td>6.13</td>
<td>6.18</td>
<td>6.16</td>
<td>0.12</td>
<td>0.86</td>
<td>0.78</td>
</tr>
<tr>
<td>Maximum</td>
<td>6.62</td>
<td>6.61</td>
<td>6.66</td>
<td>0.07</td>
<td>0.65</td>
<td>0.76</td>
</tr>
<tr>
<td>Mean</td>
<td>6.41</td>
<td>6.37</td>
<td>6.42</td>
<td>0.09</td>
<td>0.88</td>
<td>0.66</td>
</tr>
<tr>
<td>Time</td>
<td></td>
<td></td>
<td></td>
<td>0.07</td>
<td>0.65</td>
<td>0.76</td>
</tr>
<tr>
<td>pH &lt; 6.4, min/d</td>
<td>493</td>
<td>520</td>
<td>688</td>
<td>362</td>
<td>0.68</td>
<td>0.86</td>
</tr>
<tr>
<td>Area</td>
<td>76</td>
<td>86</td>
<td>77</td>
<td>61</td>
<td>0.98</td>
<td>0.88</td>
</tr>
</tbody>
</table>

1DIET. CONTROL: 0% (DM basis) DDGS, but with 19.5% corn bran, 20% sorghum silage, 60% brome hay, 0.5% trace minerals and 0.25% urea. LOW DDGS: 9.75% (DM basis) DDGS was included replacing equal percentage of corn bran. HIGH DDGS: 19.5% (DM basis) DDGS was included completely replacing corn bran.
Table 4.4. Duodenal flow measurements of DM, organic matter (OM), ash, total crude protein (CP), microbial crude protein (MCP) and residual CP for steers fed CONTROL, LOW dried distillers grains with solubles (DDGS) and HIGH DDGS treatments.

<table>
<thead>
<tr>
<th>Item</th>
<th>CONTR</th>
<th>LOW DDGS</th>
<th>HIGH DDGS</th>
<th>SEM</th>
<th>Linear</th>
<th>Quadratic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duodenal flow, g/d</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total DM</td>
<td>5406</td>
<td>5305</td>
<td>5541</td>
<td>1025</td>
<td>0.87</td>
<td>0.84</td>
</tr>
<tr>
<td>OM</td>
<td>4681</td>
<td>4588</td>
<td>4805</td>
<td>871</td>
<td>0.83</td>
<td>0.78</td>
</tr>
<tr>
<td>Ash</td>
<td>725</td>
<td>717</td>
<td>736</td>
<td>157</td>
<td>0.91</td>
<td>0.85</td>
</tr>
<tr>
<td>Total CP</td>
<td>953</td>
<td>1030</td>
<td>1168</td>
<td>201</td>
<td>0.12</td>
<td>0.79</td>
</tr>
<tr>
<td>YCP²</td>
<td>0.15</td>
<td>1.94</td>
<td>4.80</td>
<td>0.66</td>
<td>&lt; 0.01</td>
<td>0.48</td>
</tr>
<tr>
<td>BCP²</td>
<td>473</td>
<td>393</td>
<td>357</td>
<td>78</td>
<td>0.09</td>
<td>0.73</td>
</tr>
<tr>
<td>Residual CP³</td>
<td>480</td>
<td>637</td>
<td>811</td>
<td>130</td>
<td>0.02</td>
<td>0.96</td>
</tr>
<tr>
<td>BCP, % of total CP</td>
<td>49</td>
<td>38</td>
<td>30</td>
<td>3.55</td>
<td>&lt; 0.01</td>
<td>0.74</td>
</tr>
<tr>
<td>MCP⁵</td>
<td>561</td>
<td>477</td>
<td>447</td>
<td>105</td>
<td>0.11</td>
<td>0.66</td>
</tr>
<tr>
<td>BCP</td>
<td>479</td>
<td>397</td>
<td>368</td>
<td>84</td>
<td>0.14</td>
<td>0.69</td>
</tr>
<tr>
<td>PCP</td>
<td>82</td>
<td>80</td>
<td>78</td>
<td>12</td>
<td>0.64</td>
<td>0.94</td>
</tr>
<tr>
<td>Residual CP⁶</td>
<td>392</td>
<td>553</td>
<td>721</td>
<td>99</td>
<td>&lt; 0.01</td>
<td>0.96</td>
</tr>
<tr>
<td>MCP, % of total CP</td>
<td>58</td>
<td>46</td>
<td>38</td>
<td>1.65</td>
<td>&lt; 0.01</td>
<td>0.07</td>
</tr>
</tbody>
</table>

¹DIET. CONTROL: 0% (DM basis) DDGS, but with 19.5% corn bran, 20% sorghum silage, 60% brome hay, 0.5% trace minerals and 0.25% urea. LOW DDGS: 9.75% (DM basis) DDGS was included replacing equal percentage of corn bran. HIGH DDGS: 19.5% (DM basis) DDGS was included completely replacing corn bran

²YCP, yeast crude protein

³BCP, bacterial crude protein estimated using DAPA as microbial marker

⁴Residual crude protein (CP) calculated as total CP minus BCP

⁵MCP, microbial crude protein estimated by using DNA markers and is the sum of BCP and protozoal crude protein (PCP)

⁶Residual CP calculated as total CP minus MCP
Figure 4.1. Estimates of duodenal bacterial crude protein (BCP) flow averaged across treatments using DAPA or DNA as bacterial markers for steers fed CONTROL, LOW dried distillers grains with solubles (DDGS) and HIGH DDGS treatments.

Marker type, $P = 0.71$. 
CHAPTER V

Effect of reduced fat dried distillers grains with solubles on lactation performance, rumen fermentation and duodenal flow of microbial nitrogen in Holstein cows

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ABSTRACT

Sixteen multiparous lactating Holstein cows were used in 2 experiments to evaluate the effect of reduced fat dried distillers grains with solubles (RFDG) on milk production, rumen parameters, duodenal microbial N flow and nutrient digestibility. In experiment 1, RFDG was fed at 0, 10, 20 and 30% of the diet dry matter (DM) to 12 non-cannulated Holstein cows (mean and SD, 89 ± 11 DIM and 674 ± 68.2 kg BW) to determine effects on milk production. In experiment 2, the same diets were fed to 4 ruminally and duodenally cannulated Holstein cows (mean and SD, 112 ± 41 DIM; 590 ± 61.14 kg BW) to evaluate the effect on rumen parameters, duodenal flow of microbial N and nutrient digestibility. In both experiments, cows were randomly assigned in 4 × 4 Latin squares over 21-d periods. Treatments (DM basis) were 1) CONTROL, 0% RFDG; 2) 10%
RFDG; 3) 20% RFDG and 4) 30% RFDG. Feed intake and milk yield was recorded daily. In experiment 1, milk samples were collected on d 19 to 21 of each period for analysis of milk components. In experiment 2, ruminal pH was measured; samples of rumen fluid, duodenal digesta and feces were collected on d 18 to 21. Microbial N was estimated with purines or DNA as microbial markers. Milk yield was not affected by treatment and averaged 34.0 ± 1.29 kg/d and 31.43 ± 2.81 kg/d in experiment 1 and 2, respectively. Percent of milk protein tended to increase in experiment 1; estimates were 3.08, 3.18, 3.15 and 3.19 ± 0.06% when RFDG increased from 0 to 30% in diets. However, milk protein was not affected in experiment 2 and averaged 3.02 ± 0.07%. Percent milk fat was not affected and averaged 3.66 ± 0.05% and 3.25 ± 0.14% in experiment 1 and 2, respectively. Total ruminal VFA and ammonia concentrations were not affected by treatment and averaged 135.18 ± 6.45 mM and 18.66 ± 2.32 mg/dL, respectively. Duodenal microbial N flow was not affected by treatment. However, purines yielded higher estimates compared to DNA markers. When averaged across treatments, duodenal flow of microbial N was 289 ± 20 and 221 ± 18 g N/d, for purines and DNA markers, respectively. Dry matter, organic matter, neutral detergent fiber and non-fiber carbohydrate digestibilities tended to increase with RFDG inclusion. Results of these experiments indicate that dairy rations can be formulated to include up to 30% RFDG while maintaining lactation performance, rumen parameters and rumen microbial growth.

Key words: distillers grains, lactation performance, duodenal microbial N
INTRODUCTION

Over the last decade the supply of corn milling by-products such as dried distillers grains with solubles (DDGS) has increased (Klopfenstein et al., 2008; Schingoethe et al., 2009). Over that time an extensive amount research has been conducted to evaluate the effect of this by-product on lactational performance of dairy cows. Some researchers have reported that DDGS can effectively maintain milk yield with no negative effects on milk composition. For example, Ranathunga et al. (2008) reported that, compared to a control diet, the inclusion of 21% DDGS in the ration had no negative effect on milk production or composition and tended to improve feed efficiency. Janicek et al. (2008) reported a linear increase in milk production when including from 0 to 30% DDGS in diets. Furthermore, Anderson et al. (2006) demonstrated that DDGS can replace a portion of ground corn and soybean meal and maintain or enhance lactational performance. However, it has also been suggested that the inclusion of DDGS in dairy rations may potentially affect milk fat concentration response (Hollmann et al., 2011). Rapid development in the ethanol industry has spurred the development of new feed by-products. For example, the oil may be partially removed and sold separately as corn oil with the remaining DDGS is reduced in fat content, resulting in a product commonly referred to as reduced-fat DDGS (RFDG) (Mjoun et al., 2010a).

The impact of DDGS on ruminal microbial crude protein (MCP) flow has been variable. Most studies evaluating the impact of DDGS on MCP have done so by using purine derivatives as a microbial marker and measuring the concentration of this marker in the urine. Kelzer et al. (2009) reported that MCP synthesis was similar for cows fed a control diet with no DDGS, a diet containing 15% DDGS or a diet containing 15% high
protein DDGS. Chibisa et al. (2012) demonstrated that wheat-DDGS can substitute for canola meal as the major protein source in dairy cow diets without negatively affecting MCP production. In addition, Janicek et al. (2008) reported that cows consuming 30% DDGS synthesized similar amount of MCP compared to a diet containing no DDGS. However, most of this research has been focused on production performance. While the use of purine derivatives represents a non-invasive method to predict microbial N flow (Harmon and Richards, 1997), one limitation of using this method is the limited accuracy of the equations for prediction purposes (Firkins et al., 2006). In the present study, microbial N flow was measured in two ways, by using purines or DNA as microbial markers. In addition, the contribution of yeast from RFDG was assessed. The objectives of these experiments were to evaluate the effects of increasing levels of RFDG on lactation performance, rumen parameters, duodenal flow of microbial N and nutrient digestibility, and secondly to compare the estimates of microbial N using purines or DNA as microbial markers. We hypothesize that RFDG can be included in dairy rations with no negative effect on lactation performance, rumen parameters, duodenal flow of microbial N and nutrient digestibility.

**MATERIALS AND METHODS**

This study was developed in 2 separate experiments. The first experiment was conducted to evaluate the effect of RFDG on lactation performance and was conducted at the Agriculture Research and Development Center of University of Nebraska-Lincoln (Mead, NE). The second experiment was conducted in the Dairy Metabolism Facility
located in the Animal Science Complex of University of Nebraska-Lincoln (Lincoln, NE). In this study the effects of RFDG on rumen parameters, duodenal flow of microbial N and nutrient digestibility were evaluated. All experimental procedures were approved and cows were cared for according to the guidelines stipulated by the University of Nebraska Institutional Animal Care and Use Committee.

**Experiment 1: Animals, experimental design and treatments**

Twelve lactating multiparous Holstein cows (mean and SD, 89 ± 11 DIM and 674 ± 68.2 kg BW) were blocked by parity, milk yield, and DIM and randomly assigned to treatment sequences in replicated 4 × 4 Latin squares according to the method of Kononoff and Hanford (2006). The first 14 d of each period were considered an adaptation period and the remaining 7 days were used for data collection. Cows were housed in individual tie stalls equipped with rubber mats and were offered 1 of 4 treatments that differed by the level of RFDG (Poet Nutrition, Sioux Falls, SD) inclusion; 1) CONTROL, 0% RFDG; 2) 10% RFDG; 3) 20% RFDG and 4) 30% RFDG. The chemical composition of treatments is based upon chemical composition of individual feed ingredient. Treatments were formulated with the CPM-Dairy model (version 3.0) to meet or exceed requirements as estimated by the CPM-Dairy model (Boston et al., 2000).

**Experiment 2: Animal surgeries, experimental design and sample collection**

The second experiment was conducted separately after completion of experiment 1. Prior to the initiation of this experiment, 4 lactating multiparous Holstein cows (mean and SD, 112 ± 41 DIM; 590 ± 61.14 kg BW) were fitted with a ruminal and a double L-shaped duodenal cannula (Bar diamond Inc., Parma, ID).
**Ruminal cannulations.** Ruminal cannulations were conducted according to the procedure described by Noordsy and Ames (2006). Briefly, cows were restrained in a head gate with restraint chute. The left paralumbar fossa was anesthetized with a paravertebral nerve block using a modification of the Farquharson technique (Turner and McllWraith, 1989). The dorsal and ventral branches of the Thoracic 13, Lumbar 1, and Lumbar 2 were blocked with 20 cc of 2% lidocaine hydrochloride (Agri Laboratories Ltd., St Joseph, MO) at each site to desensitize the paralumbar fossa.

