

EFFECTS OF NUTRITIONAL STRATEGIES ON RUMEN ENVIRONMENT AND
PERFORMANCE IN DAIRY COWS

BY

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DISSERTATION

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ABSTRACT

Sub-acute ruminal acidosis (**SARA**) is a common condition affecting dairy cows and has up to 26% incidence in commercial dairy farms (Garrett et al., 1999; Kleen et al., 2003). Its association with DMI depression, milk yield depression, reduced feed efficiency, rumenitis, diarrhea, laminitis, inflammation, liver abscesses, and high culling and death rates in dairy cattle had been extensively reported by previous authors (Nocek, 1997; Kleen et al., 2003; Stone, 2004; Alzahal et al., 2007; Enemark, 2008). The effects of SARA on urine, fecal pH, milk yield and milk composition, and starch digestibility were determined. Six Holstein cows (**HOL**), six rumen-cannulated Holstein cows (**CAN**), and six Jersey cows (**JER**) were used in a replicated 3 × 3 Latin square design balanced to measure carry-over effects. Periods (10 d) were divided into 4 stages (S): S1, baseline, d 1-3, ad libitum TMR; S2, restricted feeding, d 4, cows fed for 50% of S1 DMI; S3, challenge, d 5, treatments applied; S4, recovery, d 6-10, all cows fed ad libitum TMR. Treatments were CON, no top dress; MOD, 10% of S1 DMI as top dress (pelleted mixture of 50:50, wheat:barley); and HIG, 20% of S1 DMI as top dress. Rumen pH and urine pH were recorded at -2 to 22 h relative to feeding on S3. Milk yield was recorded and DMI measured daily. Milk samples were obtained on d 2, 4, 5, and 8 for composition analysis. Fecal samples were obtained on d 1 afternoon (3 PM), d 4 morning (7 AM), afternoon (3 PM), night (11 PM), and the next morning before feeding (6 AM) for determination of fecal pH and starch digestibility. No treatment carry-over effect was observed for any measured variable. Mean rumen pH was depressed to 6.24 and 6.35 for cows in HIG and MOD respectively, which were lower than the 6.45 for cows in CON treatment. However, the area under curve was not different among treatments in rumen pH. Mean urine pH for HOL cows was lower on HIG (8.46) compared with 8.54 and 8.51 for CON and MOD, respectively. Mean urine pH of Jersey cows did not differ among treatments. Fecal pH was not different among treatments either for JER and HOL cows. However, fecal pH was lower 15 and 23 h after feeding in HOL cows

when compared with other time points. Fecal pH was lower at 7, 15, and 23 h after feeding in JER cows than other time points. Milk yield and composition were not different among treatments. Milk urea nitrogen was elevated in S2 and S3 on all treatments for both HOL and JER cows. No treatment effects were found for fecal pH and starch digestibility. During S3, fecal pH was depressed from 7 to 23 h and 15 to 24 h post feeding for JER and HOL cows. Rumen pH can be predicted by urine pH within 2 h after feeding. Fecal pH may be used as a tool to predict rumen pH depression as early as 7 and 15 h after feeding for JER and HOL cows respectively.

One alternative to modulate rumen environment is the utilization of direct-fed microbials (DFM) that has become common in the dairy industry, but questions regarding their value and mode of action remain prevalent. The objective of this study was to evaluate the effects of a DFM (*Bacillus pumilus* 8G-134) on pre-partum performance and prevalence of health disorders in early lactation. Thirty-nine multiparous Holstein cows were fed a TMR according to NRC (2001) recommendations and assigned to 2 treatments in a completely randomized block design. Cows in the direct-fed microbial treatment (**DFMt**, n = 21) received 5.0×10^9 cfu of *B. pumilus* in 28 g of media, whereas, cows in the control treatment (**CON**, n = 22) received 28 g of media. Treatments were top-dressed on the TMR daily. Treatments were applied from 21 d before expected calving date to 21 d after calving. Blood samples were analyzed for beta-hydroxybutyrate at d 5 and 14 after calving. Cows that had blood beta-hydroxybutyrate concentrations higher than 1.2 mmol/L were classified as experiencing sub-clinical ketosis. Treatment DFM tended to have lower haptoglobin concentration than DFMt cows on d 14. Treatment DFM had higher IgA concentration than CON cows on the first week after calving. Other health disorders recorded were retained placenta, displaced abomasum, clinical ketosis, and pneumonia. Fecal scores were recorded daily. Statistical analysis was performed using the MIXED and FREQ procedures of SAS. The DMI, BW, and BCS were not different between

treatments through experiment. Cows fed DFMt had higher milk yield, fat corrected milk, energy correct milk, fat production and protein production on the second week of lactation, however there was no differences between treatments on milk yield and any milk components. Cows fed DFMt tended to have higher feed efficiency than cows fed CON. Cows fed DFMt tended to have less incidence of sub-clinical ketosis at d 5 but not at d 14 when compared to cows receiving CON. There were no treatment effects on the incidence of displaced abomasum, clinical ketosis, or pneumonia. Although occurrence of retained placenta was low, cows fed CON tended to have higher incidence of retained placenta than cows fed DFMt. Cows that received DFMt tended to have higher fecal score than CON. In conclusion, cows receiving DFMt tended to have decreased incidence of sub-clinical ketosis than cows receiving CON, potentially by reducing negative-energy balance after calving. Cows receiving DFMt tended to have higher feed efficiency than CON. Dry matter intake, BW, BCS were not affected by DFM supplementation. Cows receiving DFMt tended to have firmer fecal and potential better immunity than CON cows.

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CHAPTER 1

Literature Review

Ruminant animals

Ruminant animals are classified in the subclass referred to as ungulates (hooved mammals) and in the order Artiodactyla (even-toed) and the suborder, Ruminantia (Church, 1988). Ruminants are mammals that are able to acquire nutrients from plant-based food by fermenting it in a specialized stomach prior to digestion, principally through bacterial actions. The process typically requires regurgitation of fermented ingesta (known as cud), and chewing it again. The process of rechewing the cud to further break down plant matter and stimulate digestion is called "Rumination". The word "ruminant" comes from the Latin ruminare, which mean "to chew over again". Zoologists have described more than 180 species of ruminants, but human efforts in ruminant domestication have largely been devoted to cattle (bovines), sheep (ovines), and goats (caprines) (Russell, 2002). Modern societies tend to emphasize their technologies and industries, but these developments are in large part a result of increased agricultural efficiency. Ruminant animals provide the bulk of our meat and milk (Russell, 2002).

Ruminal environment

Fermentation in the rumen is the process by which microbiological activities convert components of the diet to products that are useful [volatile fatty acids (VFA), microbial protein, B-vitamins], useless (CH_4 , CO_2), or even harmful (ammonia, nitrate) to the host animal (Owens and Goetsch, 1988). The ruminal fermentation occurs under anaerobic conditions with optimum environment (pH, osmolality, and temperature). The host animal maintains the environment by supplying substrate for microbial fermentation, secreting saliva as buffer, absorbing VFA through

rumen epithelium, mixing contents, and removing products and indigestible residues by eructation or to passage to the lower digestive tract.

pH

The maintenance of a stable hydrogen ion concentration ($[H^+]$) in body fluids is essential to life. The concept of $p[H]$ was first introduced by Danish chemist Søren Peder Lauritz Sørensen at the Carlsberg Laboratory in 1909. The pH of a solution is defined as the logarithm to the base 10 of the reciprocal of the H^+ concentration ($[H^+]$), that is, the negative logarithm of the $[H^+]$. The pH of water at 25°C, in which H^+ and OH^- ions are present in equal numbers, is 7.0. For each pH unit less than 7.0, the $[H^+]$ is increased 10-fold; for each pH unit above 7.0, it is decreased 10-fold. The pH is scale from 0-14, acidic is pH lower than 7 and basic is pH higher 7.

Widespread and longtime usage of pH has caused it to be considered mathematically equivalent to other biological variables. This misconception, coupled with expanded use of computer programs to statistically analyze experimental data, has increased the frequency with which pH is misused. Murphy (1982) reported that hydrogen ion concentration need to be used when conduct statistical analysis. Since the pH is negative logarithm transformation of H^+ concentration, the mean of two pH values is equal to logarithm transformation of mean of corresponding H^+ concentrations. The error is increased as H^+ concentration increases (pH decrease), and the error is up to 19% (Murphy, 1982).

Physiology of rumen pH

Normal rumen pH can range from 5.7 to 7.2. It is a common practice to feed diets with high fermentable carbohydrate content in beef feedlot and commercial dairy farms. However, rumen pH can drop dramatically if the fermentation acid production exceeds the cow's buffering capacity.

Subacute ruminal acidosis (SARA) has been defined as the pH depression between pH 5.2 and 5.6 for at least 3 h per day (Gozho et al. 2005). Subacute ruminal acidosis is usually associated with DMI depression, milk production depression, decreased feed efficiency, rumenitis, diarrhea, laminitis, inflammation, liver abscesses, higher culling, and death rate in dairy cattle (Nocek, 1997; Kleen et al., 2003; Stone, 2004; Alzahal et al., 2007; Enemark, 2008).

Acid production

Rumen pH depression usually caused by ingesting large amounts of rapidly fermentable carbohydrate. Soluble carbohydrates have a faster fermentation rate than pectin and cellulose in the rumen environment (Figure 1.1). Baldwin et al. (1977) reported that soluble carbohydrates have the greatest fermentation rate during the last 2 h of the feeding period and decreased rapidly thereafter, due to depletion of substrate. Rumen pH was depressed by the VFA accumulated from the fast fermentation. Oetzel et al (1999) observed that increased dry mater intake (DMI) was associated with lower ruminal pH. Other researchers (Wolin and Miller, 1983, Krause and Oetzel, 2005) found that increased days in milk was associated with increasing risk of low rumen pH after about the 3rd month of lactation. These results demonstrate that large amounts of soluble carbohydrates ingested in the rumen can produce a large amount of VFA in a short time, but the production can exceed the absorption rate of the cattle and therefore depress rumen pH until the VFA are absorbed.

Most carbohydrates consumed by ruminants are polymers of glucose present in the form of cellulose or starch (Fahey and Berger, 1988). Carbohydrates must undergo hydrolysis to provide glucose as fermentation substrate. Fermentation of glucose is mainly through glycolysis, and generates 2 ATP and 2 NADH₂ per glucose. The energy generated is mainly used by the bacteria, and this process doesn't affect intracellular or extracellular pH. The product of glycolysis is

pyruvate, which is the common intermediate before being converted to propionate, acetate, butyrate, CO₂, and CH₄ (Fahey and Berger, 1988). However the ratio of end products can change, depending on the type of substrates, rumen environment, and bacterial population structure.

Under a normal rumen pH environment, acetate can be generated by two pathways from pyruvate. First, the major pathway is converting pyruvate to acetyl-CoA and formate with the enzyme pyruvate formate-lyase (PFL), which is sensitive to low pH (Russell and Hino, 1985), then acetyl-CoA and formate are converted to acetate, CO₂ and H₂, with generation of ATP for bacterial use. Another pathway involves pyruvate-ferredoxin oxidoreductase, yielding reduced ferredoxin, CO₂ and acetyl-CoA (Fahey and Berger, 1988).

Propionate can be generated from pyruvate through 2 pathways, the succinate pathway and acrylate pathway. The succinate pathway contributes 80 to 90% of total propionate production, whereas, the acrylate pathway contributes up to 40% of the production. Three enzymes are involved in the succinate pathway: phosphoenolpyruvate (PEP) carboxykinase, which converts PEP plus ADP or GDP plus CO₂ to oxaloacetate (OAA) + ATP or GTP; pyruvate carboxylase, which converts pyruvate plus CO₂ plus ATP to OAA plus ADP; and methylmalonyl-CoA carboxyltransferase, a biotin-containing enzyme involved in the conversion from succinate to propionate with methylmalonyl-CoA as intermediate (Baldwin and Allison, 1983). The acrylate pathway involves the formation of lactate, conversion of lactate to acrylyl-CoA via the intermediate phospholactyl-CoA, and reduction of acrylyl-CoA to propionyl-CoA via a NADH₂-linked crotonyl-CoA reductase (Baldwin and Milligan, 1964).

Butyrate and higher fatty acids are normally formed via a reversal of the β-oxidation pathway. An alternative pathway is malonyl-CoA combined with acetyl-CoA, followed by generation of butyrate via acetoacetyl CoA. The second pathway is the major way to generate

valerate and longer chain fatty acids.

Compared with multiple pathways to generate VFA, lactate is only generated via a single pathway under anaerobic conditions. Pyruvate reduced by NADH to lactate, catalyzed by lactate dehydrogenase (LDH). This pathway produces a relatively small amount of energy. However, this results in regeneration of NAD^+ , which is required for glycolysis, and thus allows the continued production of ATP. Under a normal rumen environment, lactate does not accumulate in the rumen at concentrations above 5 μM (Owens et al., 1998). It will instead go through the acrylate pathway to generate propionate as described above.

From a practical standpoint, a large amount of rapid fermentable carbohydrate ingested in the rumen will provide sufficient substrate for glycolysis and further fermentation. This will produce large amount of VFA, which will accumulate as the absorption rate is exceeded, and eventually change the rumen environment by lowering the rumen pH.

Changes of H^+ concentration affect the biochemical pathway of glycolysis and lactate production and, therefore, change the molar proportion of VFA. More free hydrogen ions will present in the rumen as pH decreases. The free H^+ will slow down glycolysis by inhibiting NAD oxidation. The accumulation of small amounts of H_2 inhibits the production of H_2 from NADH (Wolin, 1983; Russell, 1998' USDA, 2013), which might be the reason why glycolysis is blocked during acidic conditions. Therefore, the acetyl-CoA has to be converted to ethanol or lactate to regenerate NAD^+ , instead of generating acetate. The ethanol then be converted to higher carbon VFA: butyric, valeric, and caproic acids (Durix et al., 1991).

In the pyruvate to lactate pathway, Russell and Hino (1985) reported that LDH has the greatest activity at pH 5.5, and decreased activity as pH increases. In contrast, pyruvate formate-lyase has optimum activity at pH higher than 6, and activity decreases as pH decreases (Figure

1.3). Therefore, the decrease in pH and the accumulation of lactate inhibits PFL and the subsequent formation of formate and acetate, and benefits LDH, thus favoring lactate production (Figure 1.3). The NAD^+ generated in this pathway will favor glycolysis as well. As the pH decreases, the pathway becomes the major donor of NAD^+ , which leads to the accumulation of lactate acid. For the conversion of lactate to pyruvate, LDH is not only a pH-sensitive enzyme, but also a NAD-dependent enzyme. The role of lactate in rumen metabolism can be summarized as the following: when the production of reduced equivalents in anaerobic rumen microorganisms is higher than the utilization in the normal end-products, production of lactic acid is a possible way to remove reduced equivalents from the cell into the rumen fluid (Counotte et al., 1981). This typically happens when glycolytic flux (hexose units fermented per unit time per microorganism) is high, which is typical during diet adaptation (Counotte et al., 1981).

Acid absorption, utilization and removal from the rumen

Fermentation acids are removed from the rumen by absorption across the rumen wall and by passage from the rumen through the omasal orifice (Allen, 1997). Volatile fatty acids are fermentation end products, and the majority of VFA are absorbed through the rumen and intestinal epithelium. About 20 to 40% of all VFA produced in the rumen passes into the lower digestive tract (Tamminga and Van Vuuren, 1988; Dijkstra, 1993; Penner et al., 2009). The rest is absorbed through the rumen epithelial cells.

Fermentation acids have undissociated and dissociated forms in the normal rumen environment. The proportion of each form is different for each fermentation acid, because of different pKa values. Lactic acid has a lower pKa (3.86) than acetic acid (pKa = 4.76), propionic acid (pKa = 4.87) or butyric acid (pKa = 4.82). The three major VFAs have similar pKa values,

and a $pK_a = 4.8$ is commonly used for the mixed VFA in rumen liquid.

The proportion of VFA absorbed through the rumen wall is increased at lower ruminal pH values (Dijkstra et al., 1993). Absorption rates are in the order of acetic < propionic < butyric acid at rumen pH from 4.5 to 7.2 (Dijkstra, 1993). However, production of 1 mole of butyric acid from 1 mole of hexose would reduce pH relatively less than the production of 2 moles of propionic or acetic acids from 1 mol of hexose (Dijkstra et al., 2012). Therefore, the effect of pH is acetic > propionic > butyric acid (Allen, 1998). The VFA absorption rate depends on the chain length, because of the lipophilic permeability of these VFA (Walter, 1986). Research shows that the ability of propionic acid to permeate through egg phosphatidylcholine decane bilayers is 5.1 times that of acetic acid, and the permeation coefficient of n-butyric acid is 2.7 times that of propionic acid (Walter, 1986).

As noted already, most of the VFA produced from the rumen fermentation are absorbed through the epithelium, and the VFAs exist in undissociated form and dissociated form (Gäbel, 2002). Three mechanisms of absorption are critical to maintain the rumen pH. These mechanisms have been well reviewed by Gäbel (2002) and Aschenbach et al. (2009), and are summarized in Figure 1.4. The proportion of VFA absorbed by different mechanisms is unclear.