After anesthesia was applied, a 17 cm linear skin incision was performed to place a 4 inch rumen cannula (Bar Diamond, Inc. Parma, ID) in the middle of the paralumbar fossa with a #20 scalpel. The external abdominal oblique, internal abdominal oblique and transversus muscles were incised parallel to their muscle fibers with sharp or blunt dissections. The peritoneum was entered by blunt dissection and then the peritoneal opening was extended to the size of the skin opening by sharp dissection. The rumen serosa was secured to the underside of the skin with interrupted horizontal mattress sutures. The rumen was resected with a 1 cm margin from the suture line; cannula was inserted and closed with cap. Then, cows were returned to their normal diet and closely monitored during the following 10 days post-surgery.

**Duodenal cannulations.** Duodenal cannulations were performed according the procedures described by Robinson et al. (1985) and Titgemeyer (1997). Briefly, right paralumbar fossa was anesthetized following the same method used for ruminal cannulation. Recumbent anesthesia and sedation was induced (Anderson and Rings, 2009).
Upon becoming recumbent, cow was positioned in left lateral recumbency with legs restrained forward and back. A 15 cm skin incision was made in the cranial paralumbar fossa parallel to the last rib with a #20 scalpel. The external abdominal oblique, internal abdominal oblique and transversus muscles and peritoneum were incised by sharp dissection. Upon entry to the abdomen, the pylorus and proximal 20 cm of the cranial part of the duodenum were exteriorized and separated from the remainder of the incision by moist towels. Site of cannula insertion was centered approximately 10 cm distal to the pylorus. The omentum was elevated from the duodenum for a distance corresponding to the length of the cannula barrel. Care was taken to preserve major branches of duodenal vessels and, thus, to minimize effects of cannulation on blood flow to the area. A 5 to 6-cm enterotomy was made along the right side of the duodenum in the area previously freed of omentum. The body of the double L-shaped cannula (Bar Diamond, Inc, Parma ID) was inserted into the intestinal lumen. The enterotomy was closed adjacent to the barrel of the cannula with 2-0 polydioxanone in a simple interrupted pattern. The cut edges of the enterotomy were inverted, and two purse string sutures of the same type of suture material were placed snugly around the cannula barrel. In preparation for the cannula exteriorization, a 2 cm diameter of skin was removed from the right body wall in the 10th intercostal space at the level of the frontal plane of the shoulder joint. The cannula barrel was pushed through the body wall at this site and the outer collar was inserted to hold the cannula in place. The peritoneum and transversus were apposed in a simple continuous pattern with #3 chromic gut on a ½ circle taper needle. The internal and external abdominal muscles were apposed as a single layer in a simple continuous pattern with #3 gut on a ½ circle taper needle. The skin edges were
apposed in a Ford continuous interlocking pattern with # 3 Braunamid with a “S” curved needle.

Cows were hospitalized and closely monitored until they recovered from the surgical procedure. Body temperature was taken once every day during the following 10 d post-surgery. A single dose of 25 cc of Excenell or Exceed (Pfizer Inc., New York City, NY) was administered, when body temperature exceeded normal range.

These ruminally and duodenally cannulated cows were assigned to a single Latin square and randomly assigned to the experimental treatments. Data on DMI and lactation performance were collected from these cows. In addition, rumen parameters, duodenal microbial N flow and total tract digestibility of major nutrients were measured. Cows were housed in individual tie stalls having dimensions of 164 cm × 111 cm that were equipped with rubber mats. Rations were formulated to contain the same level of RFDG included in experiment 1.

Chromic oxide (Cr$_2$O$_3$) was used as a digesta marker for the estimation of duodenal flow (Hutton et al., 1971; Harvatine et al., 2002; Sylvester et al., 2005). Seven and a half grams of Cr$_2$O$_3$ was weighed (Taylor and Allen, 2005) and placed in gelatin capsules (Torpac Inc., Fairfield, NJ). Then capsules were dosed into the rumen via the ruminal cannula twice daily at 0700 and 1900 during d 10 through 21 of each experimental period to provide a marker to estimate duodenal digesta flow.

**Ruminal pH.** Ruminal pH was measured according to the protocol described by Rolfe et al. (2009). Briefly, during d 18 through 21 of each period ruminal pH was measured once every minute continuously using a wireless pH probe (Dascor Inc.,
Escondido, CA) placed into the ventral sac of the rumen of each cow. Each probe contained a data logger, 9-volt battery, and an electrode cable housed in a watertight capsule constructed out of PVC material. Each pH electrode was enclosed in a PVC cover. Two 900-g weights were fastened to the bottom of the probe to maintain the electrode in the ventral sac of the rumen. These pH probes were calibrated in pH buffers 4 and 7 and recorded before inserting them into the rumen and after downloading pH data collected into calculation spread sheets at the end of each period. The initialization of the data logger and the transfer of data were performed by a Microsoft Windows-compatible software package supplied by Dascor (M1b version 6.1.2h). Ruminal pH measurements were averaged across the 4 collection days so that a period of 24 h beginning at 0930 is represented. This starting time point was chosen given that TMR were offered at 0930 after cows returned from a sand surfaces exercise pen, where they were temporarily transferred during stall cleaning and TMR mixing. From the averaged pH data, minimum, maximum and mean ruminal pH were calculated for each cow. The time and area below pH 6.5 and 6.3 are presented as well.

**Ruminal fluid and duodenal digesta sampling.** Ruminal fluid and duodenal digesta were collected on d 18 through 21 every 4 h. Whole rumen contents were collected from 4 different regions of the rumen (caudal ventral sac, cranial ventral sac and two samples from the feed mat in the dorsal rumen) and composited. Rumen contents were then strained through 4 layers of cheesecloth; 40 mL of strained rumen fluid was placed in a 45 mL vial and immediately frozen at -20 °C for later analysis for ruminal VFA and Ammonia. Duodenal digesta contents (200 mL) were collected and placed in 250-mL Nalgene bottles (Thermo Scientific Inc., Waltham, MA). Then samples were
composited by cow, by day within period and frozen at -20°C for subsequent analyses of duodenal flow of microbial N. Collection time of ruminal fluid and duodenal digesta was advanced one h in subsequent collection day, so that every 60-minute interval in a 24-h period was represented (6 samples per cow per d and a total of 24 samples per cow per period). Specifically, samples were collected on d 18 at 0700, 1100, 1500, 1900, 2300 and 0300; d 19 at 0800, 1200, 1600, 2000, 0000 and 0400; d 20 at 0900, 1300, 1700, 2100, 0100 and 0500; and d 21 at 1000, 1400, 1800, 2200, 0200 and 0600.

**Ruminal bacteria, ruminal protozoa and yeast sample.** On d 20 and 21 of each experimental period, 1.5 L of whole ruminal digesta contents were collected from 4 different locations within the rumen at 1000 and 1600 (d 20) and 1200 and 1800 (d 21). Samples were comprised of equal volumes of rumen contents taken from the caudal ventral sac, cranial ventral sac and two samples from the feed mat in the dorsal rumen. Then mixed ruminal bacteria were isolated according to the procedure described by Hristov et al. (2005). Briefly, whole ruminal contents were composited and squeezed through 2 layers of cheesecloth and the filtrate was retained. Solids remaining on the cheesecloth were added to a volume of cold buffer (McDougall, 1948) equal to the volume of filtrate, and shaken manually in a screw-capped jar to dislodge the ruminal microorganisms loosely associated with feed particles. This suspension was then squeezed through 2 layers of cheesecloth and the 2 filtrates were combined (1:1) and preserved with 5% (vol/vol) formalin. From this sample, bacteria were harvested via differential centrifugation (Hristov and Broderick, 1996) with an initial low-speed centrifugation at 400 × g for 5 min at 4 °C and a subsequent high-speed centrifugation at 20,000 × g for 15 min at 4 °C. Samples were maintained on ice while being processed.
Then, supernatant was discarded and the isolated bacterial pellets were composited by steer and period and frozen at −20 °C for later analysis. Rumen protozoa were isolated using a separation funnel according to the procedure described by Shabi et al. (2000). Briefly, the strained ruminal digesta was mixed with one volume of warm 0.9% saline and held in a separation funnel for 1.5 h at 39 °C. The precipitate was then removed. The protozoal pellet was mixed with 500 mL of warm saline and kept in a separation funnel for another 1.5 h at 39 °C. Protozoa were then collected and frozen at -20 °C. A sample of dried yeast cells (*Saccharomyces cerevisiae*) utilized in the ethanol production process was obtained and frozen at -20 °C until analysis.

**Fecal samples.** Fecal grab samples (approximately 250 g) were taken every 8 h on d 18 to 21 of each period (3 samples per cow per day and a total of 12 samples per cow per period). Samples were then composited by cow, by day, within period. Then, samples were stored at -20 °C for later analysis of Cr₂O₃ and chemical composition.

**Animal care and measurements, milk collection and feed sampling**

In experiment 1, cows were milked using a double 5 Herringbone parlor (Bowmatic LLC, Madison, WI) at 0730 and 1930 h, milk production was recorded and milk samples were collected during the AM and PM milkings of d 19, 20 and 21. In experiment 2, cows were milked at 0700 and 1800 using individual DELPRO™ 480 DeLaval portable milking units through a pipeline system (DeLaval International AB, Tumba, Sweden). Production was recorded and milk samples were taken on the AM and PM milkings on d 18, 19, 20 and 21. Milk samples were stored at room temperature in a vial containing a micro-tab (2-bromo-2-nitropropane-1,3 diol,) provided by the Dairy
Herd Improvement Association (DHIA) (Manhattan, KS). Samples were then analyzed for fat, milk urea nitrogen (MUN), lactose and true protein (AOAC, 2000) using a B2000 Infrared Analyzer (Bentley Instruments, Chaska, MN) by Heart of America DHIA (Manhattan, KS). During the last week of each period, daily DMI and milk yield were averaged.

In both experiments, cows were individually fed once daily at 0930 to allow for approximately 5% refusals using a Calan Data Ranger (American Calan Inc., Northwood, NH). Cows were allowed access to feed at all times except during milking in experiment 1. In experiment 1, forages were premixed using a 354-12B mixer wagon (Roto-Mix LLC, Dodge City, Kansas). The concentrate mix (control mix, RFDG-added mix or a portion of both) was added to the Calan Data Ranger after addition of the premixed forages. Feed intake for individual cows was measured daily. Water was available for *ad libitum* consumption. Samples of corn silage, alfalfa haylage, alfalfa hay, brome hay, cotton seed, each concentrate mix, RFDG, and each TMR were collected on d 20 and 21 of each experimental period and stored at −20 °C until analysis. Dry matter concentrations of corn silage and alfalfa haylage were determined weekly by heating samples at 100 °C for 24 h. Using resulting DM data, diets were then adjusted to ensure proper inclusion of components.

In experiment 1, BW and Body Condition Score (BCS) (1 to 5 scale) were measured on d 20 and d 21 of each period. Body condition score was measured by a single, trained individual, and the scoring method used was similar to that of Wildman et al. (1982), but reported to the quarter point. In experiment 2, BCS of cows was not recorded. Body weight of cows was recorded only at the initiation of the trial; however
BW was not recorded during the experiment due to difficulties experienced when handling cows through the scale chute. Specifically, scale chute was too narrow to handle the duodenally cannulated cows and there was a potential risk that the cannula would be disrupted when animals go through.

**Feed and sample analysis**

**Feed particle size and feed chemical analysis.** The Penn State Particle Separator was used to measure the distribution of particle size for TMR according to Heinrichs and Kononoff (2002). Collected feed ingredients and TMR samples were dried for 48 h at 60 °C in a forced air oven, ground to pass through a 1-mm screen (Wiley Mill, Arthur A. Thomas Co., Philadelphia, PA) and analyzed for chemical composition by an external laboratory (Cumberland Valley Analytical Services, Hagerstown, MD), which included DM (AOAC, 2000), N (Leco FP-528 Nitrogen Combustion Analyzer, Leco corp. St. Joseph, MI), NDF (Van Soest et al., 1991), starch (Hall, 2009), ether extract (AOAC, 2006) and ash (AOAC, 2000). In addition, the concentration of yeast (S. cerevisiae) protein was measured from subsamples of collected RFDG. This was conducted by using DNA markers as described by Castillo-Lopez et al. (2010).

**Ruminal VFA.** At the end of experiment 2, 10 samples of rumen fluid collected from each cow in each period (over the course of the 4 d and representing a 24 h period) were analyzed for VFA by an external laboratory (Rumen Fermentation Profile Laboratory, West Virginia University, Morgantown, WV). Specifically, rumen samples analyzed for VFA were those collected at 1000, 1100, 1200, 1400, 1600, 1800, 2100, 0000, 0400 and 0900. Briefly, analysis of VFA concentrations in effluents was performed
according to the gas chromatographic separation procedure (Anonymous, 1975). The gas chromatograph was a Varian model 3300 with an FID detector (Varian, Inc., Palo Alto, CA). The column was a 2-m2-mm glass column packed with 10% SP-1200/1% H3HPO4 on 80/100 chromosorb WAW (Supelco, Inc. Bellefonte, PA).