(1) Passive, lipophilic diffusion is the major way of VFA absorption. The VFA in undissociated form are highly lipid soluble and, consequently, may permeate biological membranes directly (Leo et al., 1971; Walter, 1986). Passive diffusion leads to elimination of protons from the ruminal contents (Rechkemmer et al., 1995). The concentration of undissociated VFA is dependent on pH; the lower the pH, the higher the percentage of each organic acid in non-dissociated form and the greater the absorption rate (Owens et al., 1998). This stimulation of absorption has been reported from both cattle and sheep research (Stevens,

1966; Weigand, 1972; Thorlacius and Lodge, 1973; Dijkstra, 1993; Kramer, 1996; Pitt, 1996; Sehested et al., 1999). The dissociated forms of VFAs are not lipophilic (Gäbel and Aschenbach, 2006), and therefore need specific transport mechanisms, which are discussed in the following.

(2) Bicarbonate-dependent VFA absorption involves the non-dissociated form exchange for HCO_3^- , this process probably being mediated by an anion exchange system. The HCO_3^- is derived from the dissociation of H_2CO_3 , whose production is catalysed by intracellular carbonic anhydrase. After uptake, VFA can be extruded as undissociated acids on the basolateral side, extruded as dissociated acids on the basolateral side with the aid of a (suspected) transport mechanism, or metabolized through oxidative or anaerobic pathways (Gäbel, 2002).

(3) Bicarbonate-independent VFA absorption, i.e. Na^+/H^+ exchanger (Martens, 1991; Sehested et al., 1999), is the remaining mechanism. The VFA are metabolized to ketone bodies, lactate, and CO_2 through oxidative or anaerobic pathways. Acetate may be partially metabolized to acetoacetate by the rumen epithelium. Most of butyrate is converted to BHBA, which a more important fuel source for the rumen epithelium than acetic or propionic acids (Kristensen, 2005). Beta hydroxybutyrate exerts mitotic effects on the rumen epithelium (Mentschel et al., 2001) that can help stimulate acid removal from the rumen through the rumen wall. CO_2 from the oxidative breakdown of VFA can be used for conversion to H_2CO_3 . Carbonic acid dissociates into H^+ and HCO_3^- . After dissociation of H_2CO_3 , the HCO_3^- may be used to drive the anion exchanger and H^+ can be extruded to the lumen side by Na^+/H^+ exchange systems (Gäbel, 2002). However, a correlation between the activity of Na^+/H^+ exchange and transport of VFA could not be demonstrated either on ovine or bovine ruminal epithelium (Kramer, 1996; Sehested et al., 1999).

Ruminal lactic acid has 2 major sources. First, a considerable amount is produced from feed ingested into the rumen, mainly from silage. The other source is rumen fermentation by lactate

producers. Lactate acid is an intermediate of rumen fermentation, which is rapidly metabolized by lactate utilizers to VFA when pH is higher than 5.0 (Therion, 1982). The same author reports that the lactate utilizers have slow growth rate under acidic conditions, whereas the growth rate of lactate producers increases as pH decreases. As the pH nears 5.0 or below for a sustained period, the growth of lactate utilizers is inhibited, and hence lactate begins to accumulate rapidly (Nagaraja and Titgemeyer, 2007). This situation is called acute acidosis, which is fatal to the animal unless treated.

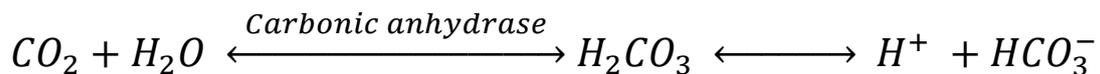
In addition, $\text{NH}_3/\text{NH}_4^+$ ($\text{pK}_a = 9.21$) also has a role in rumen pH balancing and is important for VFA absorption. Under acidic rumen conditions, urea enters the rumen via secretion across the ruminal epithelium. Bacterial urease converts urea to NH_3 , which is a very potent buffer. The NH_3 binds H^+ immediately to form NH_4^+ in the rumen environment. In dairy cows, the absorption of ammonia can reach 25 mol/d (Delgado Elorduy, 2002), whereas up to 10 mol/d of urea is secreted (Gozho et al., 2008). Therefore, rumen N absorption removes net protons from the system. The interaction of ammonia and VFA has been well demonstrated by Aschenbach (2011) and is depicted in Figure 1.5.

Acid-base metabolism

Physiological pH for cattle arterial blood is 7.4 and venous blood is 7.3. Conversely, gastric fluid pH is quite acidic (on the order of 3.0) and pancreatic secretions can be quite alkaline (on the order of 8.0). Enzymatic activity and protein structures are frequently sensitive to pH. In any given body or cellular compartment, pH is maintained to allow for maximal enzyme and protein efficiency (Barrett and Ganong, 2012).

Carbonic anhydrase is important in acid-secreting epithelia as well as in other cells, such as red blood cells, in which rapid interconversion of large quantities of CO_2 to HCO_3^- is required.

In proximal tubule, carbonic anhydrase exists not only within cells but also on the luminal brush border membrane, where it accelerates the dehydration of carbonic acid to CO₂, thereby allowing the large amount of HCO₃⁻ reabsorption in that segment. However, intracellular carbonic anhydrase is present in the tubule cells that transport CO₂ and HCO₃⁻ in the kidney (Cecil et al., 2012).



When a strong acid is added to the blood, the major buffer reactions are driven to the left. The blood levels of three “buffer anions” Hb⁻ (hemoglobin), Prot⁻ (protein), and HCO₃⁻ consequently drop (Barrett and Ganong, 2012). The anions of the added acid are filtered into the renal tubules. They are accompanied (“covered”) by cations, particularly Na⁺, because electrochemical neutrality must be maintained. The tubules replace the Na⁺ with H⁺ and in so doing reabsorb equimolar amounts of Na⁺ and HCO₃⁻, thus conserving the cations, eliminating the acid, and restoring the supply of buffer anions to normal (Barrett and Ganong, 2012).

The two main compensatory systems are respiratory compensation and renal compensation. The liver is involved in the acid-base balance system as well (Haussinger, 1990). Amino acid metabolism and acid-base homeostasis are intimately related (Patience, 1990).

The respiratory system compensates for metabolic acidosis by altering ventilation, and consequently, the Partial Pressure of Carbon Dioxide (Pco₂), which can directly change blood pH (Barrett and Ganong, 2012). Respiratory mechanisms take place as a fast response since in response to metabolic acidosis, ventilation is increased, resulting in a decrease of Pco₂ (Barrett and Ganong, 2012).

As large amounts of acid are ingested and produced, acids in the blood are quickly increased. The H₂CO₃ that is formed is converted to H₂O and CO₂, and the CO₂ is rapidly excreted via the

lungs. In actuality, as soon as metabolic acidosis begins, respiratory compensation is invoked and the large shifts in pH depicted do not occur (Barrett and Ganong, 2012). For complete compensation from acidosis, renal compensatory mechanisms are invoked. The kidney responds to acidosis by actively secreting fixed acids while retaining filtered HCO_3^- (Barrett and Ganong, 2012).

Within the kidney, the functional unit is the nephron. The kidneys play an essential role in the regulation of water homeostasis, electrolyte composition (e.g. Na, Cl, K, HCO_3^-), regulation of extracellular volume, and acid–base homeostasis. The cells of the proximal and distal tubules, like the cells of the gastric glands, secrete H^+ ions (Barrett and Ganong, 2012).

The secreted H^+ ion combines with filtered HCO_3^- to form H_2CO_3 and the presence of carbonic anhydrase on the apical membrane of the proximal tubule catalyzes the formation of H_2O and CO_2 from H_2CO_3 . The apical membrane of epithelial cells lining the proximal tubule is permeable to CO_2 and H_2O , and they enter the tubule rapidly. About 80% of the filtered load of HCO_3^- is reabsorbed in the proximal tubule. Inside the cell, carbonic anhydrase is also present and can catalyze the formation of H_2CO_3 from CO_2 and H_2O . The H_2CO_3 dissociates into H^+ ions and HCO_3^- ; the H^+ is secreted into the tubular lumen, as mentioned above, and the HCO_3^- that is formed diffuses into the interstitial fluid. Thus, for each H^+ ion secreted, one Na^+ ion and one HCO_3^- ion enter the interstitial fluid (Barrett and Ganong, 2012).

This is in contrast to what occurs in the distal tubules and collecting ducts, where H^+ secretion is relatively independent of Na^+ in the tubular lumen. In this part of the tubule, most H^+ is secreted by an ATP-driven proton pump. In acidosis, the number of H^+ pumps will increase (Barrett and Ganong, 2012).

Renal acid secretion is altered by changes in the intracellular Pco_2 , K^+ concentration,

carbonic anhydrase level, and adrenocortical hormone concentration. When the P_{CO_2} is high, more intracellular H_2CO_3 is available to buffer the hydroxyl ions and acid secretion is enhanced (Barrett and Ganong, 2012).

The amount of acid secreted depends upon the subsequent events that modify the composition of the tubular urine. The maximal H^+ gradient against which the transport mechanisms can secrete in humans corresponds to a urine pH of about 4.5; that is, an H^+ concentration in the urine that is 1000 times the concentration in plasma. A pH 4.5 is thus the limiting pH, which is normally reached in the collecting ducts. If there were no buffers that “tied up” H^+ in the urine, this pH would be reached rapidly, and H^+ secretion would stop (Barrett and Ganong, 2012).

Renal mechanisms of urinary acidification are adaptable. Three buffers of importance in the renal handling of acid and its secretion into the lumen are bicarbonate, dibasic phosphate, and ammonia. Because the amount of phosphate buffer filtered at the glomerulus cannot be increased, urinary excretion of acid via the phosphate buffer system is limited, as the concentration of phosphate in the plasma, and consequently in the glomerular filtrate, is normally only 1.5 mEq/L, whereas HCO_3^- is about 24 mEq/L (Barrett and Ganong, 2012). Therefore, the major buffers to maintain acid-base homeostasis are bicarbonate and ammonia systems.

In acidosis, the capacity to reabsorb HCO_3^- increases, including increased expression of the transporters involved in acidifying the urine. At the same time, increased glutamine uptake into proximal cells and ammonia production enables increased acid excretion and the generation of new HCO_3^- in the distal nephron (Barrett and Ganong, 2012).

When the plasma HCO_3^- concentration is low, all the secreted H^+ is being used to reabsorb HCO_3^- , and more H^+ becomes available to combine with other buffer anions. Therefore, the

lower the plasma HCO_3^- concentration drops, the more acidic the urine becomes and the greater its NH_4^+ content. In human, when the diet calls for excretion of acids, urine pH will fall to a value as low as 5.0, and the urine will become nominally free of bicarbonate (Cecil et al., 2012).

The ammonia buffering system allows secreted H^+ to combine with NH_3 , and this occurs in the proximal tubule and in the distal tubules. Reactions in the renal tubular cells produce NH_4^+ and HCO_3^- . The principal reaction producing NH_4^+ in cells is conversion of glutamine to glutamate. This reaction is catalyzed by the enzyme glutaminase, which is abundant in renal tubular cells. Glutamic dehydrogenase catalyzes the conversion of glutamate to α -ketoglutarate, with the production of more NH_4^+ . Subsequent metabolism of α -ketoglutarate utilizes 2 H^+ , freeing 2 HCO_3^- . During acidosis HCO_3^- is spared in an attempt to maintain homeostasis. This is accomplished by reduced ureagenesis, which spares HCO_3^- and increases renal ammonia synthesis (Guder et al., 1987). Renal and hepatic N metabolism are linked by an inter-organ glutamine flux, coupling both renal ammoniogenesis and hepatic urea production to systemic acid-base regulation (Guder et al., 1987).

Buffer

A "buffer" may be defined as a salt of a weak acid or oxide or hydroxide, which neutralizes acids present in feedstuffs or acids produced during nutrient digestion and metabolism (Chalupa and Schneider, 1985). The cow has three primary means of buffering either acid ingested or acid produced by rumen microorganisms. These include: 1) buffers naturally occurring in saliva, 2) buffering capacity of ingested feed, and 3) added dietary buffers (Erdman, 1988).

Salivary Function and production

Church (1988) summarized ruminant saliva based on studies from multiple laboratories. Saliva is produced in copious amount by five sets of paired glands and three unpaired glands,

with the parotid glands apparently accounting for 40 to 50% of total production. Dry matter content is said to range from 1.0 to 1.4% and ash content from 0.7 to 1.5%. The major inorganic components in the parotid saliva are P, and CO₂ with lesser amounts of Cl and K, small amounts of Ca and Mg, and variable amount of N and S.

The major functions of saliva are to aid in mastication, providing buffering, anti-frothing, and N recycling. There has been a lot of attention on the buffering capacity of the saliva, due to the study of acidosis. This section is mainly a review of the studies on buffering function.

It is common practice to increase energy density in lactation and finishing beef diets, which stimulates production rates of VFA. However, the VFA produced in the rumen has to be absorbed or buffered as soon as possible, in order to reduce the risk of rumen acidosis. Saliva contains inorganic buffers, such as sodium bicarbonate, that contribute to the neutralization of the organic acids produced during fermentation in the rumen (Church, 1988). If a net of ~5 mol of VFA are produced in the rumen per kilogram of DMI, > 4.6 mol H⁺ are released in parallel at a ruminal pH > 6. A small fraction of these protons would be sufficient to induce a fatal drop in ruminal pH. The pH drop during periods of intensive fermentation, however, is usually very mild. This has long been attributed solely to proton buffering by salivary secretion of HCO₃⁻ and HPO₄⁻ (Gaebel and Aschenbach, 2006). Dairy cows saliva production ranges up to 308 L/d (Cassida and Stokes, 1986). Woodford et al. (1986) reported that 18% of drinking water bypassed the rumen when water was withheld for 4.5 h following feeding. Some bypass of saliva also could be expected because of incomplete mixing in the rumen, although saliva contributes nearly 70% of the liquid content of rumen. The pH of saliva is slightly alkaline, ranging from 7.7 to 8.7.

Eating and ruminating are the major stimuli for saliva production (Church, 1988). Intake of physically effective fiber stimulates chewing activity (Mertens, 1997). Feeding more grain and

less fiber, as well as reducing forage particle size, also reduces the amount of time spent chewing (Woodford et al., 1986; Mertens, 1997; Yang et al., 2001; Maekawa et al., 2002; Beauchemin et al., 2003b; Fairfield et al., 2007; Yang and Beauchemin, 2007).

Researchers gave different fiber definitions to predict their capacity to stimulate chewing. Effective NDF (eNDF) used to be considered a key factor to stimulate chewing. The eNDF is related to the total ability of a feed to replace forage or roughage in a ration so that the percentage of fat in milk produced by cows eating the ration is effectively maintained (Mertens, 1997). But eNDF as defined may not stimulate chewing, since grain has some eNDF, but it might not be sufficiently long to stimulate rumination. To overcome this problem, Mertens (1997) defined physically effective NDF (peNDF) as that which influences chewing activity and the biphasic nature of ruminal contents (floating mat of large particles on a pool of liquid and small particles). The Penn State Particle Separator has been used to determine the peNDF, because it provides a good description of dietary particle length and its effects on chewing time, chewing index, rumen pH, and the ratio of total chewing activity to peNDF (Yang and Beauchemin, 2006).

Studies suggested that increased chewing time did not increase total daily saliva secretion, because increased saliva during eating and rumination was associated with decreased saliva production during resting, because of reduced resting time (Maekawa et al., 2002). This indicates that an over-supply of coarse fiber is not necessary to maintain saliva secretion. Total saliva flow can be predicted as a linear function of time spent resting, eating, and ruminating and their respective rates of flow (Allen, 1997):

$$SBF = [(IT \times IF) + (ET \times EF) + (RT \times RF)] \times BCS$$

where;

SBF = salivary buffer flow (milliequivalents per day),

IT = time spent resting (minutes per day),

IF = resting flow (liters per minute),

ET = time spent eating (minutes per day),
EF = eating flow (liters per minute),
RT = time spent ruminating (minutes per day),
RF = ruminating flow (liters per minute), and
BCS = Bicarbonate of the saliva (152 meq/L).

Dietary buffer

Feedstuffs influence the ruminal acid-base status through their pH, buffering capacity, stimulation of salivation, and the components that they release during fermentation (Le Ruyet et al., 1992). Work primarily dealing with silage fermentation has shown that forages have inherent buffering or acid-consuming capacities (Playne and McDonald, 1966; Wilkins, 1982). It is likely that in addition to the role that forages play in secretion of saliva during eating and rumination, the inherent buffering capacity of forage results in changes in rumen pH and also whole animal acid-base metabolism (Erdman, 1988). For example, the ingestion of corn silage, which has organic acids from fermentation, at roughly pH 3.9 by a cow with average rumen pH of 6.0, would require an equivalent of 33 g of NaHCO_3 /kg silage DM intake to maintain rumen pH at 6.0, whereas fresh forages or hays with pH at or near 6.0 would not require added NaHCO_3 (Erdman, 1988).

Additive buffer is a material that when present in aqueous solution, causes an effective resistance to change in pH of that solution when a strong acid or base is added (Erdman, 1988). For a compound to act as a dietary buffer under physiological conditions, Erdman (1988) proposed the following criteria that have to be met: 1) it must be water soluble, 2) it must be a weak acid or base or salt thereof, 3) its equivalence point (pKa) must be near the physiological pH of the system to be buffered, and 4) in addition to chemical properties and cost, physical properties and palatability would be important consideration for buffer selection by dairy producers and feed manufacturers (Erdman, 1988).

Published experiments documented that the use of NaHCO_3 , MgO , KHCO_3 , K_2CO_3 , and other buffering agents in low forage diets are effective in increasing rumen pH, rumen acetate:propionate molar ratio, and milk fat percentage (Erdman, 1988). Other chemicals which proved to be good alkalizing agents included sodium carbonate, potassium carbonate, sodium hydroxide, and calcium hydroxide (Staples and Lough, 1989).

Consequences of low rumen pH

Sub-acute ruminal acidosis can lead to nutritional and health consequences. Nutritional consequences include reduced fiber digestion, depressed feed intake, decreased milk fat, and reduced absorption of end-products. Health consequences include hindgut acidosis, increased heart rate, increased respiratory rate, inflammation or parakeratosis of the rumen wall, diarrhea, liver abscesses, laminitis, lameness, and reproductive failure. As a result, culling rates may be increased (Plaizier et al., 2008).

Changes in volatile fatty acids and milk composition

Fermentation products differ with diet composition because different microbes have higher affinities for and prefer to digest specific carbohydrates (Owens and Goetsch 1988). In general, rumen microbes can be grouped into either cellulolytic or amylolytic microbes. Cellulolytic microbes mainly produce acetate with high roughage diet, whereas amylolytic microbes produce a greater proportion of propionate with high concentrate or starch diets.

In the situation of SARA, rumen pH is depressed after large amounts of fermentable carbohydrate are ingested. Dijkstra (2012) reported that pH significantly affects the type of VFA formed, and the effects of pH need to be included in models if type of VFA and VFA profile are to be predicted accurately (Dijkstra et al., 2012). In response to the decreases in pH, acetic acid proportions decreased, whereas propionic and butyric acid molar proportions increased

(Marounek et al., 1985).

It has been known that increased propionic acid availability can promote insulin secretion, and therefore more energy deposited in body tissue, whereas increased acetic acid availability can promote milk fat, contents of C12, C14, and C16 fatty acids in milk fat, and decreased C18:1 in milk fat. Changes in the VFA profile are related to milk yield and composition (Thomas and Martin, 1988). The results obtained by Griinari et al. (1998) support the theory that a low rumen pH caused by feeding a low fiber diet results in incomplete biohydrogenation of fatty acids and increases formation of many isomers of *trans*-octadecenoic acids, and especially the *trans*-10, *cis*-12 conjugated linoleic acid that causes milk fat depression (Bauman and Griinari, 2001). Possible reasons for milk fat depression have been suggested to be mechanism involving coordinate regulation of key lipogenic enzymes in the mammary gland. Peroxisome proliferator-activated receptors (PPAR) and sterol regulatory element binding proteins (SREBP) might be the key regulators in the fatty acid uptake and transport, both regulated by polyunsaturated fatty acids (Bauman and Griinari, 2003).

Addition of buffering substances or monensin can prevent the formation of *trans*-C18:1 fatty acids (Kennelly et al., 1999) and lessen the depression of milk fat content (Mutsvangwa, 2003).

Osmolality

Ruminal osmolality normally ranges from 240 to 265 mOsm/L with roughage diets and 280 to 300 mOsm/L with concentrate diets (Garza and Owens, 1989). Osmotic pressure pulls or pushes water through membranes depending on relative concentrations of dissolved materials. As rumen pH is depressed, the rate of fermentation production exceeds the limitation of individual cattle's physical absorption capacity. There is significant increase of glucose, lactate acid, and VFA concentrations in the rumen contents, which can increase osmolality in the rumen. When

ruminal osmolality is markedly greater than blood osmolality, water from blood is drawn rapidly inward through the rumen wall. Rapid influx to neutralize osmotic pressure swells the ruminal papillae and can pull patches of the ruminal epithelium into the rumen by stripping the internal surface layers of the rumen wall from the underlying layers (Eadie et al., 1970).

Rumenitis, liver abscess, and inflammation

Subacute ruminal acidosis (SARA) is regularly connected to many health disorders. In the situation of SARA, rumen microbial populations are shifted, acid is accumulated, pH is depressed, and osmolality is elevated. The change of rumen environment leads to physical status changes that eventually create health problems.

Acid accumulation and increased osmolality of digesta can lead to damage and inflammation of the rumen epithelium and allow for systemic entry of endotoxins, bacteria, or amines that can cause inflammation or infection (Owens et al., 1998; Plaizier et al., 2008). The low pH from acidosis creates lesions in the rumen wall (Nocek, 1997; Kleen et al., 2003). This is believed to lead to a parakeratosis of the ruminal epithelium, exaggerated by the presence of processed, e.g. pelleted, feedstuffs. Parakeratosis may lead to rumenitis and inhibit the rate of VFA absorption for months or years (Krehbiel et al., 1995), particularly due to the presence of micro-abscesses within the ruminal mucosa (Szemerédy and Raul, 1976).

After a period of increased VFA absorption, the pathological alterations of the rumen epithelium eventually hinder the absorption activity. The function of the ruminal mucosa as a barrier between ruminal environment and bloodstream is impaired, enabling bacteria to translocate via the ruminal mucosa into the portal blood flow, colonizing the liver tissue and from there spreading to other tissues of the body such as heart, lungs, and kidneys (Nordlund et al., 1995; Nocek, 1997).

Several studies have shown that grain-induced SARA causes an increase in acute-phase proteins in blood, which is an indicator of inflammation (Gozho et al., 2005, Gozho et al., 2006, 2007). Rumen inflammation mainly caused by high rumen osmolality and translocation of free bacterial lipopolysaccharide endotoxin (LPS) (Plaizier et al., 2012), which is a toxin for the animal.

High ruminal osmolality can rapidly draw water from blood into the lumen, and disrupt the structure of the rumen epithelium (Eadie et al., 1970). This allows LPS transfer to the blood stream to induce immune response. However, large amount of rumen LPS can transfer to the blood stream whenever rumen mucosa barrier function reduced. The topic related to LPS is discussed separately.

Decrease in DMI and fiber digestion

Depression of DMI in cattle can be caused by either physical fill (distention) or metabolic mechanisms. In the SARA situation, an elevation in osmotic pressure in the rumen may be sensed by receptors in the rumen wall to inhibit feed intake (Carter and Grovum, 1990) and increase the digesta passage rate. However, distention of the abomasum inhibits the outflow, which complicates removal of fluid and acid from the rumen (Owens et al., 1998). Inflammation triggered by SARA can be considered as metabolic signal to reduce feed intake (Weingarten, 1996; Andersen, 2003).

The reduction in fiber digestion that occurs during SARA is most likely the result of the acid sensitivity of the cellulolytic rumen bacteria (Shi and Weimer, 1992). Subacute ruminal acidosis induced by adding grain pellets to the TMR of lactating dairy cows reduced the 24- and 48-h in situ NDF degradability of forages by an average of 20.5% and 24.8%, respectively (Krajcarski-Hunt et al., 2002). Plaizier et al. (2001) found that grain-induced SARA reduced the 24- and 48-h

in situ NDF degradability of mixed hay by 19.6% and 21.8%, respectively. Cellulolytic bacteria generally cannot tolerate a rumen pH below 6.0, which will reduce their numbers in the rumen and, subsequently, reduce fiber digestion (Shi and Weimer, 1992). The reduced fiber digestibility caused by SARA reduces the net energy content of the diet and might impact feed intake as well (Allen, 2000).

Laminitis

The scientific name for laminitis is *pododermatitis aseptic diffusa*, which is an aseptic inflammation of the dermal layers inside the foot (Nocek, 1997). Feet and leg problems have been reported as the fourth largest reason for disposal of dairy cows (Canwest, 2004). Laminitis has a multi-factored etiology and is thought to be associated with several, largely interdependent factors (Nordlund and Garrett, 1994). Nutritional management has been identified as a key component in the development of laminitis, particularly the feeding of increased fermentable carbohydrate, which results in an acidotic state (Nocek, 1997).

The mechanism of laminitis has been well summarized by Nocek (1997). Rumen pH depression has been considered as one of the metabolic insults, since SARA triggers the release of vasoactive substances, such as histamine and LPS of bacterial origin that damage the capillaries of the lamellae in the foot and cause hemorrhage, inflammation, and lameness (Nocek, 1997). The initial mechanical damage triggers a worsening sequence if the metabolic insult in the rumen persists. Eventually the laminar layer separates, and the pedal bone takes on a different configuration in relationship to its position in the corium and dorsal wall (Nocek, 1997), causing lameness.

Bacterial LPS

Bacterial LPS is a component of the outer membrane of gram-negative bacteria that functions

as a permeability barrier (Plaizier et al., 2012). The function of LPS is to prevent the penetration of gram-negative bacteria by bile salts, lysozymes, or antimicrobial agents from the digestive tract (Rietschel et al., 1994). Although LPS is bound to the outer membrane, it is shed during the growth and stationary phases of bacterial growth, and is released following cell disintegration and lysis (Hurley, 1995; Wells and Russell, 1996). This shedding of LPS occurs during the turnover or expansion of the cell wall from regions in which newly synthesized proteins are inserted into the outer membrane (Mugopstelten and Witholt, 1978). The rate of LPS release varies among different species and strains of bacteria (Russell, 1976). In its free form, LPS is referred to as endotoxin, because they are toxic and capable of eliciting an immune response in the host once they enter the interior circulation (Alexander and Rietschel, 2001).

Translocation of LPS from the rumen into blood stream could happen in any of the following situations.

1. Increased rumen osmolality: high concentrations of fermentation acids elevates rumen osmolality, which can cause rapid influx of water into the lumen from blood circulation, causing swelling and rupture of rumen papilla (Owens et al., 1998).

2. Rumen parakeratosis: rumen parakeratosis could also result in the translocation of rumen bacteria via the rumen epithelium into interior of the body (Plaizier et al., 2012). Parakeratosis can create a similar situation as high osmolality to allow LPS pass through the rumen wall.

3. Rumen barrier function reduction: the quantity of carbohydrate consumed and its fermentability correlates with increased ruminal osmotic pressure. Sodium is typically absorbed via a Na^+ / H^+ exchange mechanism in the luminal membrane (Chien and Stevens, 1972; Martens et al., 1991; Sehested et al., 1996). Sodium transport is stimulated by the luminal presence of VFA (Gabel et al., 1991; Sehested et al., 1996), and ammonia is transported via a cation channel

in the luminal membrane that in turn stimulates electroneutral Na transport via intracellular release of protons (Abdoun et al., 2003). These activities contribute to a declining barrier function (degradation of gap junctions and tight junctions) of the rumen epithelial tissue, which predisposes the ruminal lining to transport of endotoxin (Plaizier et al., 2012).

Hindgut acidosis and diarrhea

Hindgut acidosis occurs when excessive carbohydrate fermentation in the large intestine leads to an accumulation of organic acids (Gressley et al., 2011). When dietary, animal, or environmental factors contribute to abnormal, excessive flow of fermentable carbohydrates from the small intestine, hindgut acidosis can occur (Gressley et al., 2011). Hindgut acidosis is characterized by increased rates of production of short-chain fatty acids including VFA and lactic acid, decreased digesta pH, and damage to gut epithelium as evidenced by the appearance of mucin casts in feces (Gressley et al., 2011). A breach in the gut epithelium in response to exposure to fermentation acids produced during SARA and hindgut acidosis can allow for systemic entry of bacteria, amines, or toxins and result in inflammation (Gressley et al., 2011).

Fecal consistency changes in response to hindgut acidosis, including diarrhea, frothy feces, and presence of mucin casts, which can be used in the field as indicators of SARA (Hall, 2002; Plaizier et al., 2008). The changes are described as alterations in color, which appears brighter and yellowish. The pH of the feces is lower than normal, usually slightly acidic (Dirksen, 1986). The smell of the feces is said to be sweet–sour (Oetzel, 2000). The size of ingesta particles may be too large, being around 1–2 cm instead of less than 0.5 cm. Whole cereal grains may be present. The alterations are usually transient in nature (Garry, 2002). Excessive carbohydrate fermentation in the hindgut can also elevate osmolality, which can lead to soft feces due to binding of fluid in the intestinal lumen (Garry, 2002).

Direct fed microbial (DFM) – nutritional alternative to maintain digestive function

Using probiotics, or direct-fed microbials (DFM), began 120 years ago. But the study about benefits on livestock just received attention a few decades ago. Probiotics have been defined as “a live microbial feed supplement, which beneficially affects the host animal by improving its intestinal microbial balance” (Fuller 1989). Probiotic is a generic and all-encompassing term used for microbial cultures, extracts, and enzyme preparations; it is commonly used when the product is for human consumption (Elam et al., 2003).

The definition of DFM is very broad and may include viable microbial cultures, culture extracts, enzyme preparations, specific and nonspecific yeast, fungi, bacteria, cell fragments, filtrates, or various combinations of the above (Beharka et al., 1991). The preferred term when used in reference to products fed to livestock is DFM. The U.S. Food and Drug Administration (FDA) and the Association of American Feed Control Officials (AAFCO) have required feed manufacturers to use the term “direct-fed microbial” instead of probiotic in animal feeds (Miles and Bootwalla 1991; AAFCO 1999; FDA 2003). Furthermore, the FDA has gone on to define DFM more narrowly as “a source of live, naturally occurring microorganisms” (Yoon and Stern, 1995).

Currently, there are at least 42 individual species of microorganisms that are approved for use in DFMs by the FDA and AAFCO (Alliance Animal Health 2009). These bacterial DFM may be classified as lactic acid producing bacteria, lactic acid utilizing bacteria, yeast and fungal, and other microorganisms. Reported beneficial effects on cattle, pigs, and chickens include improved general health, more efficient feed utilization, faster growth rate, and increased milk and egg production (Hyronimus et al., 2000a).

Gaggia et al (2010) summarized the expected health-promoting characteristics and safety

criteria of probiotics (Gaggia et al., 2010), which are shown in Table 1.1.

In cattle, the gastrointestinal tract is a fully mature ecosystem comprising more than 600 known species of bacteria as well as protozoa and fungi (Hungate 1966). The rumen is the first organ that DFM reach upon ingestion. Direct-fed microbials may grow in the rumen and beneficially modify its microbial ecosystem and (or) fermentation characteristics (Seo et al., 2010). A symbiotic relationship between the host animal and its resident gastrointestinal microbial ecosystem is critical to animal health and production efficiency (Jayne-Williams and Fuller 1971; Savelkoul and Tijhaar 2007).

Microorganisms used in DFM for cattle include species of *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, *Streptococcus*, *Bacillus*, *Saccharomyces*, *Aspergillus*, and *Propionibacterium*, all of which are commonly used in probiotics for human and monogastric animals or as inocula for dairy product processing (Seo et al., 2010). Yeast and/or fungal products are some of the most widely utilized DFM products in the dairy industry currently (Callaway and Martin 2006; Dawson 1992). The industry has used various DFM for years primarily to increase growth rate, milk production, and/or production efficiency (Dawson, 1990; Lehloenya et al., 2008). Other beneficial responses observed when providing DFM to cattle include increases in average daily gains and improved feed efficiency in feedlot cattle; improved health, increased immunity, and increased performance in young calves; decreased potential for ruminal acidosis; increased propionate concentration in the rumen; and altered rumen microflora populations (Krehbiel et al. 2003; Guillen 2009). This section is mainly focused on effects of traditional and newly developed DFM on the performance and health benefits in mature dairy cows, as well as mode of actions.

Performance benefits of DFM - Feed efficiency and CH₄ production

Direct-fed microbial are included in dairy rations primarily to improve milk production efficiency (Isik et al., 2004; Jouany, 2006; Oetzel et al., 2007), thus improving dairy farm profitability (Desnoyers et al., 2009). Improved dairy feed efficiency (energy corrected milk yield divided by DMI) is accompanied by changes in body composition, protein turnover, digestibility, heat loss, physical activity, and mitochondrial function.

Propionibacteria (e.g. *M. elsdenii*) seems to be the major ruminal lactate utilizer, who can complement lactate-producing organisms by simultaneously using lactate, glucose, and maltose to generate propionate (Russell and Baldwin, 1978). Propionate spares glucogenic amino acids in gluconeogenesis, and consequently reduces the maintenance cost of metabolizable protein and possibly heat increment (Van Soest, 1994). Methane production is decreased by a decreased acetate:propionate ratio, since increased propionate decreases hydrogen available for methane production in the rumen (Seo et al., 2010).

As much as 12% of the energy in feed may be converted to methane and is lost via eructation in ruminant animals (Martin and Nisbet, 1992). Therefore, energy retention by cattle would theoretically increase if methane production declines (Wolin, 1960). This suggests the potential for more efficient energy utilization by supplying DFM to dairy rations.

Periparturient period, DMI and milk yield

The periparturient period (usually referenced as the transition period) is defined as the period from 3 wk before parturition until 3 wk postpartum. During the transition period from late gestation through early lactation, the dairy cow undergoes tremendous metabolic adaptations (Bell, 1995). Sudden changes of diet to more rapidly fermented carbohydrates that occur during this time may cause SARA (Oetzel et al., 2007; Chiquette et al., 2012). As peak milk yield

increases, the transition period for dairy cows becomes much more challenging with most infectious diseases and metabolic disorders occurring during this time (Grummer et al., 1995, Drackley, 1999). Milk production increases faster than energy intake in the first few weeks postpartum, and thus high producing cows will incur negative energy balance. Hepatic oxidation of NEFA in the liver likely suppresses feed intake in the days following parturition when cows are in a lipolytic state (Allen et al., 2009).