**Ruminal ammonia.** A portion of the 10 samples of rumen fluid analyzed for VFA were also analyzed for ammonia-N concentration using a phenol-hypochlorite assay (Yang and Varga, 1989). Briefly, 1.5 mL of rumen fluid was centrifuged at 11,000 × g and then 40 µL of supernatant was taken in duplicate and combined with 2.5 and 2.0 mL of phenol color reagent and alkaline hypochlorite reagent, respectively. After incubation for 10 minutes at 37 °C, 300 µL of sample was taken and placed in a 96-well polystyrene plate (Becton Dickinson and Company, Franklin Lakes, NJ), then absorbance was read at 550 nanometers. Analysis was repeated whenever the coefficient of variation between duplicates exceeded 4.9%.

**Duodenal flow of microbial N using purines as microbial marker.** Collected duodenal contents were lyophilized and ground to pass through a 1-mm screen using a Wiley Mill (Arthur H. Thomas, Philadelphia, PA). Then, ground samples were analyzed for DM (100 °C oven for 24 h). Subsamples of isolated ruminal bacterial pellets were ground with a mortar and pestle and analyzed for N (Leco FP-528 Nitrogen Combustion Analyzer, Leco corp. St. Joseph, MI).

Purines (Zinn and Owens, 1986; Broderick and Merchen, 1992; Obispo and Dehority, 1999; Reynal and Broderick, 2009) were used as a microbial marker to measure duodenal flow of total microbial N. The analysis of purines was conducted
according to the procedure described by Makkar and Becker (1999). Briefly, approximately 50 mg of lyophilized microbial and duodenal samples were placed in Pyrex crew cap tubes. Then samples were combined with 0.5 mL of allopurinol internal standard (3 mM allopurinol) and 2.5 mL 0.6 M HClO₄, then incubated for 1 h. After samples cooled down, they were combined with 7.5 mL of 10 mM NH₄H₂PO₄, then pH was adjusted between 6.6 and 6.9 using concentrated KOH (8 M). Samples were centrifuged at 3,000 × g and supernatant was filtered through a 13 mm disposable syringe filter (0.45 µm pore size) with GHP membrane (Whatman Inc., Florham Park, NJ) into HPLC vial. Total purines were measured using a 717 HPLC system (Waters Corporation Inc., Milford, MA). Calculation of microbial N was based on the ratio purine:N obtained from the isolated rumen bacterial pellet and on the concentration of purines in duodenal samples.

**Duodenal flow of microbial N using DNA as microbial markers.** In this study the sum of bacterial and protozoal N was considered as microbial N. Separate estimates of bacterial N and protozoal N were measured based on 1) the ratio DNA marker:N in the isolated bacterial or protozoal pellet and 2) the abundance of the targeted bacterial or protozoal DNA marker measured from duodenal samples. From those values the amount of N originating from bacteria and protozoa was calculated. To do so, subsamples of isolated bacterial and protozoal pellets were analyzed for DM (AOAC, 2000) and N (Leco FP-528 Nitrogen Combustion Analyzer, Leco corp. St. Joseph, MI). Then, DNA was extracted from the remaining portion of the pellets and from digesta samples according to the extraction method for PCR-quality DNA from digesta samples described by Yu and Morrison (2004). The concentration of DNA (ng/µL) in each sample was
measured by spectrophotometry (NanoDrop ND-1000 Spectrophotometer, NanoDrop Technologies, Inc. Wilmington, DE) and stored at -20°C in aliquots of 25 µL for later analysis using real-time PCR.

Bacterial, protozoal and yeast DNA markers utilized as wells as real-time PCR reactions and conditions were conducted as described by Castillo-Lopez et al. (in press). Briefly, for each microbial type, 4 µL of DNA sample were combined with 1 µL of 10 µM forward primer, 1 µL of 10 µM reverse primer, 0.25 µL of 10 µM TaqMan probe, 7.5 µL of TaqMan Master Mix (Applied Biosystems, Foster City, CA, USA) and 1.25 µL of nanopure water. Two samples with no DNA were included and used as non-template controls. Each sample was run in duplicate in separate wells of the 384-well real-time PCR plate.

**Duodenal digesta flow and total tract digestibility measurements.** Collected fecal samples were composited by cow, by day, within period. Samples were oven dried and ground to pass through a 1-mm screen (Wiley Mill, Arthur A. Thomas Co., Philadelphia, PA). Dried fecal samples were analyzed by an external laboratory for chemical composition (Cumberland Valley Analytical Services, Hagerstown, MD). Duodenal and fecal samples were analyzed for Cr₂O₃ by an external laboratory (Servi-Tech Laboratories, Hastings, NE) by sample digestion in 10 mL nitric acid and 3 mL peroxide, with a hydrochloric acid addition and analyzed by inductive coupled plasma using a Varian 720-ES spectrophotometer (Varian, Inc., Palo alto, CA. The average amount of Cr₂O₃ dosed daily was divided by the concentration of Cr₂O₃ in the duodenal or fecal samples to determine daily flow of DM to the duodenum and rectum (Harvatine et al., 2002). Apparent total tract digestion of DM, OM, NFC, NDF, N and P were
calculated as follows: 

\[ \frac{\text{intake of nutrient} - \text{fecal output of nutrient}}{\text{intake of nutrient}} \times 100 \] 

(May et al., 2010).

**Experiment 1: Statistical analysis**

Performance data were analyzed as a replicated 4 × 4 Latin square using the MIXED procedures of SAS (Version 9.1, SAS Institute Inc., Cary, NC). Fixed model effects included square, period within square, and treatment; and the random effect was cow within square. Linear and quadratic effects of treatments were tested. The model for this experiment is written as follows:

\[ Y_{ijkm} = \mu + \tau_m + \beta(\tau)_im + \rho(\tau)_jm + \alpha_k + \varepsilon_{ijkm} \]

where \( Y_{ijkm} \) represents observation \( ijkm \); \( \mu \) represents the overall mean; \( \tau_m \) represents the fixed effect of square \( m \); \( \beta(\tau)_im \) represents the random effect of cow \( i \) within square \( m \); \( \rho(\tau)_jm \) represents the fixed effect of period \( j \) within square \( m \); and \( \alpha_k \) represents the fixed effect of treatment \( k \). The residual term \( \varepsilon_{ijkm} \) was assumed to be normally, independently, and identically distributed, with variance \( \sigma^2 \varepsilon \).

**Experiment 2: Statistical analysis**

Data collected on lactation performance, rumen pH and duodenal flow were analyzed as a 4 × 4 Latin square using the MIXED procedure of SAS (Version 9.1; SAS Institute, Inc., Cary, NC). Fixed model effects included treatment and period with cow as the random effect. The model for this experiment is written as follows:

\[ Y_{ijk} = \mu + \beta_i + \rho_j + \alpha_k + \varepsilon_{ijk} \]
where $Y_{ijk}$ represents observation $ijk$; $\mu$ represents the overall mean; $\beta_i$ represents the random effect of cow $i$; $\rho_j$ represents the fixed effect of period $j$; and $\alpha_k$ represents the fixed effect of treatment $k$. The residual term $e_{ijk}$ was assumed to be normally, independently, and identically distributed, with variance $\sigma^2_e$.

For experiment 2, repeated measurements of rumen ammonia and VFA concentration were analyzed by including a REPEATED model statement, linear and quadratic effects of treatments were also tested. Treatment means are presented as least square means and the largest standard error of the mean (SEM) is reported. Statistical significance was declared at $P < 0.05$ and tendency was declared if $P > 0.05$ and $\leq 0.15$.

**RESULTS**

**Feed chemical composition and particle size of diets**

The ingredient composition of experimental treatments fed in experiment 1 and 2 is listed in Table 5.1. The chemical composition of those treatments is listed in Table 5.2. Table 5.3 lists the chemical composition of feed ingredients utilized in the formulation of treatments. Rations contained similar levels of CP and fat. However, the level of NDF increased with the inclusion of RFDG in the diets. In contrast, the percent of starch decreased with increasing levels of RFDG in treatments. The percent of CP, NDF and fat in the RFDG averaged 31.95, 34.85 and 5.42%, respectively. The particle size distribution of dietary treatments fed in both experiments is listed in Table 5.4. In experiment 1, a linear decrease ($P < 0.01$) of TMR particle size was observed with the inclusion of RFDG. Specifically, the proportion of particles greater than 19.0 mm
decreased from 9.38 to 6.75%, the proportion of particles between 19.0 and 8.0 mm decreased from 25.58 to 19.89 %, the proportion of particles between 8.0 and 1.18 mm decreased from 37.86 to 30.20%. However, the proportion of particles smaller than 1.18 mm increased from 27.16 to 43.14%. In experiment 2, a similar pattern in TMR particle size distribution was observed. There was a linear decrease ($P < 0.05$) of TMR particle size with the inclusion of RFDG. Specifically, the proportion of particles greater than 19.0 mm decreased from 6.50 to 3.64%, the proportion of particles between 19.0 and 8.0 mm decreased from 26.20 to 18.01%, the proportion of particles between 8.0 and 1.18 mm decreased from 39.10 to 32.06%. However, the proportion of particles smaller than 1.18 mm increased from 27.51 to 46.35% when the inclusion of RFDG increased from 0 to 30%.

**DMI, BW, BCS and lactation performance**

Data collected in experiment 1 on DMI, BW, BCS and lactation performance are listed in Table 5.5. Table 5.6 lists DMI and lactation performance data collected in experiment 2. In experiment 1, total DMI increased linearly ($P < 0.01$) with the inclusion of RFDG. Total DMI were 25.0, 23.8, 25.9 and 27.9 ± 1.38 kg/d for CONTROL, 10, 20 and 30% RFDG, respectively. In this study, an increase in BW ($P < 0.01$) and BCS ($P = 0.05$) was also observed with the inclusion of RFDG in diets. Average BW was 687.2, 687.8, 693.4 and 696.7 ± 18.4 kg; and BCS was 3.06, 3.10, 3.14 and 3.18 ± 0.06 for CONTROL, 10, 20 and 30% RFDG, respectively. In experiment 2, total DMI was not affected ($P = 0.73$) by the inclusion of RFDG, which averaged 21.56 ± 1.65 kg/d across treatments.
In experiment 1, milk yield was not affected \((P = 0.78)\) by increasing levels of RFDG and averaged \(34.1 \pm 1.29\) kg/d across treatments. The percent of milk protein tended \((P = 0.07)\) to increase with RFDG inclusion with estimates of 3.08, 3.18, 3.15, and 3.19 ± 0.06% for CONTROL, 10, 20 and 30% RFDG treatments, respectively. The yield of milk protein, however, was similar \((P = 0.23)\) across treatments with an average of 1.05 ± 0.04 kg/d. The percent \((P = 0.66)\) and the yield \((P = 0.53)\) of milk fat were not affected by treatment and averaged 3.66 ± 0.09% and 1.24 ± 0.05 kg/d across treatments, respectively. Milk urea N was not affected \((P = 0.85)\) by the inclusion of RFDG in diets and averaged 15.98 ± 0.86 mg/dL cross treatments. In experiment 2, one cow was removed because of ruminal acidosis and the presence of excessive levels of foam in the rumen. For this reason one extra period was added to the trial (a total of 5 periods). Statistical differences for lactational performance were not expected, the primary reason for conducting experiment 2 was the study of rumen fermentation, microbial N flow to the duodenum and major nutrient digestibility. In experiment 2, milk yield was not affected \((P = 0.65)\) by increasing levels of RFDG and averaged 31.43 ± 2.81 kg/d across treatments. The percent \((P = 0.35)\) and yield \((P = 0.84)\) of milk protein were not affected by RFDG and averaged 3.02 ± 0.07% and 0.95 ± 0.07 kg/d, respectively. In addition, the percent \((P = 0.28)\) and yield \((P = 0.23)\) of milk fat were not affected by RFDG inclusion and averaged 3.25 ± 0.14% and 1.02 ± 0.11 kg/d across treatments, respectively. Milk urea N tended to increase \((P = 0.14)\) when including RFDG in diets with estimates of 12.64, 13.00, 13.07 and 13.42 ± 0.93 mg/dL.
Rumen VFA and ammonia

Table 5.7 lists the concentration of VFA in the rumen. There was no significant effect ($P = 0.40$) of treatment on total rumen VFA concentration with an average of $135.18 \pm 6.45$ mM across treatments. The molar proportion of acetate decreased ($P < 0.01$) and the molar proportion of propionate increased ($P < 0.01$) with the inclusion of RFDG in diets. There was also a significant ($P < 0.01$) increase of the molar proportions of isobutyrate and valerate. The molar proportion of butyrate tended to decrease ($P = 0.07$). However, no significant change was observed in the molar proportion of isovalerate. The acetate to propionate ratio in the rumen decreased with RFDG inclusion with values of 2.98, 2.94, 2.52 and 2.39 $\pm$ 0.23 for CONTROL, 10, 20 and 30% RFDG, respectively. The concentration of ruminal ammonia (Table 5.7) was similar ($P = 0.18$) with an average of $18.66 \pm 2.32$ mg/dL across treatments.