Lactate acid producers, lactate utilizers, yeast, and/or any combination of above have been used as DFM to improve performance of dairy cows in the transition period. In some cases, DFM increased DMI, milk yield, and milk protein content during the postpartum period. Blood glucose and insulin levels were higher and NEFA levels were lower for cows receiving DFM during the postpartum period (Nocek et al., 2003). Depending on the species or combination of species, they can decrease time below subacute ruminal pH (Krehbiel et al., 2003).

However, use of DFM has been reported to have inconsistent effects on performance (Elam et al., 2003). Many of the benefits of DFM appear to make their greatest contribution to improving production in situations where animals are exposed to hot weather (Yu et al., 1997), low-quality diets, transitions, or other stresses.

Animal health and food safety

Pathogenic bacteria such as enterohemorrhagic *Escherichia coli* (including *E. coli* O157:H7), *Salmonella*, *Campylobacter*, and *Listeria* have all been isolated from cattle (Harvey et al., 2004; Oliver et al., 2005; Callaway et al., 2006). Enterotoxin-producing strains of *E. coli* attach to intestinal epithelial cells and mucus to induce diarrhea (Jones and Rutter, 1972). *Salmonella* can also cause severe disease in cattle and be transmitted along the food chain and can be the source of human illness. It is a problem both from food safety and animal health perspectives (Coburn et

al., 2007). Both *E. coli* and *Salmonella* are considered as zoonoses and used to be treated by antibiotics, which is a worldwide concern because of development of antimicrobial resistance and possible transference of antibiotic resistance genes from animal to human microbiota (Salyers et al., 2004; Mathur and Singh, 2005). Due to the rising public concern about the contamination of antibiotics in animal products, there continues to be interest in the feeding of “natural” feed additives such as DFM. According to Nocek and Kautz (2006), the inclusion of a DFM in dairy cow diets has become a generally accepted practice.

Data suggest that feeding a DFM to cattle decreases the fecal shedding of *E. coli* O157:H7 (Brashears et al., 2003; Elam et al., 2003; Younts-Dahl et al., 2004; Tabe et al., 2008; Callaway et al., 2009). In other experiments, lactate producers were able to adhere to the intestinal tracts of mice, protecting animals against *Salmonella dublin* DSPV 595T (Frizzo et al., 2010), but there have been no such data published in ruminants.

In addition to its role in the digestion and absorption of nutrients, the gastrointestinal tract (GT) provides its host a protective defense against a constant presence of antigens from food and microorganisms in the gut lumen (Krehbiel et al., 2003). Immune cells in the GT consist of natural killer cells, macrophages, neutrophils, dendritic cells, and T and B lymphocytes that are aggregated in Peyer’s patches, lamina propria, and intraepithelial regions. Upon infection by an antigen via the oral route, immune cells are rapidly activated, leading to enhanced phagocytosis as well as the production of a vast array of humoral mediators (Zhang and Ghosh, 2001). Some DFM have been shown to affect the innate, humoral, and cellular arms of the immune system (Krehbiel et al., 2003). However results are mixed, which may be because of different strains, dose, and duration of supplementation.

Mode of action

Direct-fed microbials have potential beneficial effects on the rumen and post-ruminal GT. With regard to specific roles in the rumen, the DFM can be classified into lactate producer, lactate utilizer, and fungi.

The presence of lactate producing bacteria is thought to help the ruminal microflora adapt to the presence of lactic acid (Ghorbani et al., 2002), whereas the presence of lactate utilizer bacteria is thought to prevent accumulation of lactate (Nisbet and Martin, 1994; Kung and Hession, 1995). Therefore, continual inoculation with certain bacterial DFM might help the ruminal environment adapt to acidosis (Elam et al., 2003). The most well-documented example would be use of lactate-producing bacteria such as *Lactobacillus acidophilus* in combination with lactate-utilizing bacteria such as *Propionibacterium freudenreichii* (Raeth-Knight et al., 2007). In this particular example, the presence of the lactate-producing *L. acidophilus* may help the ruminal microorganisms adapt to the presence of lactic acid (Ghorbani et al., 2002; Beauchemin et al., 2003a). The presence of the lactate utilizer *P. freudenreichii* may help to prevent lactate from accumulating in the rumen (Kung and Hession, 1995). These results suggest that bacterial DFM supplementation might be useful to decrease ruminal acidosis.

Modes of action of DFM in the rumen and post-rumen GT (Seo et al., 2010) are summarized in Table 1.2. The principal fungi that have been used and studied are *Saccharomyces cerevisiae* and *Aspergillus oryzae*. *S. cerevisiae* was able to compete with starch-utilizing bacteria for fermentation of starch (Lynch and Martin, 2002), leading to the prevention of lactate accumulation in the rumen (Chaucheyras et al., 1996). Chaucheyras et al. (1995) also reported that *S. cerevisiae* had the ability to provide growth factors, such as organic acids or vitamins, thereby stimulating ruminal populations of cellulolytic bacteria and lactate utilizers. However,

results in lactating cow diets have been quite variable, with some trials showing significant increases in milk production and other positive indications such as increased DMI (Fuller, 1997). In addition, some studies have reported higher nutrient digestibility, reduced rectal temperatures during heat stress, improved reproductive status, higher ruminal pH and a greater stability of ruminal fermentation (Fuller, 1997). On the other hand, some studies have shown no effect or have been slightly negative (Swatz et al., 1994). Generally, the fungal cultures have proven more effective in cows fed diets containing medium to large amounts of concentrate, or during early lactation. The reason for greater response under such conditions might be related to an increased need for maintaining rumen stability when feeding high-lactation cows (Fuller, 1997).

Seo et al (2010) summarized the modes of action (Table 1.2), and the mechanisms have been discussed in this section or the previous sections.

Spore forming bacteria – Bacillus as DFM

Bacterial spores are produced in nature as a means to survive extreme environmental conditions enabling long-term survival in conditions that could otherwise kill vegetative bacteria (Nicholson et al., 2000). The spore contains a core, an inactive chromosome, and one or more layers of proteinaceous spore coat layers surrounding them (Henriques and Moran, 2007). Together these protect the spore from UV radiation, extremes of heat (typically up to 80-85°C in most species), exposure to solvents, hydrogen peroxide, and enzymes such as lysozyme (Nicholson et al., 2000). The spore itself is dehydrated but if exposed to appropriate nutrients will germinate, a process taking just a few minutes, allowing water to enter the spore, cause breakage and removal of the spore coats, and stimulate outgrowth and resumption of vegetative cell growth (Moir, 2006).

Bacterial spore formers are used as probiotic supplements in animal feeds, for human dietary

supplements, and in registered medicines (Cutting, 2011). The scientific interest in *Bacillus* species as probiotics, however, has only occurred in the last 15 years (Cutting, 2011). The species that have been most extensively examined are *Bacillus subtilis*, *Bacillus clausii*, *Bacillus cereus*, *Bacillus coagulans*, and *Bacillus licheniformis*.

Spore forming bacteria were suggested to be the best DFM candidates for ruminant animals (Seo et al., 2010) because of the following advantages. Products made from spore forming bacteria have higher resistance to stresses during feed production and storage processes (Hyronimus et al., 2000b) and can be stored at room temperature in a desiccated form without any deleterious effect on viability, as they are heat stable. In general, most yeast and lactate producers and utilizers are destroyed by heat during pelleting. The second advantage is that the spore is capable of surviving the low pH of the gastric barrier (Spinosa et al., 2000, Barbosa et al., 2005), which is not the case for all species of *Lactobacillus* (Tuohy et al., 2007)

In addition to the practical advantages of spore forming bacteria, strong cellulolytic activity may support the potential of this specie as DFM for ruminant or nonruminant animals by improving fiber digestion in the rumen and/or in the GT by supplying oligosaccharides to beneficial microorganisms (Seo et al., 2010).

Numerous studies have shown that more than 10% of an inoculum of *B. subtilis* spores can germinate in the small intestine, grow, proliferate, and then re-sporulate (Hoa et al., 2001; Tam et al., 2006). Furthermore, they can elicit potent immune responses in the GT of mouse models and this immune stimulation may be the underlying reason why spores exert a probiotic effect (Duc et al., 2004).

Spore probiotics are being used extensively in humans as dietary supplements, in animals as growth promoters and competitive exclusion agents, and in aquaculture for enhancing the growth

and disease-resistance of cultured shrimps, most notably the Black Tiger shrimp (Cutting, 2011).

In ruminants, reports demonstrated the *Bacillus* species could increase milk yield, fat corrected milk, and milk protein (Kritas et al., 2006; Qiao et al., 2010). However, it should be cautioned that all these experiments were published as abstracts, and therefore information was limited and not peer reviewed.

In summary, rumen environment is important to animal health, welfare and producer economic profile. The objective of this project focused on proposing methods to predict on-site ruminal acidosis condition, and effect of DFM on animal performance.

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Tables and Figures

Table 1.1. Expected characteristics and safety criteria of probiotics (Gaggia et al., 2010).

Non-toxic and non-pathogenic

Accurate taxonomic identification

Normal inhabitant of the targeted species

Survival, colonization and being metabolically active in the targeted site, which implies:

Resistance to gastric juice and bile

Persistence in the GIT

Adhesion to epithelium or mucus

Competition with the resident microbiota

Production of antimicrobial substances

Antagonism towards pathogenic bacteria

Modulation of immune responses

Ability to exert at least one scientifically-supported health-promoting properties

Genetically stability

Amenability of the strain and stability of the desired characteristics during processing, storage and delivery

Viability at high populations

Desirable organoleptic and technological properties when included in industrial processes

Table 1.2. Mode of actions (Seo et al., 2010).

Rumen mechanisms

Lactic acid producing bacteria

1. Provision of a constant lactic acid supply
2. Adaptation of overall microflora to the lactic acid accumulation
3. Stimulation of lactate utilizing bacteria
4. Stabilization of ruminal pH

Lactic acid utilizing bacteria

1. Conversion of lactate to VFA (e.g., *Megasphaera elsdenii*)
2. Production of propionic acid rather than lactic acid (e.g., *Propionibacterium* spp.)
3. Increase of feed efficiency
4. Decrease of methane production
5. Increase of ruminal pH

Fungal DFM

1. Reduction of oxygen in the rumen
2. Prevention of excess lactic acid in the rumen
3. Provision of growth factors such as organic acid and vitamin B
4. Increase of rumen microbial activity and numbers
5. Improvement of ruminal end products (e.g., VFA, rumen microbial protein)
6. Increase of ruminal digestibility

Gastrointestinal tract mechanisms

1. Production of antibacterial compounds (acids, bacteriocins, antibiotics)
 2. Competition with pathogens for colonization of mucosa and/or for nutrients
 3. Production and/or stimulation of enzymes
 4. Stimulation of immune response by host
 5. Metabolism and detoxification of desirable compounds
-

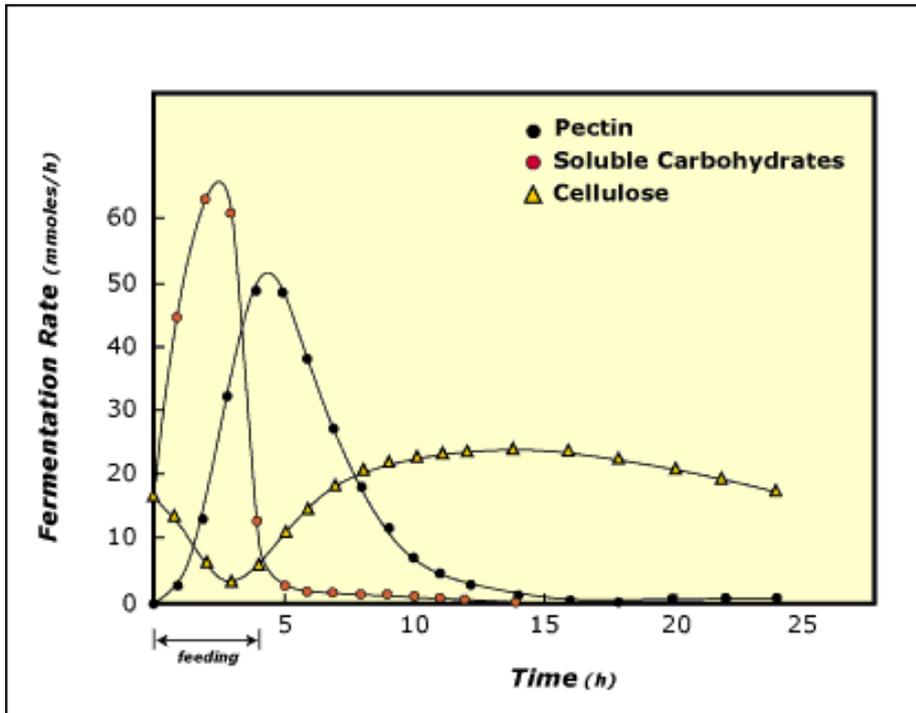


Figure 1.1. Rate of fermentation of alfalfa components in the rumen (adapted from Baldwin et al., 1977).

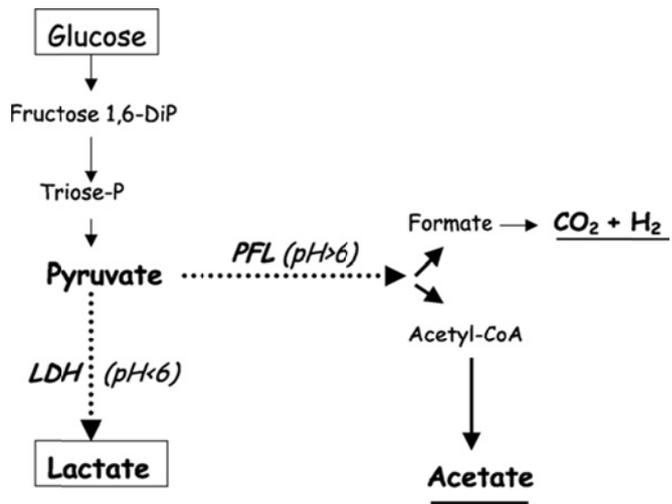


Figure 1.2. Regulation of lactate production by *Streptococcus bovis* and *Lactobacillus casei* (adapted from Russell and Hino, 1985).

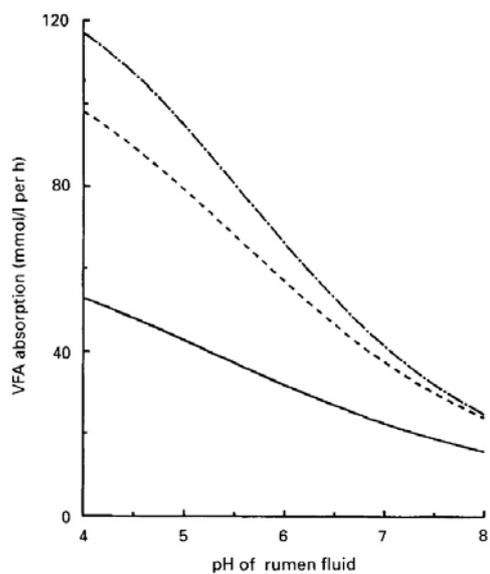


Figure 1.3. Influence of pH of rumen fluid on absorption of acetic (____), propionic (-----) and butyric (-.-.-) acids at a concentration of each acid of 50 mM for lactating dairy cows (adapted from Dijkstra, 1993).

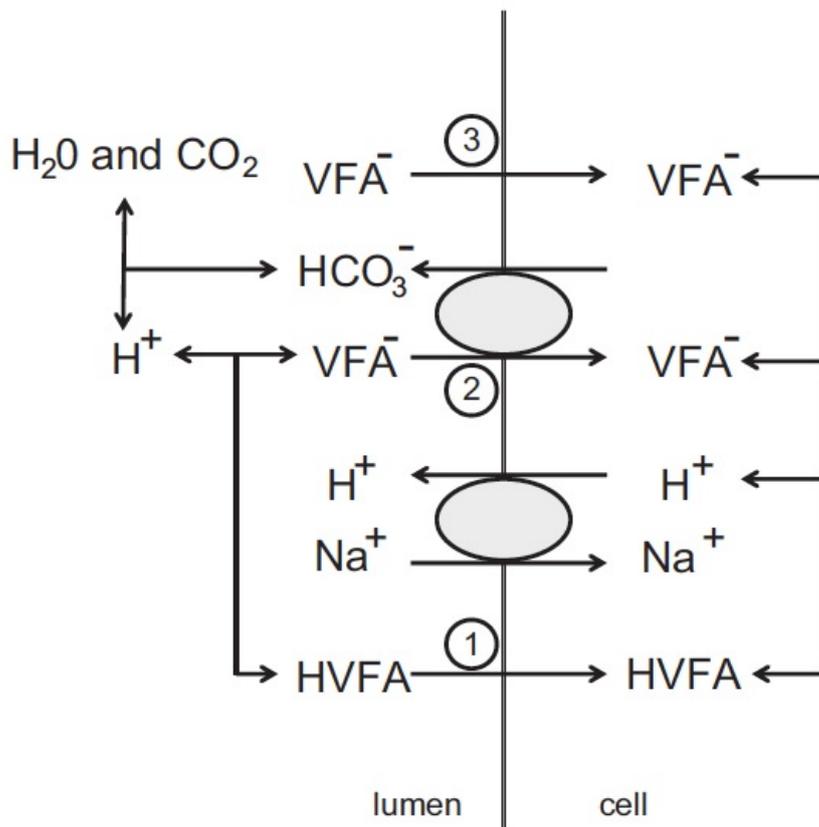


Figure 1.4. Simplified representation of VFA absorption pathways. (1), passive diffusion of undissociated VFA; (2) bicarbonate dependent VFA uptake; (3) bicarbonate independent VFA uptake (adapted from Dijkstra et al., 2012).