Ruminal pH

Figure 1 illustrates ruminal pH measurements collected and averaged over the course of 4 d. In addition, Table 5.7 lists minimum, maximum and mean ruminal pH values. A decrease ($P = 0.02$) in mean ruminal pH was observed with DDGS inclusion, with estimates of 6.53, 6.49, 6.38 and 6.35 $\pm$ 0.12 for CONTROL, 10, 20 and 30% RFDG, respectively. In addition, the time ($P < 0.01$) and area ($P = 0.03$) below pH 6.5 increased, estimates of time below pH 6.5 were 546, 834, 941 and 1040 $\pm$ 293 min/d; estimates of area below pH 6.5 were 126, 158, 357 and 334 $\pm$ 92 pH $\times$ min/d, for CONTROL, 10, 20 and 30% RFDG, respectively. Furthermore, the time below pH 6.3 increased ($P = 0.03$), estimates were 279, 382, 936 and 946 $\pm$ 208 min/d, respectively.
The area below pH 6.3 tended to increase \((P = 0.13)\) with estimates of 45, 47, 180 and 169 ± 64 pH × min/d, respectively.

**Apparent nutrient digestibility of TMR**

Major nutrient digestibilities of TMR are listed in Table 5.8. Dry matter digestibility tended to increase \((P = 0.14)\), estimates were 65.5, 65.4, 73.0 and 73.4 ± 4.8% for CONTROL, 10, 20 and 30% RFDG, respectively. Similarly, OM digestibility tended to increase \((P = 0.14)\) with DDGS inclusion, estimates were 67.7, 67.7, 74.9 and 75.2 ± 4.4% respectively. Likewise, NFC digestibility tended \((P = 0.07)\) to increase, estimates were 89.7, 90.1, 92.6 and 92.7 ± 1.2%, respectively. The digestibility of NDF tended to increase \((P = 0.11)\), estimates were 44.0, 43.2, 57.0 and 58.0 ± 7.5%, respectively. In addition, N digestibility tended to increase \((P = 0.18)\), estimates were 64.3, 67.7, 74.7 and 76.9 ± 5.1%, respectively. Table 5.8 also lists the output of N and P of cows fed increasing levels of RFDG. The excretion of N tended to decrease \((P = 0.09)\) with estimates of 0.202, 0.197, 0.152 and 0.146 ± 0.02 kg N/d for CONTROL, 10, 20 and 30% RFDG, respectively. Excretion of P was not affected \((P = 0.86)\) and averaged 51.29 ± 8.29 g of P/d.

**Duodenal microbial N**

As listed in Table 5.9, duodenal microbial N flow was not affected by treatment when estimated using purines \((P = 0.57)\) or DNA \((P = 0.45)\) markers. When averaged across treatments, duodenal microbial N flow was 289 ± 20 and 221 ± 18 g N/d, for purines and DNA markers, respectively. However, purines yielded higher \((P < 0.01)\) microbial N flow compared to DNA markers. Duodenal protozoal N flow was not
affected ($P = 0.77$) by treatment and averaged $38 \pm 7$ g N/d. Duodenal yeast N flow, however, increased ($P = 0.02$) with RFDG inclusion, estimates were 0.34, 0.987, 1.329, 1.836 $\pm$ 0.22 g N/d for CONTROL, 10, 20 and 30% RFDG, respectively.

**DISCUSSION**

**Lactation performance and DMI**

One of the main objectives of this study was to evaluate the effect of increasing levels of RFDG on lactation performance. Previous research has demonstrated that DDGS can be effectively included in dairy rations with no negative effects on milk yield and milk components (Mjoun et al., 2010a; Mjoun et al., 2010b; Janicek et al., 2008; Nichols et al., 1998). Results obtained in this study agree with data reported by these researchers. The inclusion of RFDG in rations of lactating dairy cows had no negative effect on milk yield and tended to increase the percent of milk protein. In spite of these results, potential challenges related to milk fat depression have been suggested when including DDGS in dairy rations (Hollmann et al., 2011; Bauman and Griinari, 2003). Milk fat depression may be caused by diets containing large concentrations of unsaturated fatty acids, which are metabolized generating intermediates like the trans-10 cis-12 C$_{18:2}$ (Baumann and Griinari, 2003). In this study, the low level (5.53%) of fat in the RFDG used in the formulation of the diets lowered the risk of milk fat depression.

The increase in DMI observed in experiment 1 may reflect greater palatability of diets containing RFDG compared to the CONTROL and may also be a consequence of the reduction in TMR particle size with the RFDG inclusion. The decrease in TMR
particle size allowed more feed to be consumed before reaching rumen fill (Allen, 2000). In addition, based on the CPM dairy model predictions, the concentration of metabolizable energy of diets decreased from 2.53 to 2.47 Mcal/kg when RFDG increased from 0 to 30% possibly because of the reduction in the level of non-fiber carbohydrates in the rations. Therefore, the increase in DMI may have been a response to compensate for the lower metabolizable energy ration being consumed maintaining the level of total nutrient intake. The demand for nutrients, particularly glucose, increases during lactation (Wiltrout and Satter, 1971; Akers, 2002). Most of the glucose that dairy cows need to meet their requirements is originated from hepatic gluconeogenesis from propionate (Nafikov and Beitz, 2007). The availability of glucose to the mammary gland has an important impact on milk yield because lactose is the major osmoregulator in mammary uptake of water (Lemosquet et al., 2004). In this study, the increase in the proportion of ruminal propionate supported glucose and lactose synthesis, which may have contributed in maintaining milk yield through osmoregulation.

**VFA and rumen pH**

The relative concentration of individual ruminal VFA can also affect milk composition (Thomas and Martin, 1988). Specifically, increases in supply of acetic acid are associated with increased milk yield and milk fat concentration (Firkins, et al., 2006). In the present study, however, the decrease in the concentration of ruminal acetate and butyrate was not severe enough to have a negative impact on the percent and yield of milk fat. In addition, the type of VFA produced plays an important role in energy utilization by the dairy cow. In the rumen, fermentation to propionate conserves more energy than fermentation to either acetate or butyrate. Therefore, increasing the amount
of energy available to the cows per unit of feed consumed (Van Maanen et al., 1978). The higher concentration of ruminal propionate observed in cows consuming RFDG in this reflects an efficient production of energy when feeding RFDG. The type of carbohydrate digested by ruminants is a decisive factor in determining the ratios of the resultant ruminal VFA. Specifically, increasing the concentration of starch in diets typically yield more propionate (Evans et al., 1975). In the current study, the inclusion of RFDG decreased the percent of starch in TMR. In spite of such change, the molar proportion of ruminal propionate increased with RFDG inclusion, which agrees with reports of Zhan et al. (2010), but contrasts the observations of Anderson et al. (2006) and Ranathunga et al. (2010). This suggests that diets containing RFDG can support or enhance ruminal propionate production.

The reduction of TMR particle size with RFDG inclusion likely decreased the time cows spent chewing producing less saliva needed to buffer pH in the rumen. In addition, the highly fermentable fiber provided by this by-product (Rolfe et al., 2011) may have contributed to the decrease in rumen pH. Overall, measurements indicate that ruminal pH was highest just before feeding and declined for approximately 8 to 9 h thereafter before gradually increasing and return to similar values prior to the next feeding. During this time, gradual decrease in acid production and increased VFA absorption (Whitelaw et al., 1970), combined with salivary buffer production during rumination (Maekawa et al., 2002), returned pH to near its previous value before feeding (Palmonary et al., 2010).

In dairy rations, adequate forage particle length is necessary for proper rumen function (Yang and Beauchemin, 2009). The reduction in TMR particle size may
represent risk for the development of ruminal acidosis (Pereira et al., 1999). In the present study, the observed values of particle size distribution differed from the guidelines for TMR for high producing dairy cows, where the recommendations are 2 to 8% of particles greater than 19.0 mm, 30 to 50% of particles between 19.0 and 8.0 mm or between 8.0 and 1.18 mm and no more than 20% of particles smaller than 1.18 mm (Heinrichs and Kononoff, 2002). The greatest difference in particle size distribution between our treatments and the suggested guidelines was observed with the 30% RFDG TMR. In this treatment, the proportion of particles smaller than 1.18 mm in experiment 1 and 2 averaged 44.8%, this is considerably higher than the established guidelines suggesting that this proportion should not be higher than 20%. However, it is important to point out that these guidelines were established without taking into consideration the inclusion of distillers grains in ration for lactating cows. This experiment indicates that even in spite of the reduction in particle size of diets, no negative effects on rumen fermentation and nutrient digestibility was observed.

**Nutrient digestibility and duodenal microbial N flow**

In this study, an increase in major nutrient digestibility was observed. The NDF from DDGS is highly digestible (Getachew et al., 2004), we suggest that the low fat levels of the RFDG improved NDF digestion because fat usually inhibits microbial digestion of fiber (Ueda et al., 2003). In addition, smaller particle size of RFDG increased the surface area available for microbial attachment, enhancing digestibility of major nutrients (Kitessa et al., 1999) allowing for increased DMI (Oba and Allen, 1999). In contrast to our expectations, the enhanced nutrient digestion in the rumen did not increase the concentration of ruminal VFA. The similar concentration of ruminal VFA across
treatments observed in the current study may be explained by higher absorption rate of VFA due to lower ruminal pH when RFDG was fed (Van Maanen et al., 1978). The enhanced nutrient digestibility and the sustained production of rumen ammonia when feeding RFDG may have contributed to maintain the growth of rumen microorganisms, which prefer ammonia as their source of nitrogen (Hristov and Ropp, 2003).

The beef NRC (2000) suggests that MCP can supply from 50% to essentially all the MP required by beef cattle. Clark et al. (1992) reported that microbial N supplies an average of 59% of the non-ammonia N that passes to the small intestine of dairy cow. One of the factors that may affect rumen microbial growth is protein degradability of the diet (Stern and Hoover, 1970). Based on intestinal flow of feed protein, estimates of rumen undegradable protein of diets were 47.8, 46.4, 49.6 and 50.7% for CONTROL, 10, 20 and 30% RFDG, respectively. In this study, the percent of metabolizable protein of microbial origin was not affected by treatment; estimates were 53.0, 54.0, 50.0 and 49.0% for CONTROL, 10, 20 and 30% RFDG diets, respectively. However, duodenal microbial N flow measured with DAPA tended to decrease with the inclusion of DDGS in a forage based diet fed to beef steers (Castillo-Lopez et al., in press). When averaged across treatments, duodenal protozoal N flow represented 13.2% of total duodenal microbial N. The contribution of protozoa N observed in this study was similar to that observed by Sylvester et al. (2005), who reported that protozoal N accounts for 11.9% of the duodenal flow of microbial N. In contrast to what we expected, the lower starch levels of RFDG containing diets had no effect on the presence of protozoa, which are known to utilize starch granules as a major substrate for engulfment and intracellular digestion (Coleman, 1992; Fondevila and Dehority, 2001). Yeast N flow ($P < 0.01$)
represented 0.17, 0.27, 0.56 and 0.64 ± 0.04% of total microbial N for CONTROL, 10, 20
and 30% RFDG diets, respectively. This result is lower compared to that of Castillo-
Lopez et al. (in press), who reported that yeast N represents 1.06% of total microbial N
flowing to the duodenum of steers fed 20% DDGS. Based on the concentration of yeast
based N in RFDG, animals consumed 6.5, 13.0 and 19.0 g of yeast N/d when feeding 10,
20 or 30% RFDG, respectively. Therefore, our results indicate that yeast cells contained
in DDGS are extensively degraded in the rumen. Estimates of duodenal microbial N flow
obtained in this study are lower compared to values predicted by the CPM dairy model,
which predicted 340, 360, 353 and 335 g of microbial N/d for CONTROL, 10, 20 and
30% RFDG diets, respectively. Other researchers have reported that the CPM model may
overpredict microbial N flow (Pacheco et al., 2001; Hristov and Ropp, 2003) due to mean
biases of the model (Pacheco et al., 2001). The higher yield of duodenal microbial N
obtained in this study when using purines as microbial marker may indicate that non-
microbial purines are also reaching the duodenum (Belanche et al., 2011a). However, the
great difference in microbial yield observed between these two markers more likely
indicates that the DNA markers used in the current study are not present in all rumen
bacterial and protozoal species, therefore underestimating the amount of microbial N
reaching the duodenum. Analytically speaking, the procedures using either purines or
DNA as microbial markers for the estimation of microbial N take approximately the same
length of time. However, lyophilizing samples required for purine analysis takes several
days. If the purpose is the estimation of bacterial and protozoal N separately, the use
DNA markers though real-time PCR represent an alternative option.
**Phosphorous and N excretion**

The efficiency in the utilization of N and P by ruminants may have both economic and environmental impacts. An increase in N captured and a reduction in N lost would lead to an improvement in N efficiency (Jonker et al., 2002; Rius et al., 2010). In addition, N and P contamination of surface water may cause algae blooms, which shade aquatic vegetation, reducing photosynthetic activity (Burkholder et al., 2004). The NRC (2001) indicates that the requirement of P for lactating Holstein cows should range from 0.32 to 0.38% of diet. With the inclusion of RFDG in diets, the concentration of P increased from 0.35 to 0.51%. Our observed values indicate that the inclusion of RFDG in diets does not increase the amount of N and P output, and therefore does not exacerbate the negative environmental effects that these two elements may cause when they are excreted in excess. It is important to mention that the data on N and P output were collected in experiment 2, where DMI was not different across treatments. Results on fecal N output obtained in this present experiment are lower compared to those reported by Gehman et al. (2010), who observed that N excretion was 0.287 kg N/d for cows consuming 25% wet distillers grains and solubles vs. 0.165 kg N/d for cows consuming RFDG in this study. This difference in fecal N output may be explained by higher N intake of cows consuming DDGS observed by those researchers compared to that observed in the present study, 0.719 kg N/d vs. 0.628 kg N/d.

**CONCLUSIONS**

This study was designed to evaluate the effect of RFDG on lactation performance,
rumen parameters and duodenal microbial N supply; and secondly to compare the estimates of microbial N using purines or DNA as microbial markers. Results indicate that DNA markers yield lower microbial N compared to purines possibly because the DNA markers utilized in this study are not accounting for all rumen microbial species reaching the small intestine. In addition, when RFDG was included in dairy rations, lactation performance was maintained, no negative effects on rumen parameters and rumen microbial growth was observed and nutrient digestibility tended to increase. Furthermore, the excretion of N and P was not affected, which represents an economical and environmental benefit. Overall, this study demonstrates that RFDG is an effective alternative energy and protein feed source for the dairy industry.
Literature cited


Chibisa, G. E., D.A. Christensen, and T. Mutsvangwa. 2012. Effects of replacing canola meal as the major protein source with wheat dried distillers grains with solubles on
ruminal function, microbial protein synthesis, omasal flow, and milk production in cows. J. Dairy Sci. 95(2):842-841).


Table 5.1. Ingredient composition of formulated diets containing increasing levels of reduced fat dried distillers grains with solubles (RFDG) fed in experiment 1 and 2.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>CONTROL</th>
<th>10% RFDG</th>
<th>20% RFDG</th>
<th>30% RFDG</th>
</tr>
</thead>
<tbody>
<tr>
<td>RFDG¹</td>
<td>--</td>
<td>9.7</td>
<td>19.2</td>
<td>29.4</td>
</tr>
<tr>
<td>Corn silage</td>
<td>21.4</td>
<td>20.7</td>
<td>20.1</td>
<td>18.7</td>
</tr>
<tr>
<td>Alfalfa haylage</td>
<td>9.7</td>
<td>8.4</td>
<td>7.1</td>
<td>5.8</td>
</tr>
<tr>
<td>Brome hay</td>
<td>4.9</td>
<td>5.2</td>
<td>5.3</td>
<td>5.5</td>
</tr>
<tr>
<td>Alfalfa hay</td>
<td>9.7</td>
<td>8.4</td>
<td>7.1</td>
<td>5.8</td>
</tr>
<tr>
<td>Cottonseed</td>
<td>7.8</td>
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<td>2.6</td>
</tr>
<tr>
<td>Ground corn</td>
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<td>17.8</td>
<td>13.8</td>
<td>10.0</td>
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<tr>
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<td>15.5</td>
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<td>2.8</td>
<td>1.4</td>
</tr>
<tr>
<td>Soybean meal</td>
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<td>4.0</td>
<td>2.0</td>
<td>--</td>
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<tr>
<td>Megalac</td>
<td>--</td>
<td>0.6</td>
<td>1.2</td>
<td>1.8</td>
</tr>
<tr>
<td>Bloodmeal</td>
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<td>0.6</td>
<td>0.3</td>
<td>--</td>
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<td>1.05</td>
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<td>0.44</td>
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</tr>
<tr>
<td>Magnesium oxide</td>
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<tr>
<td>Trace mineral premix³</td>
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<td>0.11</td>
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<tr>
<td>Vitamin premix⁴</td>
<td>0.11</td>
<td>0.11</td>
<td>0.11</td>
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</tr>
</tbody>
</table>

¹RFDG: reduced fat dried distillers grains with solubles (Poet Nutrition, Sioux Falls, SD).
²LignoTech, Overland Park, KS.
³Formulated to contained 1.0% Ca, 0.50 % P, 0.36% Mg, 1.3% K.
⁴Formulated to supply approximately 120, 000 IU/d vitamin A, 24, 000 IU/d of vitamin D, and 800 IU/d Vitamin E in total ration.
Table 5.2. Chemical composition of formulated diets containing increasing levels of reduced fat dried distillers grains with solubles (RFDG) fed in experiment 1 and 2.

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>CONTROL</th>
<th>10% RFDG&lt;sup&gt;1&lt;/sup&gt;</th>
<th>20% RFDG</th>
<th>30% RFDG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemical&lt;sup&gt;2&lt;/sup&gt;, % of DM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CP</td>
<td>18.3</td>
<td>18.2</td>
<td>18.2</td>
<td>18.0</td>
</tr>
<tr>
<td>NDF</td>
<td>35.4</td>
<td>36.5</td>
<td>37.9</td>
<td>38.3</td>
</tr>
<tr>
<td>Starch</td>
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<td>22.3</td>
<td>20.0</td>
<td>17.6</td>
</tr>
<tr>
<td>Ether extract</td>
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<td>4.0</td>
<td>4.1</td>
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<tr>
<td>NFC&lt;sup&gt;3&lt;/sup&gt;</td>
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<td>33.1</td>
<td>32.1</td>
<td>30.7</td>
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<td>Ash</td>
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<td>8.2</td>
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<td>8.4</td>
</tr>
<tr>
<td>Phosphorous</td>
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<td>0.41</td>
<td>0.46</td>
<td>0.52</td>
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<tr>
<td>ME&lt;sup&gt;4&lt;/sup&gt;, Mcal/kg</td>
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<td>2.51</td>
<td>2.49</td>
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<tr>
<td>NE&lt;sub&gt;L&lt;/sub&gt;&lt;sup&gt;4&lt;/sup&gt;, Mcal/kg</td>
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<td>1.62</td>
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<tr>
<td>Experiment 2</td>
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<tr>
<td>CP</td>
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<td>18.2</td>
<td>18.3</td>
<td>18.2</td>
</tr>
<tr>
<td>NDF</td>
<td>33.3</td>
<td>34.5</td>
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</tr>
<tr>
<td>Starch</td>
<td>25.8</td>
<td>22.9</td>
<td>20.1</td>
<td>17.2</td>
</tr>
<tr>
<td>Ether extract</td>
<td>4.08</td>
<td>4.2</td>
<td>4.3</td>
<td>4.4</td>
</tr>
<tr>
<td>NFC&lt;sup&gt;3&lt;/sup&gt;</td>
<td>35.9</td>
<td>34.8</td>
<td>33.7</td>
<td>31.8</td>
</tr>
<tr>
<td>Ash</td>
<td>8.5</td>
<td>8.4</td>
<td>8.4</td>
<td>8.3</td>
</tr>
<tr>
<td>Phosphorous</td>
<td>0.35</td>
<td>0.40</td>
<td>0.44</td>
<td>0.51</td>
</tr>
<tr>
<td>ME&lt;sup&gt;4&lt;/sup&gt;, Mcal/kg</td>
<td>2.58</td>
<td>2.56</td>
<td>2.53</td>
<td>2.51</td>
</tr>
<tr>
<td>NE&lt;sub&gt;L&lt;/sub&gt;&lt;sup&gt;4&lt;/sup&gt;, Mcal/kg</td>
<td>1.66</td>
<td>1.65</td>
<td>1.63</td>
<td>1.62</td>
</tr>
<tr>
<td>RUP&lt;sup&gt;4&lt;/sup&gt;</td>
<td>41.66</td>
<td>42.26</td>
<td>44.22</td>
<td>49.29</td>
</tr>
</tbody>
</table>

<sup>1</sup>RFDG: reduced fat dried distillers grains with solubles (Poet Nutrition, Sioux Falls, SD).

<sup>2</sup>Calculated based on analysis of individual feed ingredient and the rate of inclusion to the diet.

<sup>3</sup>NFC = Nonfiber carbohydrate calculated by difference 100 – (%NDF + % CP + % Fat + % Ash).

<sup>4</sup>According to the CMP Dairy Ration Analyzer v3.0.8.1
Table 5.3. Analyzed chemical composition (mean and standard deviation) of feedstuffs used in the formulation of diets containing increasing levels of reduced fat dried distillers grains with solubles (RFDG) fed in experiments 1 and 2.

<table>
<thead>
<tr>
<th>FEED INGREDIENT</th>
<th>Component</th>
<th>Corn Silage</th>
<th>Alfalfa Haylage</th>
<th>Brome Hay</th>
<th>Alfalfa Hay</th>
<th>Cotton Seed</th>
<th>RFDG&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Control Mix</th>
<th>RFDG-added Mix</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DM</td>
<td>32.83 ± 1.04</td>
<td>32.50 ± 2.58</td>
<td>88.25 ± 0.86</td>
<td>87.80 ± 0.88</td>
<td>89.20 ± 1.49</td>
<td>90.30 ± 0.14</td>
<td>88.98 ± 0.74</td>
<td>90.53 ± 0.62</td>
<td></td>
</tr>
<tr>
<td>CP</td>
<td>8.70 ± 0.42</td>
<td>22.00 ± 0.72</td>
<td>10.25 ± 0.60</td>
<td>20.68 ± 1.18</td>
<td>21.98 ± 1.46</td>
<td>31.85 ± 0.49</td>
<td>21.80 ± 0.37</td>
<td>20.88 ± 0.51</td>
<td></td>
</tr>
<tr>
<td>NDF</td>
<td>39.30 ± 1.28</td>
<td>42.85 ± 2.07</td>
<td>69.85 ± 4.13</td>
<td>41.88 ± 1.70</td>
<td>48.60 ± 2.20</td>
<td>33.90 ± 3.54</td>
<td>25.03 ± 1.72</td>
<td>34.77 ± 0.25</td>
<td></td>
</tr>
<tr>
<td>Starch</td>
<td>35.00 ± 2.38</td>
<td>1.45 ± 0.86</td>
<td>1.53 ± 0.83</td>
<td>1.88 ± 0.67</td>
<td>1.05 ± 0.39</td>
<td>7.50 ± 0.57</td>
<td>36.03 ± 1.18</td>
<td>17.63 ± 0.61</td>
<td></td>
</tr>
<tr>
<td>Ether extract</td>
<td>2.84 ± 0.17</td>
<td>3.13 ± 0.20</td>
<td>2.04 ± 0.31</td>
<td>1.78 ± 0.37</td>
<td>19.57 ± 0.43</td>
<td>5.53 ± 0.53</td>
<td>2.66 ± 0.25</td>
<td>4.05 ± 0.24</td>
<td></td>
</tr>
<tr>
<td>NFC&lt;sup&gt;2&lt;/sup&gt;</td>
<td>43.31 ± 1.21</td>
<td>20.04 ± 2.55</td>
<td>8.42 ± 3.72</td>
<td>24.03 ± 1.72</td>
<td>5.54 ± 1.29</td>
<td>22.75 ± 4.44</td>
<td>42.30 ± 1.43</td>
<td>31.76 ± 0.55</td>
<td></td>
</tr>
<tr>
<td>Ash</td>
<td>5.86 ± 0.31</td>
<td>11.98 ± 0.39</td>
<td>9.44 ± 0.42</td>
<td>11.64 ± 0.95</td>
<td>4.32 ± 0.42</td>
<td>5.98 ± 0.91</td>
<td>8.22 ± 0.20</td>
<td>8.56 ± 0.20</td>
<td></td>
</tr>
<tr>
<td>Phosphorous</td>
<td>0.32 ± 0.03</td>
<td>0.32 ± 0.01</td>
<td>0.30 ± 0.01</td>
<td>0.30 ± 0.03</td>
<td>0.61 ± 0.04</td>
<td>0.99 ± 0.05</td>
<td>0.37 ± 0.02</td>
<td>0.64 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>YCP&lt;sup&gt;3&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.97 ± 0.04</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DM</td>
<td>35.32 ± 2.52</td>
<td>50.24 ± 2.83</td>
<td>87.98 ± 1.33</td>
<td>86.70 ± 1.77</td>
<td>88.74 ± 0.98</td>
<td>90.25 ± 0.21</td>
<td>88.52 ± 0.62</td>
<td>91.16 ± 1.36</td>
<td></td>
</tr>
<tr>
<td>CP</td>
<td>8.28 ± 0.40</td>
<td>22.38 ± 1.03</td>
<td>10.34 ± 0.58</td>
<td>19.40 ± 2.46</td>
<td>21.72 ± 1.14</td>
<td>32.05 ± 0.21</td>
<td>21.98 ± 0.80</td>
<td>21.18 ± 0.70</td>
<td></td>
</tr>
<tr>
<td>NDF</td>
<td>37.22 ± 2.38</td>
<td>30.44 ± 1.06</td>
<td>67.66 ± 2.16</td>
<td>43.86 ± 4.37</td>
<td>47.56 ± 4.52</td>
<td>35.80 ± 0.85</td>
<td>23.90 ± 1.30</td>
<td>34.02 ± 1.34</td>
<td></td>
</tr>
<tr>
<td>Starch</td>
<td>37.30 ± 1.81</td>
<td>1.60 ± 0.26</td>
<td>2.06 ± 0.45</td>
<td>2.02 ± 0.19</td>
<td>1.18 ± 0.50</td>
<td>7.55 ± 0.49</td>
<td>37.08 ± 4.03</td>
<td>16.36 ± 0.87</td>
<td></td>
</tr>
<tr>
<td>Ether extract</td>
<td>3.29 ± 0.25</td>
<td>3.53 ± 0.41</td>
<td>2.42 ± 0.34</td>
<td>1.88 ± 0.27</td>
<td>18.61 ± 1.57</td>
<td>5.32 ± 0.23</td>
<td>2.77 ± 0.38</td>
<td>4.28 ± 0.51</td>
<td></td>
</tr>
<tr>
<td>NFC</td>
<td>45.74 ± 3.09</td>
<td>29.48 ± 2.01</td>
<td>9.84 ± 1.50</td>
<td>23.33 ± 1.90</td>
<td>7.29 ± 2.91</td>
<td>20.72 ± 1.53</td>
<td>42.79 ± 1.19</td>
<td>32.09 ± 1.55</td>
<td></td>
</tr>
<tr>
<td>Ash</td>
<td>5.47 ± 0.40</td>
<td>14.17 ± 0.44</td>
<td>9.74 ± 0.46</td>
<td>11.53 ± 0.40</td>
<td>4.83 ± 0.31</td>
<td>6.12 ± 0.71</td>
<td>8.56 ± 0.69</td>
<td>8.43 ± 1.47</td>
<td></td>
</tr>
<tr>
<td>Phosphorous</td>
<td>0.28 ± 0.02</td>
<td>0.24 ± 0.01</td>
<td>0.30 ± 0.01</td>
<td>0.30 ± 0.03</td>
<td>0.65 ± 0.02</td>
<td>0.96 ± 0.01</td>
<td>0.38 ± 0.02</td>
<td>0.63 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>YCP&lt;sup&gt;3&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.91 ± 0.04</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1RFDG: reduced fat dried distillers grains with solubles (Poet Nutrition, Sioux Falls, SD).
2NFC = Nonfiber carbohydrate calculated by difference 100 − (%NDF + % CP + % Fat + % Ash).
3YCP, Yeast crude protein determined with real-time PCR according to Castillo-Lopez et al. (2010).
Table 5.4. Particle size distribution of formulated diets containing increasing levels of reduced fat dried distillers grains with solubles (RFDG) fed in experiments 1 and 2.