CHAPTER 2

Effect of Induced Subacute Ruminant Acidosis on Ruminant, Urine and Fecal pH, Digestibility and Milk Parameters in Holstein and Jersey Cows

ABSTRACT

The effect of sub-acute ruminal acidosis (SARA) on urine, fecal pH, milk yield and milk composition, and total tract apparent digestibility of starch were determined. Six Holstein cows (**HOL**), six rumen-cannulated Holstein cows (**CAN**), and six Jersey cows (**JER**) were used in a replicated 3 × 3 Latin square design balanced to measure carry-over effects. Periods (10 d) were divided into 4 stages (S): S1, baseline, d 1-3, ad libitum TMR; S2, restricted feeding, d 4, cows fed for 50% of S1 DMI; S3, challenge, d 5, treatments applied; S4, recovery, d 6-10, all cows fed ad libitum TMR. Treatments were CON, no top dress; MOD, 10% of S1 DMI as top dress (pelleted mixture of 50:50, wheat: barley); and HIG, 20% of S1 DMI as top dress. Rumen pH and urine pH were recorded at -2 to 22 h relative to feeding on S3. Milk yield was recorded and DMI measured daily. Milk samples were obtained on d 2, 4, 5, and 8 for composition analysis. Fecal samples were obtained on d 1 afternoon (3 PM), d 4 morning (7 AM), afternoon (3 PM), night (11 PM), and the next morning before feeding (6 AM) for determination of fecal pH and starch digestibility. No treatment carry-over effect was observed for any measured variable. Mean rumen pH was depressed to 6.24 and 6.35 for cows in HIG and MOD respectively, which were lower than the 6.45 for cows in CON treatment. However, the area under curve was not different among treatments in rumen pH. Mean urine pH in HOL cows was lower on HIG (8.46) compared with 8.54 and 8.51 for CON and MOD, respectively. Mean urine pH of Jersey cows

did not differ among treatments. Fecal pH was not different among treatments either for JER and HOL cows. However, fecal pH was lower 15 and 23 h after feeding in HOL cows when compared with other time points. Fecal pH was lower 7, 15, and 23 h after feeding in JER cows than other time points. Milk yield and composition were not different among treatments. Milk urea nitrogen (MUN) was elevated in S2 and S3 on all treatments for both HOL and JER cows. No treatment effects were found for fecal pH and starch digestibility. During S3, fecal pH was depressed from 7 to 23 h and 15 to 24 h post feeding for JER and HOL cows. Rumen pH can be predicted by urine pH within 2 h after feeding. Fecal pH may be used as a tool to predict rumen pH depression as early as 7 and 15 h after feeding for JER and HOL cows respectively.

INTRODUCTION

Sub-acute ruminal acidosis (**SARA**) is a common condition affecting dairy cows and has up to 26% incidence in commercial dairy farms (Garrett et al., 1999; Kleen et al., 2003). Its association with DMI depression, milk yield depression, reduced feed efficiency, rumenitis, diarrhea, laminitis, inflammation, liver abscesses, high culling and death rates in dairy cattle had been extensively reported by previous authors (Nocek, 1997; Kleen et al., 2003; Stone, 2004; Alzahal et al., 2007; Enemark, 2008). It is important to diagnose SARA in order to improve animal welfare and prevent potential economic loss due to the aforementioned conditions. However, the clinical signs might be dismissed, as the signs are delayed from the time of the acidotic insult, and can be caused by other problems or diseases besides SARA (Nocek, 1997; Enemark, 2008).

It is still unclear how SARA affects feed digestibility throughout the digestive tract. Sub-acute ruminal acidosis can decrease rumen NDF digestibility and impact fecal consistency. Krajcarski-Hunt et al. (2002) reported that SARA reduced 24- and 48-h in situ NDF degradability of forages

by an average of 20.5% and 24.8%, respectively. Similar results were reported by Plaizier et al, (2000). Feces usually had longer particles, whole grains, sweet–sour smell, and appeared to be brighter, yellowish, liquid, but actually had greater DM content in cows experiencing SARA (Oetzel, 2000; Garry, 2002; Kleen et al., 2003; Li et al., 2012). The alterations might be caused by post-ruminal fermentation in the large intestine due to a massive outflow of fermentable carbohydrates from the rumen, or binding of fluid in the intestinal lumen with high osmolarity (Oetzel, 2000; Garry, 2002), which suggests that the ruminal microbial population and passage rate can be changed in SARA.

It has long been understood that rumen pH depression is associated with decreased milk fat percentage (Byers and Schelling, 1988). Prolonged low rumen pH results in incomplete biohydrogenation of fatty acids and increases in *trans*-octadecenoic acids, and especially the *trans*-10 isomer of *trans*-octadecenoic acid (Griinari et al., 1998; Bauman and Griinari, 2003), which is associated with milk fat depression (Byers and Schelling, 1988; Bauman and Griinari, 2003). However, it is still not well understood how SARA affects cows' milk production and milk protein synthesis. Decreased milk production caused by SARA has been reported previously (Stone, 1999; Yang et al., 2009; Colman et al., 2013). Milk protein responses are inconsistent. Stone (1999) observed that cows had decreased milk protein yield. However, other authors (Fairfield et al., 2007; Khafipoor et al., 2007; Colman et al., 2013) observed increased milk protein yield. Plaizier et al. (2008) suggested that the change in rumen digestible organic matter from microbial protein synthesis might play a role in those findings. The literature and aforementioned observations were based on research using either Holstein cows or beef steers. Effects of SARA on Jersey cows' milk production and milk composition are unclear. It is known

that milk fat composition is different in the lactating Jersey cows and that short chain fatty acids are the major source of milk fatty acid (Drackley et al., 2001).

Effects of SARA on urine and fecal pH have been reported by others (Enemark et al., 2004; Yang and Beauchemin 2006; Morgante et al., 2009; Gianesella et al., 2010; Li et al., 2012); however, the results were inconclusive because of limited and inconsistent time points of sample collection. Therefore, the objectives of this experiment were: 1) to determine the effects of induced SARA on ruminal pH, urine pH, fecal pH, starch digestibility, and milk composition of lactating cows; and 2) determine potential differences in response to SARA by Holstein and Jersey lactating cows.

MATERIALS AND METHODS

Animals and housing

All experimental procedures were approved by the University of Illinois (Urbana) Institutional Animal Care and Use Committee. Cows were housed in tie stalls with sand bedding and ad libitum water and feed access. Cows were fed according to NRC (2001) recommendations and milked 3 times a day.

Experimental design and SARA induction procedure

Eighteen animals were selected and assigned into one of three groups. The first group had six Holstein cows (**HOL**), parity 2.67 ± 1.5 , BW $717 + 30$ kg, and 258 ± 16 days in milk (**DIM**); the second group had six rumen-cannulated Holstein cows (**CAN**), parity 3.86 ± 1.0 , BW $774 + 30.0$ kg, and 287 ± 45 DIM; and the third group had six Jersey cows (**JER**), parity $2.50 \pm$

1.5, BW 471 + 30.0 kg, and 190 ± 86 DIM. This experiment was conducted as a replicated 3 × 3 Latin square design balanced to measure carry-over effects.

The SARA induction protocol proposed by Krause and Oetzel (2005) was used. Briefly, each cow received all treatments in different periods. Each period had 10 d and was divided into 4 stages. The first stage (**S1**), d 1 to d 3, served as baseline with regular TMR ad libitum. The second stage (**S2**), d 4, served as restricted feeding, with cows offered 50% of the TMR fed on S1 (DM basis). The third stage (**S3**), d 5, served as SARA induction, in which cows were not fed (0%) or fed an addition of 10% or 20% pellet wheat-barley (1:1), based on DMI obtained in S1. The fourth stage (**S4**), d 6 to d 10, served as recovery stage with regular TMR fed ad libitum.

Experimental treatments were control (**CON**), no addition of wheat-barley (1:1) pellet; moderate SARA induction (**MOD**), addition of 10% of S1 DMI as wheat-barley (1:1) pellet top dressed; and high SARA induction (**HIG**), addition of 20% of S1 DMI as wheat-barley (1:1) pellet top dressed. Cows were fed the same TMR during all the aforementioned stages and it was delivered once daily (8 AM).

Data Collection and Sampling Procedures

Samples of feed ingredients and TMR were obtained weekly and analyzed for DM contents (AOAC International, 2000). Diet DM was adjusted weekly for changes in DM content of ingredients. Samples of TMR were taken weekly and stored at -20°C until analyzed.

Composite samples were analyzed for contents of DM, CP, SP, ADF, NDF, lignin, starch, fat, ash, NFC, Ca, P, Mg, K, Na, Fe, Zn, Cu, Mn, Mo, and S using wet chemistry methods (Dairy One, Ithaca, NY). Values for RFV, TDN, NEI, NEm, NEg, ME, and DE were provided by the lab and were based on NRC (2001). Individual intakes were measured daily.

Rumen and urine pH were measured every hour from -2 to 22 h after SARA induction on S3. Representative samples of rumen fluid (40 ml) were collected and composited from 3 sites (ventral sac, cranial sac, and caudo-ventral blind sac) through the rumen cannula using a syphon. The pH of the fluid was measured with portable pH meter (AP110 Fisher Scientific, Pittsburgh, PA) immediately after collection from the rumen.

Urinary catheters (Bardex Foley Catheter, REF 0166L22; C. R. Bard, Inc. Covington, GA) were placed in the cow's bladder for urine sample collection. A 15 × 10 cm rectangular area centered on the perineum was clipped and scrubbed with 15% Novalsan solution. The catheter was directed to the urethral orifice by putting a finger into the urethra, so that the catheter could follow the direction to the bladder. Sterile water (75 ml for Holstein cows, 65 ml for Jersey cows) was injected to the catheter balloon to keep it inflated during the collection period. In order to make sure enough sample volume was collected, the catheter was blocked 0.5 hour before sampling. The catheter was removed by extracting the sterile water out of the balloon after the 24 h sampling period.

Fecal samples (approximately 400 g, wet weight) were collected at 7 time points (**TP**): TP1, d 1, 3 PM; TP2, d 5, -1 h after feeding; TP3, d 5, 5 h after feeding (3 PM); TP4, d 5, 16 h after feeding; TP5, d 5, 23 h after feeding; TP6, d 6, 3 PM; and TP7, d 7, 3 PM. Fecal samples were collected from the rectum into suitable plastic containers, pH was measured immediately after the collection, and then samples were stored at -20°C until analyzed. Fecal samples from TP1 to TP5 were analyzed for DM, starch, and lignin contents.

Cows were milked 3 times per day at 0600, 1400, and 2100 h. Milk weights were recorded daily and samples were obtained from consecutive milkings at AM, PM, and night milking on d 2, 4, 5, and 8 of each period. Consecutive milk samples (0600, 1400, and 2100)

were composited in proportion to milk yield at each sampling and preserved (800 Broad Spectrum Mirotabs II; D&F Control Systems, Inc., San Ramon, CA). Composite milk samples were analyzed for fat, protein, lactose, urea N, total solids, and comatic cell count (SCC) using mid-infrared procedures (AOAC, 1995) at a commercial laboratory (Dairy Lab Services, Dubuque, IA).

Health conditions recorded included body temperature, heart rate, respiratory score, general appearance, rumination, and fecal score, and were evaluated daily and every 4 h on S3. Normal body temperature was considered elevated if >39.4°C, heart rate was considered elevated if >100 beats/min, respiratory rate was considered abnormal if >40 breaths/min, general appearance was considered abnormal if <2, where 1 = bright and alert, 2 = depressed, 3 = reluctant to rise, rumination rate was considered abnormal if no movement in 1 min, and fecal score was considered abnormal if <2 (Ireland-Perry and Stallings, 1993; Krause and Oetzel, 2005).

Body weight was measured and BCS was assigned in quarter unit increments (Ferguson et al., 1994) for each cow at S4 stage in each period. Three individuals assigned BCS independently at each time of scoring throughout the experiment and the average value was used.

Apparent Digestibility in the Total Tract

Lignin was used as marker to calculate the apparent nutrient digestibility. The following formula was used (D.C. Church, 1988):

$$\text{Apparent nutrient digestibility (\% of intake)} = 100 - 100 \times \left(\frac{\% \text{ lignin in feed}}{\% \text{ lignin in feces}} \right)$$

$$\text{Digestibility of nutrient (\%)} = 100 - \left(100 \times \frac{\% \text{ Lignin in feed}}{\% \text{ Lignin in feces}} \times \frac{\% \text{ Nutrient in feces}}{\% \text{ Nutrient in feed}} \right)$$

Statistical Analyses

The data were analyzed using the mixed model procedure of SAS (v 9.2; SAS Institute Inc., Cary, NC) to account carry over effect by the following model:

$$y_{ijklm} = \mu + S_i + A_{(i)j} + P_{(i)k} + T_l + C_m + e_{(ijk)l}$$

Where y_{ijklm} is the observations for dependent variables; μ is the general mean; S_i is the fixed effect of the i th treatment sequence; $A_{(i)j}$ is the random effect of the j th cow in the i th sequence; $P_{(i)k}$ is the fixed effect of the k th period; T_l is the fixed effect of the l th treatment; C_m is the fixed carry-over effect from the previous period ($C=0$, if period =1); $e_{(ijk)l}$ is the random error (R. O. Kuehl, 2000). If carry-over effects were not detected data were analyzed as a replicated Latin square by the following model:

$$y_{ijklm} = \mu + S_i + A_{(i)j} + P_{(i)k} + T_l + D_m + T \times D_{lm} + e_{(ijk)lm}$$

Where y_{ijklm} is the observations for dependent variables; μ is the general mean. S_i is the random effect of the i th square; $A_{(i)j}$ is the random effect of the j th cow in the i th square; $P_{(i)k}$ is the fixed effect of the k th period; T_l is the fixed effect of the l th treatment; D_m is the fixed effect of repeat measurement, which used as TP in pH and day in DMI analysis. $T \times D_{lm}$ is the interaction of treatment and repeat measurement, and the interaction was removed if $p > 0.3$; $e_{(ijk)l}$ is the random error. Data were subjected to ANOVA using the MIXED procedure of SAS. The estimation method was restrictive maximum likelihood (REML) and the degrees of freedom method was Kenward-Rogers (Littell et al., 2002). Repeated measurement analysis was conducted using compound symmetric covariance structure. Milk SCC data were log10 transformed to stabilize the variance before statistical analysis. The REG procedure of SAS was used to assess the association between rumen pH and urine pH in CAN cows. Breed and

treatment linear and quadratic orthogonal contrasts were tested using the CONTRAST statement of SAS. Values reported are least squares means with the standard error of the difference.

RESULTS

The ingredient composition of the diets is detailed in Table 2.1. The chemical composition analyses of the diets are shown in Table 2.2. The physical characteristic of the TMR, based on the Penn State Particle Separator (Kononoff et al., 2003), was 8.0 ± 3 % on upper, 44.5 ± 3 on middle, 36.0 ± 1 on lower, and 11.0 ± 2 % on bottom sieves. Carry-over effect was tested and was not present for any variable of interest ($P > 0.30$). We observed no differences for body temperature, heart rate, respiratory score, general appearance, rumination, and fecal score among treatments. Cows were healthy throughout the experiment and no sick animals were reported.

DMI, BW, and BCS

Dry matter intake had an overall treatment effect ($P = 0.05$), as cows in MOD and HIG treatments had higher ($P = 0.05$) DMI than cows in CON (Table 2.3). Cows in MOD treatment had similar DMI as HIG treatment. There was no treatment by stage interaction ($P = 0.13$) observed. The DMI was affected by stage ($P < 0.01$), because DMI decreased on S2 when feed restriction occurred and DMI increased on S3 (Table 2.4). Cows in CON treatment had lower ($P = 0.02$) DMI than cows in MOD treatment, but similar to cows in HIG treatment on S3. Stage had linear ($P < 0.01$) and quadratic ($P < 0.01$) effects for DMI. The overall data are combined with HOL ($n = 12$) and JER ($n = 6$) cows, so the effect was across breeds.

Jersey cows in HIG treatment had lower ($P < 0.01$) DMI than MOD cows, whereas HOL cows had similar ($P = 0.10$) DMI among treatments. We observed a quadratic effect for DMI ($P < 0.01$) in JER cows. There was a linear effect ($P = 0.04$) for DMI in HOL cows.