<table>
<thead>
<tr>
<th>Particle size</th>
<th>TREATMENT</th>
<th>SEM</th>
<th>Liner</th>
<th>Quadratic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CONTROL</td>
<td>10% RFDG</td>
<td>20% RFDG</td>
<td>30% RFDG</td>
</tr>
<tr>
<td>&gt; 19.0 mm, %</td>
<td>9.38</td>
<td>7.31</td>
<td>7.90</td>
<td>6.75</td>
</tr>
<tr>
<td>19.0 – 8.0 mm, %</td>
<td>25.58</td>
<td>24.05</td>
<td>21.40</td>
<td>19.89</td>
</tr>
<tr>
<td>8.0 – 1.18 mm, %</td>
<td>37.86</td>
<td>36.05</td>
<td>33.97</td>
<td>30.20</td>
</tr>
<tr>
<td>&lt; 1.18 mm, %</td>
<td>27.16</td>
<td>32.57</td>
<td>36.71</td>
<td>43.14</td>
</tr>
<tr>
<td>&gt; 19.0 mm, %</td>
<td>6.50</td>
<td>5.21</td>
<td>4.90</td>
<td>3.64</td>
</tr>
<tr>
<td>19.0 – 8.0 mm, %</td>
<td>26.20</td>
<td>23.32</td>
<td>19.41</td>
<td>18.01</td>
</tr>
<tr>
<td>8.0 – 1.18 mm, %</td>
<td>39.10</td>
<td>38.12</td>
<td>35.58</td>
<td>32.06</td>
</tr>
<tr>
<td>&lt; 1.18 mm, %</td>
<td>27.51</td>
<td>34.36</td>
<td>39.84</td>
<td>46.35</td>
</tr>
</tbody>
</table>

1 Determined using the Penn State Forage Particle Separator as described by (Heinrichs and Kononoff, 2002).
2 RFDG: reduced fat dried distillers grains with solubles (Poet Nutrition, Sioux Falls, SD).
3 The highest standard error of treatment means is shown.
4 P-values for effects of increasing levels of DDGS in diet.
Table 5.5. Effects of feeding increasing levels of reduced fat dried distillers grains with solubles (RFDG) on DMI, milk production and milk composition (Experiment 1).

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>CONTROL</th>
<th>10% RFDG&lt;sup&gt;1&lt;/sup&gt;</th>
<th>20% RFDG</th>
<th>30% RFDG</th>
<th>SEM&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Linear</th>
<th>Quadratic</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMI, kg/d</td>
<td>25.0</td>
<td>23.8</td>
<td>25.9</td>
<td>27.9</td>
<td>1.388</td>
<td>&lt; 0.01</td>
<td>0.02</td>
</tr>
<tr>
<td>Milk Yield, kg/d</td>
<td>34.4</td>
<td>33.2</td>
<td>34.5</td>
<td>34.2</td>
<td>1.296</td>
<td>0.78</td>
<td>0.46</td>
</tr>
<tr>
<td>3.5% FCM&lt;sup&gt;4&lt;/sup&gt;</td>
<td>35.1</td>
<td>34.4</td>
<td>35.2</td>
<td>35.3</td>
<td>1.449</td>
<td>0.66</td>
<td>0.56</td>
</tr>
<tr>
<td>Fat, %</td>
<td>3.59</td>
<td>3.74</td>
<td>3.64</td>
<td>3.67</td>
<td>0.097</td>
<td>0.66</td>
<td>0.32</td>
</tr>
<tr>
<td>Fat Yield, kg/d</td>
<td>1.24</td>
<td>1.23</td>
<td>1.25</td>
<td>1.26</td>
<td>0.056</td>
<td>0.53</td>
<td>0.80</td>
</tr>
<tr>
<td>Protein, %</td>
<td>3.08</td>
<td>3.18</td>
<td>3.15</td>
<td>3.19</td>
<td>0.062</td>
<td>0.07</td>
<td>0.43</td>
</tr>
<tr>
<td>Protein Yield, kg/d</td>
<td>1.06</td>
<td>1.04</td>
<td>1.07</td>
<td>1.09</td>
<td>0.044</td>
<td>0.23</td>
<td>0.44</td>
</tr>
<tr>
<td>MUN&lt;sup&gt;5&lt;/sup&gt;, mg/dL</td>
<td>16.24</td>
<td>15.54</td>
<td>16.23</td>
<td>15.94</td>
<td>0.863</td>
<td>0.85</td>
<td>0.41</td>
</tr>
<tr>
<td>Body Weight</td>
<td>687.2</td>
<td>687.8</td>
<td>693.4</td>
<td>696.7</td>
<td>18.4</td>
<td>&lt; 0.01</td>
<td>0.58</td>
</tr>
<tr>
<td>BCS&lt;sup&gt;6&lt;/sup&gt;</td>
<td>3.06</td>
<td>3.10</td>
<td>3.14</td>
<td>3.18</td>
<td>0.065</td>
<td>0.05</td>
<td>1.00</td>
</tr>
</tbody>
</table>

<sup>1</sup>RFDG: reduced fat dried distillers grains with solubles (Poet Nutrition, Sioux Falls, SD).

<sup>2</sup>The highest standard error of treatment means is shown.

<sup>3</sup>P-values for effects of increasing levels of RFDG in diet.

<sup>4</sup>3.5 % Fat Corrected Milk.

<sup>5</sup>MUN = Milk Urea Nitrogen.

<sup>6</sup>BCS = Body Condition Score 1 to 5 scale.
Table 5.6. Effects of feeding increasing levels of reduced fat dried distillers grains with solubles (RFDG) on DMI, milk production and milk composition (Experiment 2).

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>CONTROL</th>
<th>10% RFDG</th>
<th>20% RFDG</th>
<th>30% RFDG</th>
<th>SEM²</th>
<th>Linear</th>
<th>Quadratic</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMI, kg/d</td>
<td>21.42</td>
<td>21.78</td>
<td>21.91</td>
<td>21.13</td>
<td>1.65</td>
<td>0.73</td>
<td>0.23</td>
</tr>
<tr>
<td>Milk Yield, kg/d</td>
<td>31.41</td>
<td>32.33</td>
<td>30.86</td>
<td>31.13</td>
<td>2.81</td>
<td>0.65</td>
<td>0.76</td>
</tr>
<tr>
<td>3.5% FCM⁴</td>
<td>30.28</td>
<td>31.79</td>
<td>29.55</td>
<td>29.21</td>
<td>2.94</td>
<td>0.29</td>
<td>0.38</td>
</tr>
<tr>
<td>Fat, %</td>
<td>3.29</td>
<td>3.37</td>
<td>3.23</td>
<td>3.12</td>
<td>0.14</td>
<td>0.28</td>
<td>0.42</td>
</tr>
<tr>
<td>Fat Yield, kg/d</td>
<td>1.03</td>
<td>1.09</td>
<td>0.99</td>
<td>0.97</td>
<td>0.11</td>
<td>0.23</td>
<td>0.33</td>
</tr>
<tr>
<td>Protein, %</td>
<td>2.99</td>
<td>3.01</td>
<td>3.04</td>
<td>3.03</td>
<td>0.07</td>
<td>0.35</td>
<td>0.70</td>
</tr>
<tr>
<td>Protein Yield, kg/d</td>
<td>0.94</td>
<td>0.97</td>
<td>0.93</td>
<td>0.94</td>
<td>0.07</td>
<td>0.84</td>
<td>0.80</td>
</tr>
<tr>
<td>MUN⁵, mg/dL</td>
<td>12.64</td>
<td>13.00</td>
<td>13.07</td>
<td>13.42</td>
<td>0.93</td>
<td>0.14</td>
<td>0.98</td>
</tr>
</tbody>
</table>

¹RFDG: reduced fat dried distillers grains with solubles (Poet Nutrition, Sioux Falls, SD).
²The highest standard error of treatment means is shown.
³P-values for effects of increasing levels of RFDG in diet.
⁴3.5 % Fat Corrected Milk.
⁵MUN = Milk Urea Nitrogen.
Table 5.7. Effect of feeding increasing levels of reduced fat dried distillers grains with solubles (RFDG) on ruminal pH, concentration of ruminal VFA and ammonia.

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>CONTROL</th>
<th>10% RFDG$^1$</th>
<th>20% RFDG</th>
<th>30% RFDG</th>
<th>SEM$^2$</th>
<th>Liner</th>
<th>Quadratic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rumen pH</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minimum</td>
<td>6.08</td>
<td>6.17</td>
<td>6.06</td>
<td>6.03</td>
<td>0.14</td>
<td>0.60</td>
<td>0.53</td>
</tr>
<tr>
<td>Maximum</td>
<td>6.95</td>
<td>6.88</td>
<td>6.83</td>
<td>6.77</td>
<td>0.11</td>
<td>&lt; 0.01</td>
<td>0.94</td>
</tr>
<tr>
<td>Mean</td>
<td>6.53</td>
<td>6.49</td>
<td>6.38</td>
<td>6.35</td>
<td>0.12</td>
<td>0.02</td>
<td>0.88</td>
</tr>
<tr>
<td>Time &lt; 6.5, min/d</td>
<td>546</td>
<td>834</td>
<td>941</td>
<td>1040</td>
<td>293</td>
<td>&lt; 0.01</td>
<td>0.15</td>
</tr>
<tr>
<td>Area &lt; 6.5, pH × min/d</td>
<td>126</td>
<td>158</td>
<td>357</td>
<td>334</td>
<td>92</td>
<td>0.03</td>
<td>0.63</td>
</tr>
<tr>
<td>Time &lt; 6.3, min/d</td>
<td>279</td>
<td>382</td>
<td>936</td>
<td>946</td>
<td>208</td>
<td>0.03</td>
<td>0.72</td>
</tr>
<tr>
<td>Area &lt; 6.3, pH × min/d</td>
<td>45</td>
<td>47</td>
<td>180</td>
<td>169</td>
<td>64</td>
<td>0.13</td>
<td>0.88</td>
</tr>
<tr>
<td>Total VFA, (mM)</td>
<td>136.22</td>
<td>134.91</td>
<td>138.50</td>
<td>131.10</td>
<td>6.45</td>
<td>0.40</td>
<td>0.30</td>
</tr>
<tr>
<td>VFA mol/100 mol</td>
<td>63.36</td>
<td>63.41</td>
<td>60.84</td>
<td>59.99</td>
<td>1.48</td>
<td>&lt; 0.01</td>
<td>0.24</td>
</tr>
<tr>
<td>Acetate</td>
<td>22.12</td>
<td>21.97</td>
<td>25.00</td>
<td>26.11</td>
<td>1.60</td>
<td>&lt; 0.01</td>
<td>0.19</td>
</tr>
<tr>
<td>Propionate</td>
<td>0.81</td>
<td>0.78</td>
<td>0.70</td>
<td>0.70</td>
<td>0.08</td>
<td>&lt; 0.01</td>
<td>0.57</td>
</tr>
<tr>
<td>Isobutyrate</td>
<td>11.24</td>
<td>11.21</td>
<td>10.59</td>
<td>10.73</td>
<td>0.49</td>
<td>0.07</td>
<td>0.74</td>
</tr>
<tr>
<td>Butyrate</td>
<td>0.61</td>
<td>0.61</td>
<td>0.61</td>
<td>0.63</td>
<td>0.07</td>
<td>0.71</td>
<td>0.69</td>
</tr>
<tr>
<td>Isovalerate</td>
<td>1.77</td>
<td>1.92</td>
<td>2.08</td>
<td>2.17</td>
<td>0.12</td>
<td>&lt; 0.01</td>
<td>0.65</td>
</tr>
<tr>
<td>Valerate</td>
<td>2.98</td>
<td>2.94</td>
<td>2.52</td>
<td>2.39</td>
<td>0.23</td>
<td>&lt; 0.01</td>
<td>0.55</td>
</tr>
<tr>
<td>Ratio Acetate:Propionate</td>
<td>19.00</td>
<td>18.80</td>
<td>19.25</td>
<td>17.62</td>
<td>2.32</td>
<td>0.31</td>
<td>0.37</td>
</tr>
</tbody>
</table>

$^1$RFDG: reduced fat dried distillers grains with solubles (Poet Nutrition, Sioux Falls, SD).

$^2$The highest standard error of treatment means is shown.

$^3$P-values for effects of increasing levels of RFDG in diet.
Table 5.8. Fecal output, nutrient intake and nutrient total tract apparent digestibility by cows fed increasing levels of reduced fat dried distillers grains with solubles (RFDG).

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>SEM²</th>
<th>Linear</th>
<th>Quadratic</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Fecal output,</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DM, kg/d</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N, kg/g</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P, g/d</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DM Digestibility, %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OM Intake, kg/d</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Digestibility, %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NFC Intake, kg/d</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Digestibility, %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NDF Intake, kg/d</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Digestibility</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N Intake, kg/d</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Digestibility</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphorous Intake, g/d</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Digestibility, %</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1RFDG: reduced fat dried distillers grains with solubles (Poet Nutrition, Sioux Falls, SD).
2The highest standard error of treatment means is shown.
3P-values for effects of increasing levels of RFDG in diet.
4NFC: Non-fiber carbohydrates.
Table 5.9. Duodenal digesta and N flows in cows fed increasing levels of reduced fat dried distillers grains with solubles (RFDG).

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>CONTROL</th>
<th>10% RFDG(^1)</th>
<th>20% RFDG</th>
<th>30% RFDG</th>
<th>SEM(^2)</th>
<th>Linear</th>
<th>Quadratic</th>
</tr>
</thead>
<tbody>
<tr>
<td>DM, kg/d</td>
<td>14.8</td>
<td>15.7</td>
<td>15.0</td>
<td>14.6</td>
<td>0.79</td>
<td>0.27</td>
<td>0.98</td>
</tr>
<tr>
<td>NAN(^4), g/d</td>
<td>550</td>
<td>580</td>
<td>560</td>
<td>560</td>
<td>20.0</td>
<td>0.93</td>
<td>0.46</td>
</tr>
<tr>
<td>Purine markers</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microbial N(^5), g/d</td>
<td>287</td>
<td>311</td>
<td>282</td>
<td>276</td>
<td>20.0</td>
<td>0.57</td>
<td>0.49</td>
</tr>
<tr>
<td>Microbial N, % of NAN</td>
<td>53.0</td>
<td>54.0</td>
<td>50.0</td>
<td>49.0</td>
<td>4.93</td>
<td>0.57</td>
<td>0.86</td>
</tr>
<tr>
<td>DNA markers</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacterial N, g/d</td>
<td>181</td>
<td>179</td>
<td>209</td>
<td>146</td>
<td>16.0</td>
<td>0.56</td>
<td>0.20</td>
</tr>
<tr>
<td>Protozoal N, g/d</td>
<td>40</td>
<td>39</td>
<td>34</td>
<td>39</td>
<td>7.0</td>
<td>0.77</td>
<td>0.48</td>
</tr>
<tr>
<td>Microbial N(^6), g/d</td>
<td>225</td>
<td>223</td>
<td>243</td>
<td>194</td>
<td>18.0</td>
<td>0.45</td>
<td>0.23</td>
</tr>
<tr>
<td>Microbial N, % of NAN</td>
<td>39.7</td>
<td>38.7</td>
<td>41.0</td>
<td>35.0</td>
<td>3.0</td>
<td>0.42</td>
<td>0.44</td>
</tr>
<tr>
<td>Yeast N, g/d</td>
<td>0.324</td>
<td>0.987</td>
<td>1.329</td>
<td>1.836</td>
<td>0.22</td>
<td>0.02</td>
<td>0.87</td>
</tr>
<tr>
<td>Yeast N, % of MN</td>
<td>0.17</td>
<td>0.27</td>
<td>0.56</td>
<td>0.64</td>
<td>0.04</td>
<td>&lt; 0.01</td>
<td>0.80</td>
</tr>
<tr>
<td>Predicted MN(^7), g/d</td>
<td>340</td>
<td>360</td>
<td>353</td>
<td>335</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^1\)RFDG: reduced fat dried distillers grains with solubles (Poet Nutrition, Sioux Falls, SD).
\(^2\)The highest standard error of treatment means is shown.
\(^3\)P-values for effects of increasing levels of RFDG in diet.
\(^4\)NAN: Non-Ammonia Nitrogen.
\(^5\)Microbial N estimated using purines as microbial marker.
\(^6\)Microbial N estimated using DNA as microbial markers and it is the sum of bacterial and protozoal N.
\(^7\)Microbial N predicted by the CMP Dairy Ration Analyzer v3.0.8.1
Figure 5.1. Effect of feeding increasing levels of reduced fat dried distillers grains with solubles (RFDG) on diurnal variation of ruminal pH. Time after feeding 0 represents 0930, when TMR were offered. Mean pH, $P = 0.02$. 
CHAPTER VI

Summary and conclusions

The expansion of the corn ethanol industry during the past 3 decades has increased the supply of by-products for ruminant nutrition. Current production rates of these by-products, namely DDGS, will likely be maintained or perhaps increased in the future. In addition, with the expansion of ethanol production, new by-products are being generated. Reduced fat dried distillers grains and solubles (RFDG) is an example of these new by-products, which represents an attractive feed ingredient in rations formulated for dairy cattle. Because of the increase in the supply of by-products for ruminant nutrition, it is important to understand their effects on nutrient utilization, rumen fermentation and their subsequent effect on animal production.

In ruminants, microbial crude protein (MCP) synthesized during rumen fermentation of feedstuff contributes to the supply of metabolizable protein (MP) reaching the small intestine. It has been suggested that more than half of the MP required by the ruminant is provided by MCP. Therefore, accurate measurement of rumen microbial growth is important. In order to estimate rumen microbial growth, the measurement of microbial markers is needed. The most common approach to estimate rumen microbial growth is the analysis of purines (Adenine and Guanine) or the measurement of purine derivatives in urine. However, these approaches assume that all the purines reaching the small intestine are of microbial origin. As a consequence, purines supplied by undegraded feedstuff and sloughed cells would lead to an overestimation of MCP. The inclusion of DDGS in ruminant diets may also provide residual yeast cells used in the ethanol production process, and therefore, provide purines,
which may also lead to an overestimation of MCP if purines are used as a microbial marker. Therefore, the use of alternatives microbial markers and the comparison of MCP estimated with different markers is needed.

This investigation was focused on exploring the ability of utilizing DNA as microbial markers for the estimation of MCP through real-time PCR. To do so, *in vitro* and *in vivo* studies were conducted. Comparison of DNA markers with widely used microbial markers, namely, purines and diaminopumelic acid (DAPA) was conducted. The *in vivo* studies were also designed to investigate the effects of DDGS and RFDG on nutrient utilization, rumen fermentation and lactation performance in Holstein dairy cows. Overall, results indicate that DNA markers have the ability to detect and quantify rumen microbial growth. The growth of rumen bacteria and their subsequent supply on duodenal MCP measured with DNA or DAPA yielded similar values. However, estimates obtained with DNA markers yielded significantly lower values compared to those obtained using purines. This great difference in MCP between DNA and purine markers can be attributed to 1) undegraded feed purines reaching the small intestine and 2) DNA markers may not be present across all ruminal microbial species, therefore underestimating the amount of microbial mass. Our data indicate that the contribution of protozoa to total MCP is approximately 17%. Additionally, we observed that the contribution of yeast to total MCP is lower than 1% when animals are fed DDGS. This suggests that yeast cells used in the fermentation of corn during ethanol production are extensively degraded in the rumen and do not represent a significant contribution of intestinal purines. Furthermore, our results indicate the DDGS contains 63% rumen undegradable protein and that contribution of MCP to MP ranges from 38 to 58%. 
Overall, this research suggests that rations formulated for Holstein dairy cattle may contain up to 30% RFDG with no negative effect on lactation performance, rumen fermentation and microbial protein to the small intestine.

Several studies have been conducted to evaluate the effects of by-products on rumen microorganisms and animal performance. However, further research is needed in this field. Some potential topics worth exploring are 1) differences in chemical composition and diversity of liquid associated versus particulate associated rumen bacteria; 2) the use of SYBR Green instead of TAQMAN probes in quantitative PCR; 3) effects of DDGS versus RFDG on the generation and intestinal supply of CLA intermediates; 4) effects of DDGS or RFDG on VFA production and rumen and intestinal microbial diversity shifts at the different taxonomic levels.
APPENDIX A

ABBREVIATIONS

ADF  Acid Detergent Fiber
AOAC Association of Official Analytical Chemists
BCP  Bacterial Crude Protein
BW   Body Weight
CDS  Condensed Distillers Solubles
CGF  Corn Gluten Feed
CONT Control
CP   Crude Protein
CS   Corn Silage
DAPA Diaminopumelic Acid
DCGF Dried Corn Gluten Feed
DDGS Dried Distillers Grains with Solubles
DG   Distillers Grains
DGS  Distillers Grains with Solubles
DIM  Days In Milk
DM   Dry Matter
dNTPs Deoxyribonucleotide Triphosphates
EDTA Ethylenediaminetetraacetic Acid
EE   Ether Extract
FAM  Carboxyfluorescein
GC   Ground Corn
HPDDG High Protein Dried Distillers Grains
HPLC High Performance Liquid Chromatography
IDT  Integrated DNA technology
MCP  Microbial Crude Protein
MP   Metabolizable Protein
N    Nitrogen
NCBI National Center of Biotechnology Information
NDF  Neutral Detergent Fiber
NFC  Non Fiber Carbohydrates
NRC  National Research Council
OM   Organic Matter
PCP  Protozoal Crude Protein
PCR  Polymerase Chain Reaction
PVC  Polyvinyl Chloride
RC   Replacing Corn
RCS  Replacing Corn and Soybean Meal
RDP  Rumen Degradable Protein
RFDG Reduced Fat Dried Distillers Grains with Soluble
rRNA Ribosomal RNA
RS   Replacing Soybean meal
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>RUP</td>
<td>Rumen Undegradable Protein</td>
</tr>
<tr>
<td>SBM</td>
<td>Soybean Meal</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
</tr>
<tr>
<td>TAMRA</td>
<td>Tetramethylrhodamine</td>
</tr>
<tr>
<td>TMR</td>
<td>Total Mixed Ration</td>
</tr>
<tr>
<td>VFA</td>
<td>Volatile Fatty Acid</td>
</tr>
<tr>
<td>WDG</td>
<td>Wet Distillers Grains</td>
</tr>
<tr>
<td>WDGS</td>
<td>Wet Distillers Grains with Solubles</td>
</tr>
<tr>
<td>YCP</td>
<td>Yeast Crude Protein</td>
</tr>
</tbody>
</table>
APPENDIX B

THE PURINE ANALYSIS ASSAY FOR THE ESTIMATION OF MCP

Following is a description of the purine analysis (Makkar and Becker, 1999).