Both JER and HOL cows had a difference for stage ($P < 0.01$), due to the restriction feeding on S2. Additionally, a quadratic effect was observed for stage ($P < 0.01$). We observed a linear effect for stage ($P < 0.01$) on both HOL and JER cows. Cows in HOL had higher DMI ($P < 0.01$) on S3, and numerically higher DMI on S4, when compared with S1. However, JER cows only had numerically higher DMI on S3, and similar DMI on S4 when compare with S1. Body weight and BCS were not different ($P = 0.32$) among treatments, and no treatment by period interaction was observed.

Milk

Milk yield was not affected by treatment on HOL or JER cows. However, treatment by stage interaction for milk yield was observed for HOL. Most likely, this was because cows in CON produced ($P < 0.01$) less milk than MOD cows on S4; however, milk yield was similar among treatments from S1 to S3. As expected, we observed milk yield differences by stage ($P < 0.01$) and a quadratic ($P < 0.01$) effect for stage on both HOL and JER cows. Milk yield was unaffected on S2, when restricted feeding was conducted, but decreased on S3. We also observed stage linear effect for milk yield ($P < 0.01$) on both breeds.

There was no treatment effect for FCM, fat percentage, or fat yield for any breed. However, FCM and fat yield were affected by stage ($P = 0.03$) and showed a tendency for quadratic effect ($P = 0.09$) on both JER and HOL cows, due to restricted feeding on S2. We observed a linear effect for FCM and milk fat yield due to stage ($P < 0.01$) on JER cows, but not for HOL cows. There was no difference among treatments or stages for milk fat percentage on either HOL or JER cows. A tendency for differences for FCM due to period was observed in HOL cows.

Treatments did not affect milk protein percentage or milk protein yield, and no interactions with stage were found on JER and HOL cows. However, milk protein yield was lower ($P < 0.01$) on S3 than other stages on both JER and HOL cows. Treatment did not affect milk lactose or milk lactose yield, and no interactions with stage on JER and HOL cows were found. However, milk lactose yield was lower on S3 than other stages for both JER and HOL cows. We also observed a linear effect on milk lactose yield on JER and HOL cows.

Treatments did not affect milk urea nitrogen (MUN) and no interactions with stage for JER and HOL cows were found. However, MUN was significantly ($P < 0.01$) elevated on S2 and S3, then reduced on S4 on both HOL and JER cows. The higher MUN concentration for S2 and S3 when compared with S1 and S4 may be due to the depression of milk yield. Somatic cells count was not affected by treatment on JER or HOL cows.

Rumen, urine, and fecal pH

We observed ($P < 0.01$) a treatment effect for rumen pH (Table 2.4). Hours below ruminal pH 5.8 increased from 2.17 h to 4.33 h from CON to HIG. Cows in HIG had rumen pH depressed below 5.8 for longer ($P < 0.01$) period of time than cows in CON and MOD, but no difference was observed between CON and MOD. However, area under curve was not different among treatments ($P = 0.75$).

Overall, cows that received HIG had lower ($P < 0.01$) urine pH than cows that received CON. Cows that received MOD had numerically lower urine pH than cows that received CON. This explained the treatment linear ($P < 0.01$) effect observed for urine pH. However, JER and HOL cows had different urine pH responses to SARA induction. We observed no differences

among treatments for JER cows. However, HOL cows that received HIG and MOD had significantly lower urine pH than cows that received CON (Table 2.4).

On S3, fecal pH for JER cows' was not affected by treatment ($P = 0.32$), but by time point ($P < 0.01$). Time point 3 (TP3), TP4, and TP5 were lower ($P < 0.01$) than TP2. Cows in the HOL group had fecal pH affected by treatment ($P = 0.03$) and time point ($P < 0.01$). Also, cows in the HOL group had a treatment effect on the afternoon of S3; cows in HIGH had lower ($P = 0.02$) fecal pH than cows in CON, whereas cows in MOD had a tendency ($P = 0.07$) for fecal pH to be lower than cows in CON. Fecal pH for HOL and JER cows decreased ($P < 0.01$) linearly on S3. However, JER cows had faster response than HOL cows in terms of fecal pH. For HOL cows, time point 4 (TP4) and TP5 had lower ($P < 0.01$) fecal pH than cows in TP2 and TP3. There was no difference ($P = 0.60$) for fecal pH between TP4 and TP5. Jersey cows had lower ($P < 0.01$) fecal pH in TP3, TP4, and TP5 than cows in TP2, and TP3. There was no difference ($P = 0.80$) between TP4 and TP5 (Figure 2.1 to 2.3).

On all fecal pH values that were measured 7 h after feeding, we found that fecal pH was lower ($P < 0.01$) on S3 (TP3) and numerically lower on the first day of S4 (TP6) when compared with S1 (TP1) and the second day of S4 (TP7). Jersey cows had lower ($P = 0.02$) fecal pH on S3 than all other days, whereas HOL cows' fecal pH was depressed on the day after SARA induction rather than S3. We observed that within the 2 h after feeding (Figure 2.11), the rumen pH can be predicted from the urine pH by the formula:

$$\text{Rumen pH} = \frac{\text{Urine pH} - 6.2916}{0.2946}$$

Digestibility

There was no treatment effect for starch digestibility on any time point. However, we observed that all cows had lower ($P = 0.02$) starch digestibility at TP4. We also observed a tendency ($P = 0.06$) for a quadratic effect on S3. This may indicate that starch digestibility decreased as fecal pH decreased. There was no difference ($P = 0.33$) on starch digestibility between S1 afternoon and S3 afternoon.

DISCUSSION

Sub-acute ruminal acidosis has been defined as a condition where rumen pH is less than 5.8 for at least 3 h (Russell and Wilson, 1996). By this definition, we were able to successfully induce SARA in cows that received HIG. In the present induced SARA model, we restricted feeding before the induction, in order to ensure consumption of the amount of rapidly fermentable carbohydrate (pellets) to reduce ruminal pH. Consumption of a large amount of TMR besides the top-dress may have contributed to a lower than expected reduction of rumen pH. Feed restriction may have destabilized the ruminal microbial flora due to starvation of some bacteria (Van Kessel and Russell, 1997). Such a phenomenon of ruminal pH depression had previously been observed in experimental induced SARA in Holstein lactating cows (Dohme et al., 2008). The decrease in rumen pH is caused by the combination of destabilization and large amount of DMI in the beginning of the induction day even present in cows in CON. The similar area under curve among treatments supports this hypothesis. However, this “feeding effect” may have reduced response differences between CON and HIG treatments.

It has been assumed that DMI will be reduced by depressed rumen pH. Effects associated with this DMI depression have been summarized by Desnoyers et al (2009) and includes:

decreased frequency and amplitude of ruminal contractions inducing a relative increase in rumen load, increased rumen lactic acid and in osmotic pressure, an inflammation of the ruminal epithelium, metabolic dehydration, decreased blood bicarbonate reserves, or alteration in the metabolic acid-base status. However, we did not observe DMI depression in the present experiment, perhaps because pH was not lower than 5.5 (Oetzel, 2004). Therefore, the acidotic condition we created may not have triggered any of the above metabolic disorders in either HOL or JER cows. This finding is also supported by health observations during and after the induction.

There was no difference for DMI between S1 and S4 on both HOL and JER cows. This may have been an indication that the recovery period had appropriate length; a conclusion also supported by an absence of carry-over effects. The DMI quadratic effect ($P < 0.01$) on stage was expected, as the restricted feeding on S2, and the linear effect ($P < 0.01$) had contributed by the DMI difference between S2 and S3, since DMI on S1 and S4 were similar. Milk yield, which is strongly linked with DMI, did not recover to baseline level in 5 d after the induction, as all the experimental animals were enrolled after milk peak, so the stage of lactation would not be a major factor on the milk yield difference between baseline and recovery stage. There was no milk fat percentage depression observed, in accordance with Krause and Oetzel (2005). Milk protein percentage, milk protein yield, milk lactose percentage, and milk lactose yield had similar response to restriction feeding as milk fat and milk fat production, respectively. However, MUN increased on restriction and induction stages, then decreased on recovery stage. This may have been related to limited availability of rapidly fermentable energy in the rumen from the restricted feeding, and it might have affected the first milking on S3, therefore, decreasing the daily average MUN on S3. However, we did not analyze MUN for individual milkings to confirm this assumption.

High concentrations of VFA in the rumen could cause metabolic acidosis. Giancesella et al (2010) reported that blood pH significantly decreased during acidosis. Cows have a relatively small lung capacity, and rely heavily on the kidneys to excrete excess hydrogen ions. Positive relationships have been shown between blood pH and urinary pH and between ruminal pH and urinary pH, respectively (Roby et al., 1987; Fűrll, 1993). Cowles (2010) found a positive relationship between rumen and urine pH ($R = 0.15$, $P < 0.02$). Others (Morgante et al., 2008) have reported that there was no urine pH depression observed during SARA at 4 to 6 h post feeding. Urinary pH generally reflects the acid-base state of an animal, thus monitoring urine pH can be an inexpensive and sensitive method to monitor the effect of diet on blood pH (Goff and Horst, 1998.). As the urine pH data from the present experiment show (Figures 2.4 to 2.6), urine pH for both JER and HOL cows did not follow the same pattern as rumen pH (Figure 2.7). The urine pH shows 3 large drops during the day. The drops happened after feeding or after milking (we started to measure the urine pH at 2 h before feeding, and cows were milked 3 times at 4, 12, and 20 h after feeding). Decreased urine pH may be attributed to the decline in blood HCO_3^- and greater urine net acid excretion, implying that the acid load of lactating cows increased dramatically as fresh feed was ingested, which was excreted in urine.

We assume that buffer systems of blood and organs are able to adapt to the change 2 h after feeding, so that the urine pH starts to increase, however, as a larger size meal was ingested again (when cows had access to feed after milking), the urine pH decreased again (Figure 2.11). These results shows that the urine pH changed dramatically during the day, therefore, for SARA diagnosis purposes, urine sampling should be obtained at specific time points in the first 2 h after feeding (Figure 2.11).

Jersey cows were less responsive to rumen pH depression, because no treatment effect was detected on urine pH; whereas, the treatment effect was significant for HOL cows. However, we did not have Jersey cows' rumen pH data to develop a similar formula to describe relationship between urine and rumen pH

Hindgut acidosis occurs when excessive carbohydrate fermentation in the large intestine leads to an accumulation of organic acids (Gressley et al., 2011). Fecal consistency changes in response to hindgut acidosis, including diarrhea, frothy feces, and presence of mucin casts, which can be used in the field as indicators of SARA (Hall, 2002, Plaizier et al., 2008). The pH of the feces is usually slightly acidic (Dirksen, 1986). Li et al. (2012) reported that daily average pH decreased in the feces of cows experiencing SARA induction. We observed different time points after SARA induction, and found that the fecal pH pattern was similar to that of rumen pH but lagged in time. Also, HOL cows had stronger responses to treatments than JER cows; this may be related to the amounts of fermentable carbohydrate passed to the lower gut in each breed. Additionally, JER cows had faster response due to treatments than HOL cows, which may have happened because the smaller breed has a smaller digestive tract than larger size breed. However, JER cows (37.64 ± 3.61) had similar ($P = 0.23$) BW/DMI ratio compare to HOL cows (43.09 ± 2.53), and therefore, the rationale for such response is not clear.

In the present experiment, we found that the time points of 15 and 23 h after feeding could be used to monitor the rumen pH change based on fecal pH for HOL cows, whereas, the time points of 7, 15, and 23 h after feeding could be used to monitor the rumen pH change based on fecal pH, for JER cows. However, we didn't monitor the fecal pH continuously. More research needed to identify the best time point for practical use.

CONCLUSIONS

Holstein cows' DMI and health were not affected when rumen pH was depressed to between 5.6 and 5.8, for less than 4.3 h. Sub-acute ruminal acidosis induction increased total urine acid excretion by Holstein cows. Rumen pH can be predicted by urine pH within 2 h after feeding [Rumen pH = (Urine pH – 6.2916)/0.2946]. Fecal pH can be used to predict rumen pH depression as early as 7 and 15 h after feeding for JER and HOL cows, respectively.

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TABLES AND FIGURES

Table 2.1. Ingredient composition of the lactation diet fed to cows in control (CON), Moderate (MOD) and High (HIG) treatments throughout the experiment

Ingredient, % of DM	
Alfalfa hay	3.35
Grass hay	2.80
Corn silage	33.6
Alfalfa silage	9.31
Cottonseed	8.01
Soy hulls	4.64
Dry ground corn grain	21.0
Lactating supplement ¹	17.3

¹ Lactating supplement was formulated for 43.6% CP, 13.4% NDF, 7.1% ADF, 0.3% lignin, 5.47% crude fat, and 25.21 mEq/100g DCAD, and contained: 24.7% soybean meal, 26.16% bypass protein, 1.94% bypass fat, 7.53% blood meal, 4.3% sodium bicarbonate, 6.13% limestone, 2.26% dicalcium phosphate, 1.18% white salt, and <1% of each of the following: trace minerals, vitamin E.

Table 2.2. Mean chemical composition and standard error of mean (SEM) of diet fed throughout the experiment

Item	Period			SEM	P-value
	1	2	3		Period
DM, %	47.1	47.9	47.1	0.57	0.60
CP, % of DM	18.0	17.6	18.3	0.27	0.30
Available protein, % of DM	16.5	16.3	17.0	0.48	0.62
ADICP, % of DM	1.5	1.33	1.30	0.36	0.92
Adjusted crude protein, % of DM	17.5	17.3	18.1	0.46	0.53
ADF, % of DM	22.7	24.4	23.2	1.05	0.56
NDF, % of DM	35.6	37.3	36.3	1.71	0.78
Lignin, % of DM	4.53	4.00	3.80	0.29	0.30
NFC, % of DM	33.4	32.6	33.2	1.71	0.95
Starch, % of DM	22.3	21.4	23.6	1.73	0.68
Crude fat, % of DM	5.23	4.70	4.57	0.19	0.13
Ash, % of DM	7.79	7.77	7.59	0.25	0.82
TDN, % of DM	70.0	69.0	70.0	0.77	0.79
NE _L , Mcal/kg of DM	0.74	0.74	0.75	0.01	0.83
NE _M , Mcal/kg of DM	0.74	0.73	0.74	0.01	0.82
NE _G , Mcal/kg of DM	0.47	0.46	0.47	0.01	0.86
Ca, % of DM	0.98	1.07	1.02	0.10	0.83
P, % of DM	0.44	0.45	0.45	0.01	0.53
Mg, % of DM	0.28	0.29	0.31	0.01	0.12
K, % of DM	1.51	1.55	1.41	0.05	0.25
Na, % of DM	0.30	0.38	0.32	0.01	0.01
S, % of DM	0.24	0.25	0.27	0.01	0.18
Fe, ppm	493	955	480	231	0.35
Zn, ppm	156	210	191	18.3	0.22
Cu, ppm	27.0	38.0	33.3	3.01	0.14
Mn, ppm	120	138	133	5.39	0.17
Mo, ppm	0.83	0.87	0.77	0.10	0.77

Table 2.3. Least squares means and associated SE for DMI and milk parameters response of cows in Control (**CON**), Moderate (**MOD**), and High (**HIG**) treatments at different stages.