Reagents:

a) 10 mM NH₄H₂PO₄ (ammonium dihydrogen phosphate) – Reagent A
   i. Add 1.1503 g NH₄H₂PO₄ to 1 L volumetric flask and q.s. to 1 L with ddH₂O.
   ii. pH to 6.0 with 2.86 M NH₄OH and filter through 0.22 µm filter.

b) Acetonitrile/12.5 mM NH₄H₂PO₄ – reagent B
   i. Add 1.4378 g NH₄H₂PO₄ and 150 mL acetonitrile to 1 L volumetric flask and q.s. to 1 L with ddH₂O
   ii. pH to 6.0 with 2.86 M NH₄OH and filter through 0.22 µm filter.

c) 2.86 M NH₄OH (ammonium hydroxide)
   i. Add 48.31 mL 30% (14.8 M) NH₄OH to 250 mL volumetric flask; q.s. to 250 mL with ddH₂O


d) 0.6 M HClO₄ (perchloric acid)
   i. Add 50 mL concentrate (70%, 12M) HClO₄ to 1 L volumetric flask and q.s. to 1 L with ddH₂O

Standards:

a. Purine/Allopurinol Standard
   i. Add 0.1021 g Allopurinol (C₃H₄N₄O), 0.1133 Guanine (C₅H₄N₅O), and 0.1013 g adenine (C₅H₅N₅) to 250 mL volumetric flask; q.s. to 250 mL with ddH₂O (solution will be cloudy, mix well before using)

b. Allopurinol Internal Standard
   i. Add 0.1021 g Allopurinol to 250 mL volumetric flask and q.s. to 250 mL with ddH₂O

Sample preparation:

a. Weigh 25-100 mg of lyophilized material in duplicate into 25-50 mL pyrex tubes
b. Add 0.5 mL Purine Standard to 6 empty standard tubes
c. Add 0.5 mL Internal Standard to sample tubes excluding standard tubes
d. Add 2.5 mL 0.6 M HClO₄ to all tubes and vortex tubes until sample is wet
e. Incubate in 90-95 °C water bath for 1 h
f. Allow samples to cool, then add 7.5 mL reagent A to all tubes
g. Adjust pH between 6.6 and 6.9 with KOH
   a. Add 260 µL KOH to standard tubes
   b. Add 250 µL KOH to sample tubes
h. Vortex tubes thoroughly, then centrifuge at 3000 x g to remove precipitate
i. Filter supernatant through 13 mm disposable syringe filter with GHP membrane into HPLC vial

Chromatographic conditions:

a. Column
   i. C₁₈ reversed-phase
   ii. 5 µm pore size
   iii. 250 mm x 4.6 mm I.D.
b. HPLC
i. Injection volume – 15-50 µL
ii. Wavelength – 254 nm
iii. Analysis temperature – 22 ºC
iv. Gradient flow rate 0.8 mL/min
   1. 100% reagent A to 100% reagent B – 30min
   2. 100% reagent B – 10min
   3. 100% reagent B to 100% reagent A – 5 min
   4. Equilibrium 15 min
v. Analysis run time – 60min

Calculation:

a. Convert µMol amount to mg:
   mMol Guanine x 151.1/1000 = mg Gu; mMol Adenine x 135.1/1000 = mg Ad

b. Express total purine content as proportion of total weight:
   (mg Gu + mg Ad)/sample wt (g DM) = purine mg/g

c. Determine the amount of microbial nitrogen:
   [(Purine mg/g)purine:N ratio]/10 = % microbial N
APPENDIX C

THE DIAMINOPUMELIC ACID ASSAY FOR THE QUANTIFICATION OF BCP

Between 150-180 mg of ground duodenal digesta samples or between 28-30 mg of ground bacterial pellets is weighed and placed into screw cap culture tubes.

Then, 3 mL of metasulfonic acid is added to each tube and samples are hydrolyzed at 110ºC for 22 h.

Samples are neutralized with sodium hydroxide and pH adjusted to 7.0 – 7.5 with sulfuric acid or sodium hydroxide. This step is needed due to the tendency of the sample matrix to precipitate at lower pH and higher organic concentrations (McCalley, 2005).

The adjustment of pH is followed by two clean up procedures. First by adding HCl-washed charcoal (Sigma Chemical Co., St. Louis, MO) and centrifuging samples at 1,000 × g for 20 minutes, and then by filtering samples through a Sep-Pak C-18 cartridge filter (Waters Associates, Milford, MA). The authors of the protocol suggest that, this second clean up procedure was carried out as a precaution in order to eliminate any non-polar materials, which would negatively affect column performance.

Then, 1 mL of o-phthalaldehyde derivatization solution is added to 1.0 mL of sample in a small test tube; contents are mixed thoroughly and reacted for 14 h at 4 ºC. Although the procedure described by Webster et al. (1990) suggests a reaction time of 2 minutes, test runs conducted by Castillo-Lopez et al. (in press) demonstrated that 14 h is needed in order to obtain peaks indicating the presence of DAPA in samples.

Finally, 20 µL of derivatized sample are injected into the column.
APPENDIX D

DESCRIPTION OF QUANTITATIVE REAL-TIME PCR

Following is a general description of the working principles of real time PCR. Specific conditions and reaction concentrations are adjusted according to research objectives (Moya et al., 2009).

Components/ingredients needed:
a) Forward primer, b) Reverse primer, c) TaqMan probe, d) Polymerase, e) dNTPs, f) Buffer, g) DNA template, h) nanopure water

Temperature cycling:
b) 50 °C for 2 minutes

c) 95 °C for 10 minutes
d) 45 cycles alternating:
   1. 95 °C for 15 seconds for denaturation
   2. 60 °C for 1 minute for annealing and polymerization

I) When temperature is elevated to 95 °C the DNA is denatured.
II) Single stranded DNA allows for the primers and probe to anneal.
III) When polymerization reaches the TaqMan probe, the endonuclease activity of polymerase cleaves the probe.
IV) The probe has a quencher and a reporter (i.e. FAM and TAMRA). The signal of the reporter is blocked by the quencher as long as they are in close proximity. However, when the probe is cleaved, the reporter is no longer close to the quencher and the signal can be released and detected by the thermocycler.
V) The signal is then represented on a graph. On the Y axis is the magnitude of reporter’s signal and on the X axis are number of cycles. After a few cycles, the plot illustrates an exponential amplification, then linear amplification and finally it reaches a plateau.
VI) The efficiency of DNA amplification can be calculated as $E= 10^{(-1/\text{slope})}$. Where $E$ is efficiency, slope is the slope of the curve when plotting # of cycles against DNA concentration. The PCR amplification curve allows us to monitor how DNA is being duplicated in real-time. Figure 2.4 illustrates an example of a real-time amplification plot of bacterial DNA.
Example of a 4 inch rumen cannula

The dorsal and ventral branches of the Thoracic 13, Lumbar 1, and Lumbar 2 were blocked with 20 cc of 2% lidocaine hydrochloride at each site to desensitize the paralumbar fossa.

A 17 cm linear skin incision was performed to place the 4 inch rumen cannula

The rumen was resected with a 1 cm margin from the suture line; cannula was inserted and closed with cap.
APPENDIX F

DUODENAL CANNULATION

Example of a double L-shaped duodenal cannula

The right paralumbar fossa was anesthetized following a similar method used for ruminal cannulation

Cow was positioned in left lateral recumbency with legs restrained forward and back. A 15 cm skin incision was made in the cranial paralumbar fossa parallel to the last rib

The cannula barrel was exteriorized in the 10th intercostal space at the level of the frontal plane of the shoulder joint. Skin incision was closed and cannula plug was placed
APPENDIX G

PROCEDURE FOR RUMINAL CANNULATION

1. Eighteen to twenty-four hours preoperatively, 19.8 mg/kg of Oxytetracycline HCl (Liquamycin LA-200) will be administered subcutaneously at no more than 10 ml per injection site. **The cow must not be slaughtered for human consumption within 28 days from the date of injection.**

2. The rumen cannula and cap will be placed in a bucket of very hot (140°-160°F) tap water to make the material more pliable for placement in the body wall.

3. The cow will be restrained in a headgate with side rails or a restraint chute.

4. The left paralumbar fossa will be anesthetized with a proximal paravertebral nerve block using a modification of the Farquharson technique. The dorsal and ventral branches of T13, L1, and L2 will be blocked with 20 cc of 2% lidocaine HCl at each site to desensitize the paralumbar fossa. The block will be tested with an 18 gauge 1.5 inch needle to insure adequate desensitization of the surgical site.

5. A 17 cm linear skin incision for a 4 inch rumen cannula will be performed in the mid-paralumbar fossa with a #20 scalpel. (15 cm linear skin incision for a 3 inch rumen cannula)

6. The external abdominal oblique, internal abdominal oblique and transversus muscles will be incised parallel to their muscle fibers with sharp or blunt dissection. The peritoneum will be entered by blunt dissection and then the peritoneal opening will be extended to the size of the skin opening by sharp dissection.

7. The rumen wall will be lifted to the incision with 6 inch towel forceps or Vulsellum forceps. Approximately 4 cm of rumen wall will be lifted out of the skin incision to allow placement of the retaining sutures with minimal tension on the rumen wall. The rumen serosa will be secured to the underside of the skin with interrupted horizontal mattress sutures using #3 chromic gut or #3 Braunamid. Initially, the rumen will be secured at the 12, 6, 3, and 9 o’clock positions. Interrupted horizontal mattress sutures will continue to be placed until the rumen wall is secured throughout the complete circumference with special attention to the ventral and dorsal commissures of the incision.

8. The rumen wall will be resected with a 1 cm margin from the suture line.

9. The cannula will be removed from the hot water and one edge of the inner ring will be inverted through the center hole to form a cone. The cone will be inserted through the incision and the inner ring will be pushed back through the hole from the outside to reestablish the inner ring inside the rumen.

10. The cap will be placed in the cannula.

11. The exterior of the cow will be rinsed to remove any blood, rumen contents, and residual disinfectant.

12. The cow will be returned to her stall and returned to her normal diet.
APPENDIX H

PROCEDURE FOR DUODENAL CANNULATION

1) Feed will be withdrawn 48 h and water 24 h before surgery.

2) Eighteen to twenty-four hours preoperatively, 19.8 mg/kg of Oxytetracycline HCl (Liquamycin LA-200) will be administered subcutaneously at no more than 10 ml per injection site. **The cow must not be slaughtered for human consumption within 28 days from the date of injection.** A second injection at 48 forty-eight hours following the first injection may be administered at the discretion of the attending veterinarian, but will alter the slaughter withdrawal period. The veterinarian prescribing the second dose will be responsible for establishing the new slaughter withdrawal date.

3) The right paralumbar fossa will be clipped, scrubbed, and anesthetized with a proximal paravertebral nerve block using a modification of the Farquharson technique. The dorsal and ventral branches of T13, L1, and L2 will be blocked with 20 cc of 2% lidocaine HCl at each site to desensitize the paralumbar fossa. Additionally, a 10 cm cutaneous and muscular ring block will be performed at the tenth intercostal space at the proposed site of cannula exit. The blocks will be tested with an 18 gauge 1.5 inch needle to insure adequate desensitization of the surgical sites.

4) Recumbent anesthesia and sedation will be induced by the subcutaneous administration of 0.05 mg/kg Xylazine HCl, 0.1 mg/kg Ketamine HCl, and 0.025 mg/kg butorphanol. Upon becoming recumbent, the cow will be positioned in left lateral recumbency with the legs restrained forward and back and the head restrained by a halter in a comfortable position. The right side of the abdomen will have a final scrub and be draped by a standard four-corner draping technique.

5) A 15 cm skin incision will be made in the cranial paralumbar fossa parallel to the last rib with a #20 scalpel. The external abdominal oblique, internal abdominal oblique and transversus muscles and the peritoneum will be incised with sharp dissection.

6) Upon entry to the abdomen, the pylorus and proximal 20 cm of the cranial part of the duodenum will be exteriorized and separated from the remainder of the incision by moist towels. Site of cannula insertion will be centered approximately 10 cm distal to the pylorus.

7) Omentum will be elevated from the duodenum for a distance corresponding to the length of the cannula barrel. Care will be taken to preserve major branches of duodenal vessels and, thus, to minimize effects of cannulation on blood flow to the area. A 5 to 6-cm enterotomy will be made along the right side of the duodenum in the area previously freed of omentum.
8) Intermittent suction will be used when necessary to remove intestinal contents. The body of the cannula will be inserted into the intestinal lumen. The enterotomy will be closed adjacent to the barrel of the cannula with 2-0 PDS II in a simple interrupted pattern. The cut edges of the enterotomy will be inverted, and two purse string sutures of the same type of suture material will be placed snugly around the cannula barrel.

9) A single piece of knitted polypropylene surgical mesh (Marlex, C. R. Bard and Co., Murray Hill, NJ) will be positioned around the intestine to hold the intestinal wall against the cannula body. Mesh will be precut to correspond in size to the length of the cannula body. Mesh will be placed deep to major vessels and will be held in place and drawn snugly by interrupted imbricating sutures. The surgical field will be lavaged with sterile saline, and the inner collar will be inserted over the cannula barrel for designs that included a collar.

10) In preparation for cannula exteriorization a 2-cm diameter circular piece of skin will be removed from the right body wall in the 10th intercostal space at the level of the frontal plane of the shoulder joint. By sharp and blunt dissection, muscle layers and peritoneum will be incised. The cannula barrel will be pushed through the body wall at this site, and the outer collar will be inserted to hold the cannula in place.

11) The peritoneum and transversus will be apposed in a simple continuous pattern with #3 chromic gut on a ½ circle taper needle. The internal and external abdominal oblique muscles will be apposed as a single layer in a simple continuous pattern with #3 chromic gut on a ½ circle taper needle. The skin edges will be apposed in a Ford continuous interlocking pattern with #3 Braunamid on an “S” curved needle.

12) Cows will be hospitalized until they recover adequately from the surgical procedure (5 to 10 days).