	Treatment¹				Stage²					P-value³							
	CON	MOD	HIG	SEM	S1	S2	S3	S4	SEM	Trt	Stage	Stage×Trt	Period	Linear		Quad	
														Trt	Stage	Trt	Stage
Overall																	
DMI, kg/d	16.2 ^a	16.9 ^b	16.4 ^{ab}	0.53	16.1 ^a	7.07 ^b	18.3 ^c	16.7 ^a	0.79	0.05	<0.01	0.13	0.41	0.09	<0.01	0.03	<0.01
Milk yield, kg/d	22.6 ^a	23.7 ^b	23.5 ^b	1.89	23.5 ^a	22.9 ^{ab}	18.6 ^c	22.1 ^b	0.48	0.02	<0.01	0.24	<0.01	0.09	<0.01	0.02	<0.01
3.5% FCM, kg/d	24.0	24.7	24.6	0.95	25.3 ^a	24.6 ^a	20.9 ^b	24.5 ^a	1.90	0.50	<0.01	0.04	0.01	0.35	<0.01	0.43	<0.01
Fat, %	4.12	3.96	4.14	3.96	4.30	4.13	4.32	4.13	0.14	0.95	0.42	0.12	0.53	0.74	0.57	0.98	0.33
Fat, kg/d	0.88	0.90	0.90	0.69	0.94 ^a	0.91 ^a	0.79 ^b	0.90 ^a	0.07	0.80	<0.01	0.05	0.10	0.60	0.02	0.67	0.02
Protein, %	3.28	3.28	3.28	0.09	3.38	3.38	3.33	3.40	0.06	0.98	0.28	0.93	0.06	0.89	0.17	0.96	0.95
Protein, kg/d	0.72	0.75	0.74	0.35	0.76 ^a	0.76 ^a	0.61 ^b	0.76 ^a	0.05	0.28	<0.01	0.13	0.11	0.21	<0.01	0.31	0.02
Lactose, %	4.40	4.40	4.41	0.85	4.41	4.45	4.37	4.37	0.12	0.98	0.29	0.38	<0.01	0.87	0.44	0.96	0.72
Lactose, kg/d	1.01	1.05	1.04	0.09	1.05 ^a	1.04 ^a	0.83 ^b	1.02 ^a	0.10	0.43	<0.01	0.14	<0.01	0.37	<0.01	0.35	0.01
MUN, mg/dL	13.7	13.8	13.6	0.38	13.5 ^a	14.4 ^b	15.0 ^c	12.4 ^d	0.42	0.65	<0.01	0.82	<0.01	0.62	<0.01	0.43	0.76
SCC, x 1,000/ml	122	109	96.5	1.23	105	85.6	126	105	1.27	0.22	0.10	0.41	0.04	0.08	0.99	0.97	0.98
Jersey																	
DMI, kg/d	13.2 ^{ab}	14.0 ^a	12.4 ^b	0.63	13.9 ^a	6.16 ^b	14.7 ^a	13.8 ^a	0.79	<0.01	<0.01	0.64	0.11	0.70	<0.01	<0.01	<0.01
Milk yield kg/d	18.7	19.1	19.4	2.37	19.8 ^a	20.0 ^a	15.2 ^b	20.8 ^a	3.77	0.18	<0.01	0.08	0.22	0.30	<0.01	0.13	<0.01
3.5% FCM kg/d	22.2	21.8	22.2	3.26	23.5 ^a	22.7 ^a	18.4 ^b	23.8 ^a	3.00	0.85	<0.01	0.22	0.31	0.98	<0.01	0.58	0.09
Fat, %	4.93	4.57	4.75	0.24	4.86	4.57	4.81	4.74	0.25	0.19	0.61	0.79	0.19	0.37	0.69	0.12	0.44
Fat, kg/d	0.88	0.84	0.86	0.07	0.93 ^a	0.86 ^a	0.73 ^b	0.93 ^a	0.09	0.56	<0.01	0.59	0.16	0.68	<0.01	0.32	0.08
Protein, %	3.61	3.69	3.65	0.09	3.68	3.67	3.58	3.67	0.11	0.31	0.31	0.75	<0.01	0.50	0.35	0.17	0.58
Protein, kg/d	0.65	0.70	0.68	0.02	0.71 ^a	0.73 ^a	0.54 ^b	0.73 ^a	0.09	0.22	<0.01	0.16	0.51	0.22	<0.01	0.19	0.47
Lactose, %	4.39	4.44	4.36	0.15	4.43	4.43	4.32	4.41	0.18	0.33	0.22	0.17	<0.01	0.64	0.46	0.16	0.52

Table 2.3 continued

Lactose, kg/d	0.82	0.86	0.85	0.12	0.88 ^a	0.91 ^a	0.67 ^b	0.91 ^a	0.14	0.52	<0.01	0.13	0.34	0.41	<0.01	0.43	0.42
MUN, mg/dL	14.0	14.5	13.9	0.48	13.3 ^a	14.5 ^b	15.7 ^c	12.9 ^a	0.47	0.32	<0.01	0.72	<0.01	0.73	<0.01	0.14	0.10
SCC, x 1,000/ml	4.64	4.42	4.51	0.25	97.5	79.04	120	78.3	1.35	0.44	0.13	0.93	0.01	0.47	0.22	0.30	0.53

Holstein

DMI, kg/d	17.7	18.4	18.6	0.80	18.4 ^a	8.73 ^b	21.3 ^c	19.3 ^a	1.25	0.10	<0.01	0.18	0.11	0.04	<0.01	0.53	0.02
Milk yield, kg/d	24.6	25.9	25.6	2.03	26.9 ^a	25.9 ^{ab}	21.9 ^c	25.1 ^b	2.29	0.09	<0.01	0.03	<0.01	0.17	<0.01	0.09	<0.01
3.5% FCM, kg/d	24.9	26.1	25.7	1.81	27.1 ^a	26.4 ^a	23.0 ^b	25.8 ^a	0.29	0.34	0.34	0.09	0.06	0.34	0.10	0.27	0.02
Fat, %	3.63	3.78	3.66	0.15	3.75	3.65	3.81	3.56	0.17	0.58	0.55	0.08	0.79	0.87	0.35	0.30	0.50
Fat, kg/d	0.88	0.93	0.91	0.08	0.96 ^a	0.94 ^a	0.83 ^b	0.90 ^{ab}	0.05	0.43	0.03	0.08	0.36	0.42	0.42	0.31	0.09
Protein, %	3.11	3.07	3.10	0.08	3.09	3.09	3.06	3.12	0.08	0.69	0.65	0.93	0.67	0.79	0.30	0.41	0.78
Protein, kg/d	0.75	0.77	0.77	0.05	0.82 ^a	0.79 ^a	0.67 ^b	0.79 ^a	0.06	0.67	<0.01	0.17	0.01	0.44	<0.01	0.66	0.02
Lactose, %	4.41	4.39	4.43	0.11	4.40	4.46	4.40	4.36	0.10	0.77	0.47	0.53	<0.01	0.69	0.23	0.54	0.94
Lactose, kg/d	1.10	1.14	1.13	0.19	1.02 ^a	1.17 ^a	0.98 ^b	1.14 ^a	0.07	0.65	<0.01	0.17	<0.01	0.53	0.04	0.49	0.02
SCC, x 1,000/ml	4.88	4.82	4.60	0.30	116	94.6	137	128	1.39	0.25	0.33	0.49	0.39	0.12	0.57	0.60	0.80
MUN, mg/dL	13.6	13.53	13.47	0.52	13.4 ^a	14.2 ^{ab}	14.6 ^b	12.0 ^c	0.55	0.93	<0.01	0.76	<0.01	0.71	<0.01	0.99	0.50

¹ Treatment = Dietary treatments were control diet (CON, with 0% of top dress), moderate diet (MOD, with 10% of top dress) and High diet (HI with 20% of top dress). Top dress applied on S3 only.

² Each period had 4 stages: baseline (S1, d 1 to 3, cows fed normal TMR with ad libitum intake), restricted feeding (S2, d 4, cows fed 50% of DM as stage 1), challenge feeding (S3, d 5, cows fed TMR + treatment) and recovery (S4, d 6 to 10, cows fed normal TMR with ad libitum intake).

^{abc} Means within a row with different superscripts are different ($P < 0.05$).

Table 2.4. Least squares means and associated SE for rumen, urine and fecal pH response of Holstein and Jersey cows in control (**CON**), moderate (**MOD**), and high (**HIG**) treatments.

	Treatment ¹				P-value				
	CON	MOD	HIG	SEM	Trt	Period	Time Point	Linear	Quad
Rumen									
Mean pH	6.45 ^a	6.35 ^b	6.24 ^c	0.07	<0.01	<0.01	<0.01	<0.01	0.85
Hour < 5.8	2.17 ^a	2.00 ^a	4.33 ^b	0.78	<0.01	<0.01	---	<0.01	<0.01
Area under the curve	0.60	0.38	0.54	0.23	0.75	<0.01	---	0.85	0.48
Mean urine pH									
Overall	8.48 ^a	8.46 ^{ab}	8.43 ^b	0.02	0.04	<0.01	<0.01	0.01	0.63
Jersey	8.35	8.37	8.37	0.03	0.84	<0.01	<0.01	0.61	0.79
Holstein	8.54 ^a	8.51 ^a	8.46 ^b	0.24	<0.01	<0.01	<0.01	<0.01	0.75
Mean fecal pH									
Overall	6.61	6.54	6.56	0.05	0.30	<0.01	<0.01	0.25	0.30
Jersey	6.69	6.68	6.73	0.06	0.85	<0.01	<0.01	0.70	0.68
Holstein	6.57	6.46	6.46	0.06	0.14	<0.01	<0.01	0.09	0.33

¹ Dietary treatments were control diet (with 0% of top dress), moderate diet (with 10% of top dress) and High diet (with 20% of top dress). Top dress pellet made from 50:50 wheat-barley. Top dress amount calculated by S1 DMI. Diets were fed once daily. Treatment only applied on S3.

^{ab} Means within a row with different superscripts are different ($P < 0.05$).

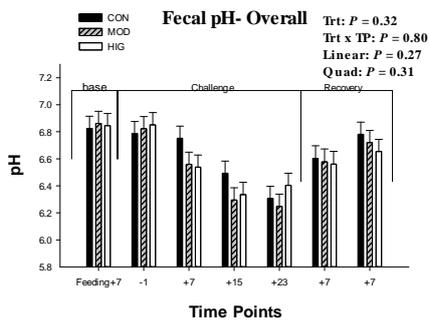


Figure 2.1.

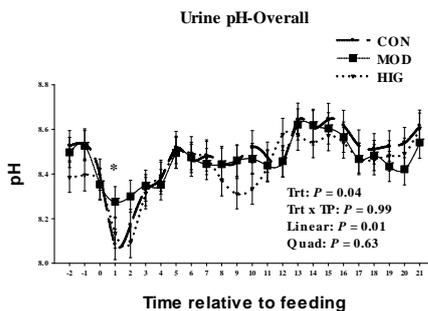


Figure 2.4.

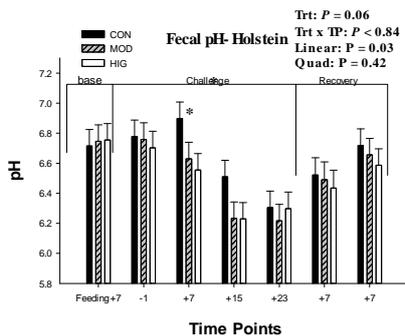


Figure 2.2.

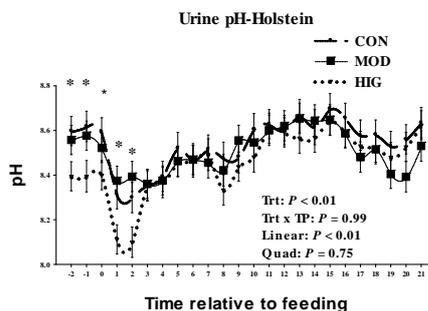


Figure 2.5.

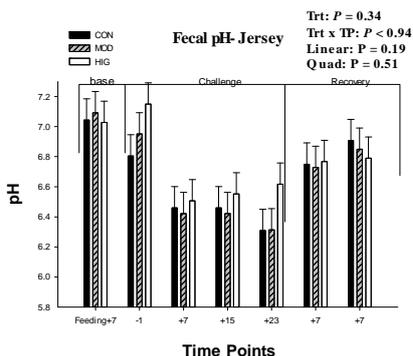


Figure 2.3

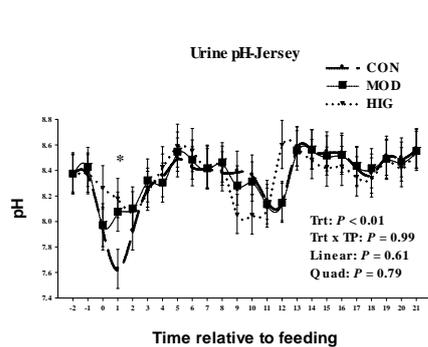


Figure 2.6.

Figure 2.1. Urine pH least squares means and SEM for cows receiving control (CON), Moderate (MOD) or HIGH (HIG) from hour – 2 to 21 relative to feeding.

Figure 2.2. Urine pH least squares means and SEM for Holstein cows receiving control (CON), Moderate (MOD) or HIGH (HIG) from hour – 2 to 21 relative to feeding.

Figure 2.3. Urine pH least squares means and SEM for Jersey cows receiving control (CON), Moderate (MOD) or HIGH (HIG) from hour – 2 to 21 relative to feeding.

Figure 2.4. Fecal pH least squares means and SEM for cows receiving control (CON), Moderate (MOD) or HIGH (HIG) from 7 hr

Figure 2.5. Fecal pH least squares means and SEM for Holstein cows receiving control (CON), Moderate (MOD) or HIGH (HIG) from 7 hr after feeding on first day of S1, -1, +7,+15 and +23 hr after feeding of S3, and +7 hr after first and second day on S4.

Figure 2.6. Fecal pH least squares means and SEM for Jersey cows receiving control (CON), Moderate (MOD) or HIGH (HIG) from 7 hr after feeding on first day of S1, -1, +7,+15 and +23 hr after feeding of S3, and +7 hr after first and second day on S4.

pH-CON

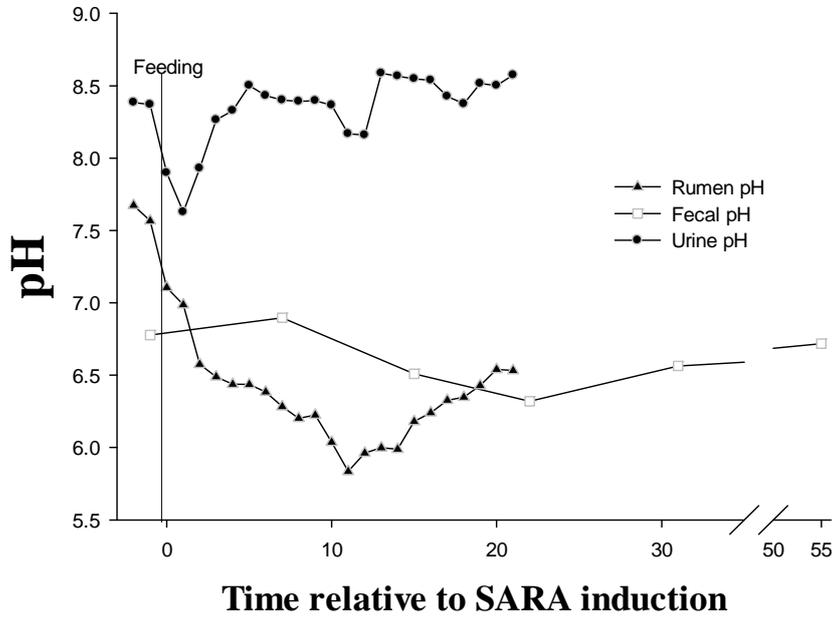


Figure 2.7. Ruminal, urine, fecal pH least squares means for cows received CON treatment during the challenge and recovery stage.

pH-MOD

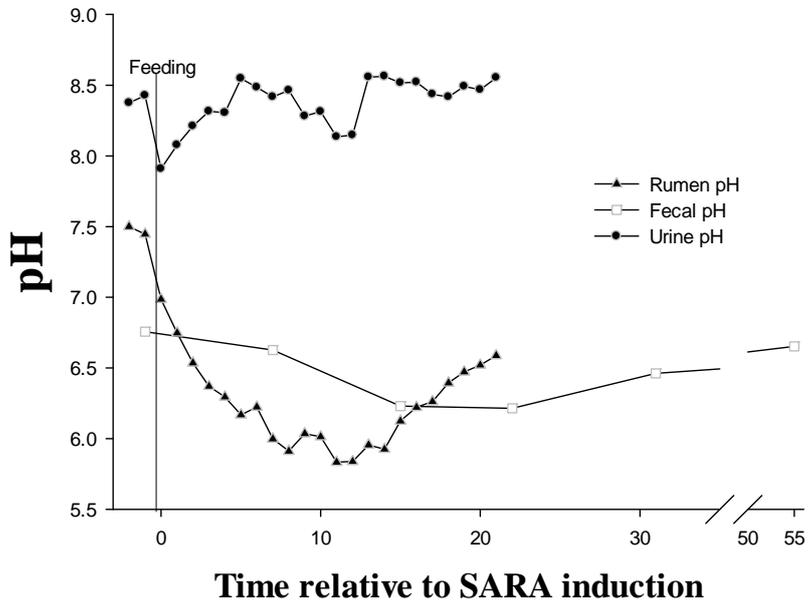


Figure 2.8. Ruminal, urine, fecal pH least squares means for cows received MOD treatment during the challenge and recovery stage.

pH-HIG

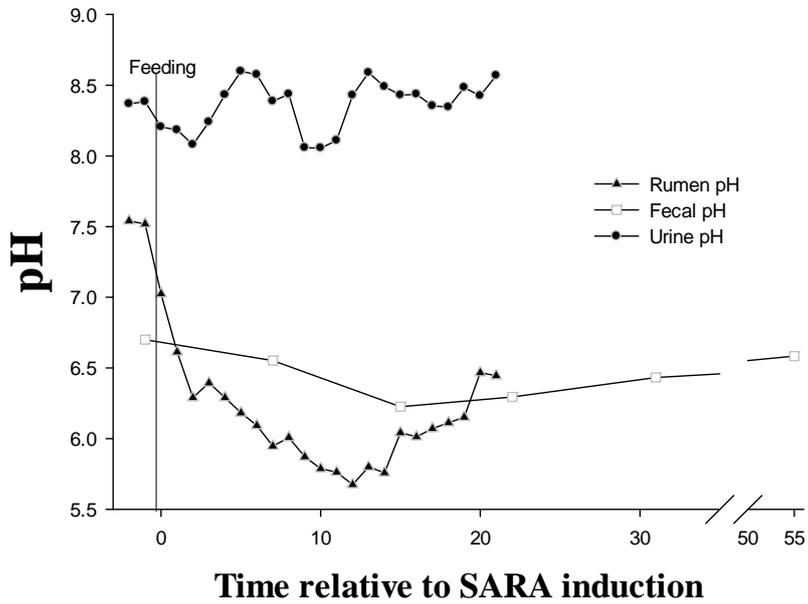


Figure 2.9. Ruminal, urine, fecal pH least squares means for cows received HIG treatment during the challenge and recovery stage.

Rumen pH

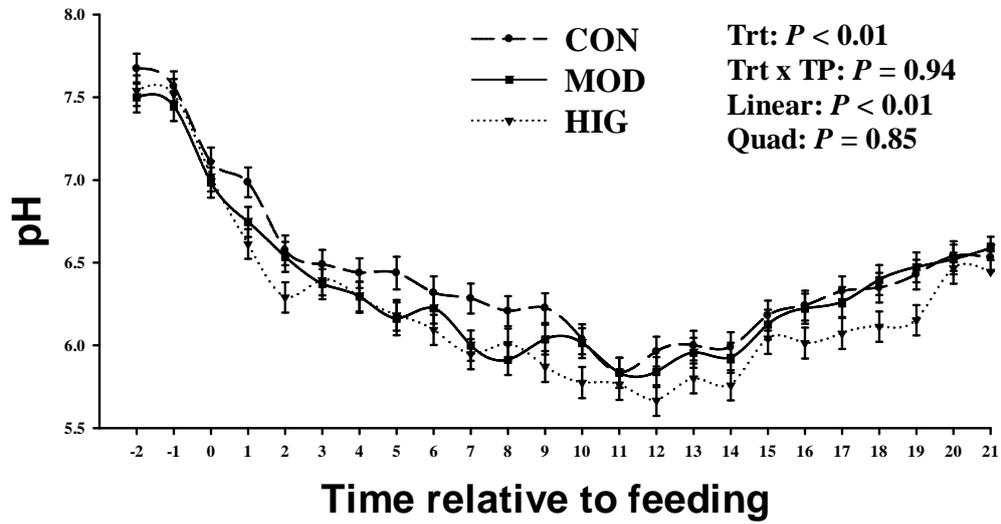


Figure 2.10. Rumen pH least squares means and SEM for cows receiving control (CON), Moderate (MOD) or HIGH (HIG) from hour - 2 to 21 relative to feeding.

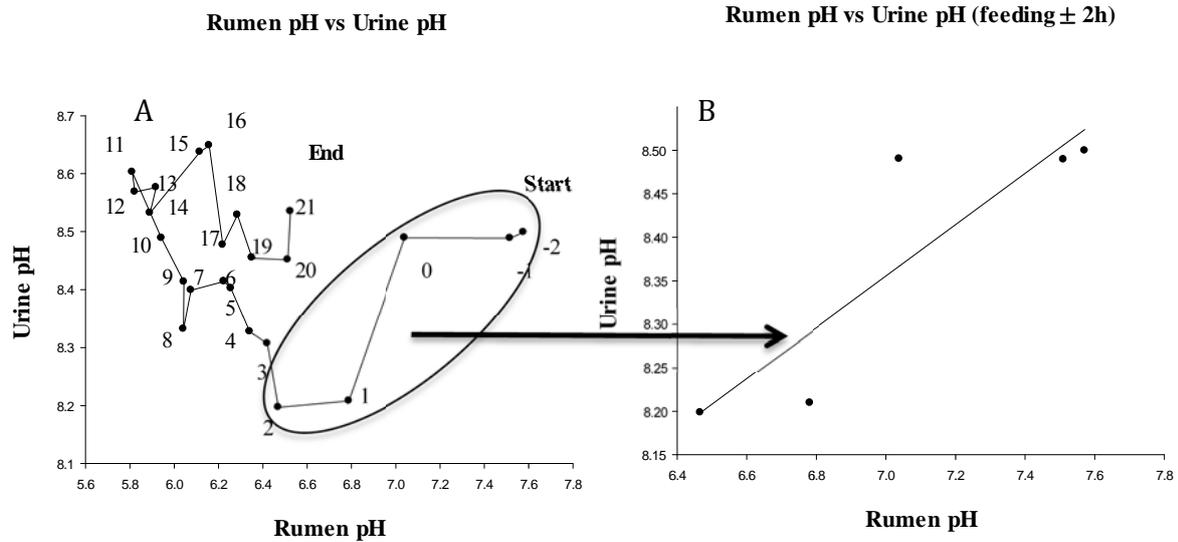


Figure 2.11. **A)** Relationship between rumen pH and urine pH between -2 to 22 hours after feeding. Data set includes all treatments observations from CAN cows. **B)** Relationship between rumen pH and urine pH between -2 to 2 hours after feeding. Urine pH = $6.2916 + 0.2949$ rumen pH; $R^2 = 0.77$, $P = 0.049$. Data set includes all treatments observations from the cannulated Holstein cows.

CHAPTER 3

Effects of Direct-fed *Bacillus pumilus* 8G-134 (NRRL B-50174) on Feed Intake, Milk Yield, Milk Composition, Feed Efficiency, and Health Condition of Pre- and Postpartum Holstein Cows

ABSTRACT

The usage of direct-fed microbials (**DFM**) has become common in the dairy industry, but questions regarding their value and mode of action remain prevalent. The objective of this study was to evaluate the effects of a DFM (*Bacillus pumilus* 8G-134) on pre-partum performance and prevalence of health disorders in early lactation. Forty-three multiparous Holstein cows were fed a TMR according to NRC (2001) recommendations and assigned to 2 treatments in a completely randomized block design. Cows in the direct-fed microbial treatment (**DFMt**, n = 21) received 5.0×10^9 cfu of *B. pumilus* in 28 g of media, whereas, cows in the control treatment (**CON**, n = 22) received 28 g of media. Treatments were top-dressed on the TMR daily. Treatments were applied from 21 d before expected calving date to 21 d after calving. Blood samples were analyzed for beta-hydroxybutyrate at d 5 and 14 after calving. Cows that had blood beta-hydroxybutyrate concentrations higher than 1.2 mmol/L were classified as experiencing sub-clinical ketosis. Treatment DFMt tended to have lower haptoglobin concentration than DFM cows on d 14. Treatment DFMt had higher IgA concentration than CON cows on the first week after calving. Other health disorders recorded were retained placenta, displaced abomasum, clinical ketosis, and pneumonia. Fecal scores were recorded daily. Statistical analysis was performed using the MIXED and GLIMMIX procedures of SAS. The DMI, BW, and BCS were not different between treatments through experiment. Cows fed DFMt had higher milk yield, fat

corrected milk, energy correct milk, fat production and protein production on the second week of lactation, however there were no differences between treatments on milk yield and milk components overall. Cows fed DFMT tended to have higher feed conversion than cows fed CON. Cows fed DFMT tended to have less incidence of sub-clinical ketosis at d 5 but not at d 14 when compared to cows receiving CON. Cows fed DFMT tended to have higher fecal score than CON. In conclusion, cows receiving DFMT tended to have lower incidence of sub-clinical ketosis than cows receiving CON. Cows fed DFMT tended to have higher feed conversion and immunity than CON. Dry matter intake, BW, BCS were not affected by DFMT supplementation.

INTRODUCTION

Direct-fed microbials (**DFM**) have been studied to manipulate the microbial ecosystem and fermentation characteristics in the rumen and intestinal tracts of livestock animals (Seo et al., 2010). Commercial DFM are widely used in the dairy industry to improve performance of lactating dairy cows.

Numerous experiments have reported DFM to have positive effects on digestion rate, DMI, milk yield, milk composition, rumen pH stability, and feed efficiency in lactating dairy cows (Nocek et al., 2002; Nocek and Kautz, 2006; Qiao et al., 2009; Oetzel et al., 2007; Stein et al., 2006; Chiquette et al., 2008; Ferguson et al., 2010). *Bacillus* has been proven as an efficient starch utilizer (Zhang et al., 2014). Most of the research was performed using lactate acid producers, lactate acid utilizers, yeast, or fungi as DFM (Nocek et al., 2002; Nocek et al., 2003; Nocek and Kautz, 2006; Stein et al., 2006; Ghorbani et al., 2002; Raeth-Knight et al., 2007; Oetzel et al., 2007; Keyser et al., 2007; Chiquette et al., 2008; Weiss et al., 2008; Lehloenya et

al., 2008). The potential mode of action of those DFMs has been summarized (Krehbiel et al., 2003; Seo et al., 2010).

Recently, research has focused on spore forming bacteria, which have higher resistance to stresses during production and storage processes (Hyronimus et al., 2000) and also higher resistance to gastric and intestinal environmental conditions (Sanders et al., 2003; Hong et al., 2005). *Bacillus* species are the most examined in this category. Reports demonstrated that *Bacillus* species could increase milk yield, fat-corrected milk (**FCM**), and milk protein (Kritas et al., 2006; Qiao et al., 2009). These observations may be explained by increased volatile fatty acids (**VFA**) production (Qiao et al., 2010) and strong cellulolytic activity (Seo et al., 2010). Ferguson et al. (2010) reported that feeding *B. pumilus* increased milk yield and milk fat for the first 22 wk postpartum. Performance improvement may be a result of altered rumen fermentation patterns, which enhance overall energy supply from the digestive tract.

Rumen fermentation pattern is physiologically altered around parturition. The transition period is defined as the period from 3 wk before parturition until 3 wk postpartum. During the transition period from late gestation through early lactation, the dairy cow undergoes tremendous metabolic adaptations (Bell, 1995). As peak milk yield increases, the transition period for dairy cows becomes much more challenging with most infectious diseases and metabolic disorders occurring during this time (Drackley, 1999; Grummer, 1995). Milk production increases faster than energy intake in the first few weeks postpartum, and thus high producing cows will incur negative energy balance.

Therefore, *B. pumilus* as a DFM may be beneficial to minimize the negative energy balance during the transition period and decrease the risk of metabolic disorders, thus improving health and performance during the transition period through lactation. The objectives of this experiment

were to 1) to evaluate the effect of *Bacillus pumilus* 8G-134 (NRRL B-50174) as a DFM supplement on DMI, milk production and composition, and feed conversion from pre-partum to mid-lactation; and 2) to evaluate the effect of the DFM on energy balance and prevalence of metabolic disorders during the transition period.

MATERIALS AND METHODS

Animal care and housing

All experimental procedures were approved by the University of Illinois at Urbana-Champaign Institutional Animal Care and Use Committee. Forty-three multiparous Holstein cows were assigned to two treatments in a randomized complete block design. During the prepartum period, cows were housed in free stalls with individual Calan feed gates (American Calan Inc., Northwood, NH). Approximately 2 d before expected parturition, cows were moved to individual maternity pens in the same barn until parturition. After parturition, cows were housed in tie stalls with mangers designed for measurement of feed intake. Cows were milked three times daily. During the experimental period, cows were fed for *ad libitum* intake. Diets (pre – and postpartum) were formulated to meet or exceed cows' requirements according to NRC (2001) and were delivered once daily as a total mixed ration (**TMR**).

Treatments and management

Cows in the DFM treatment (**DFMt**, n = 21) received 5.0×10^9 cfu of *Bacillus pumilus* 8G-134 (NRRL B-50174) DFM (Dupont Nutrition and Health, Waukesha, WI) in 28 g of media, whereas cows in the control treatment (**CON**, n = 22) received 28 g of media as placebo. Treatments were mixed with 0.45 kg ground corn and top-dressed on the close-up or lactation

TMR once daily for each cow. Treatments were applied from 21 ± 1 d before expected calving date to 154 d after calving. Cows were randomly assigned to treatments and balanced for initial body weight (**BW**; 719 ± 9.59 kg vs 715 ± 9.78 kg for CON and DFMT, respectively), parity (2.53 ± 0.42 vs 2.03 ± 0.36 for CON and DFMT, respectively), and previous lactation (305 d) milk production ($11,703 \pm 543$ kg vs $11,389 \pm 473$ kg for CON and DFMT, respectively).

Sample collection

Feed ingredients and TMR samples were obtained weekly and analyzed for dry matter (**DM**) content (AOAC, 1995) by drying for 24 h in a forced-air oven at 110°C . Dietary DM was adjusted weekly for changes in DM content. Total mixed ration samples were taken weekly, and stored at -20°C until submitted for analysis. Monthly composite samples were analyzed for contents of DM, crude protein (**CP**), acid-detergent fiber (**ADF**), neutral-detergent fiber (**NDF**), lignin, starch, fat, ash, Ca, P, Mg, K, Na, Fe, Zn, Cu, Mn, Mo, and S using wet chemistry methods (Dairy One, Ithaca, NY). Values for RFV, TDN, NEI, NEm, NEg, ME, and DE were provided by the lab and were based on NRC (2001). Intake from each cow was measured and DMI was recorded daily.

Cows were milked 3 times daily at 0600, 1400, and 2100 h. Milk weights were recorded daily and samples were obtained from 3 consecutive milkings weekly. Consecutive weekly samples were composited in proportion to milk yield at each sampling and preserved (800 Broad Spectrum Microtabs II; D&F Control Systems, Inc., San Ramon, CA). Composite milk samples were analyzed for fat, protein, lactose, urea N (**MUN**), total solid and somatic cell count (**SCC**) using mid-infrared procedures (AOAC, 1995) at a commercial laboratory (Dairy Lab Services, Dubuque, IA).

Health disorders included retained placenta (**RP**), displaced abomasum (**DA**), clinical ketosis (**CK**), mastitis (**MAST**), and metritis (**MET**). Retained placenta was defined as placenta that failed to deliver completely longer than 12 h after calf delivery; DA was diagnosed by a veterinarian; CK was diagnosed by urinalysis strip (Ketostix, Bayer Corp. Diagnostics Division, Elkhart, IN); MAST was diagnosed by altered milk composition confirmed by positive microbiological culture; and MET was defined as uterine discharge that is foul, purulent, orange-brown in color. Fecal score (**FS**) and general appearance (**GA**) were recorded daily. Fecal scores were on a 1 to 4 scale according to (Krause et al., 2009): 1 = runny: liquid consistency, splatters on impact, spreads readily; 2 = loose: may pile slightly and spreads and splatters moderately on impact and setting; 3 = soft: firm but not hard, piles but spreads slightly on impact and settling; 4 = dry: hard, dry appearance, original form not distorted on impact and settling. General appearance was scored as (Krause et al., 2009): 1: bright and alert; 2: depressed; 3: reluctant to rise. Cows with fecal score ≤ 2 were classified as experiencing transient digestive problems (**FS** ≤ 2) whereas cows with $FS > 2$ were classified as healthy (**HEALTHY**). Cows with $GA \geq 2$ were classified as sick (**ALTERED**) whereas cows with $GA < 2$ were classified as healthy (**HEALTHY**).

Body weight was measured and body condition score (**BCS**) was assigned in quarter unit increments (Ferguson et al., 1994) for each cow weekly. More than one individual assigned BCS independently at each time of scoring throughout the experiment.

Blood samples were collected on d 5 and d 14 after calving. Beta-hydroxybutyrate (**BHBA**) was measured immediately after the collection with a commercial blood ketone monitoring system (Precision Xtra, Abbott Diabetes Care Inc., Alameda, CA). Cows that had blood BHBA concentrations higher than 1.2 mmol/L were classified as experiencing sub-clinical ketosis

(**SCK**). Serum samples were analyzed for non-esterified fatty acids (**NEFA**), which was determined by enzymatic analysis (NEFA-HR(2), Wako Diagnostics, Richmond, VA). Cows that had NEFA serum concentrations higher than 0.7 mEq/L were classified as high (**HNEFA**) whereas cows that had NEFA serum concentrations lower than 0.7 mEq/L were classified as low (**LNEFA**). Both BHBA and NEFA variables were dichotomized based on cut-off points previously established by Ospina et al. (2010). The immunoglobulins, IgA, IgG and IgM were quantified in milk on the first week after calving and serum on d 5 and d 14 by an ELISA assay (Bethyl laboratories, Montgomery, TX). Haptoglobin was quantified in serum samples by an ELISA assay (ALPCO, SALEM, NH). Cows that had haptoglobin serum concentrations higher than 150 µg/ml were classified as positive (**POSITIVE**) whereas cows that had serum haptoglobin concentrations lower than 150 µg/ml were classified as negative (**NEGATIVE**).

Feed conversion calculation

There are multiple formulas to calculate feed conversion (**FE**) for different purposes. In the present manuscript, we based FE calculations on energy-corrected milk (**ECM**), fat-corrected milk (**FCM**), or milk yield along with DMI (Tyrrell and Reid, 1965).

The formulas were as follow:

$$FCM(kg) = (0.4255 \times Milk\ Yield) + 16.425 \times \left(\frac{fat\ percentage}{100} \times Milk\ Yield \right);$$

$$ECM(kg) = 12.82 \times \frac{fat\ percentage}{100} \times yield + 7.13 \times \frac{protein\ percentage}{100} \times Milk\ yield$$

$$FE\ (ECM) = \frac{ECM}{DMI}$$

$$FE(FCM) = \frac{FCM}{DMI}$$

$$FE(yield) = \frac{Milk\ Yield}{DMI}$$

Statistical Analyses

The data were analyzed using SAS (v 9.3; SAS Institute Inc., Cary, NC). The MIXED models procedure was used for the outcomes of interest DMI, BW, BCS, milk parameters, and composed variables (e.g. FE), which were averaged weekly. The model contained the fixed effects of treatment, week, and the interaction of treatment by week. Initial measurements, before treatment administration, were used as covariates when analyzing the dependent variables BW and BCS. Variables were subjected to 5 covariance structures: compound symmetry, autoregressive order 1, autoregressive heterogeneous order 1, unstructured, and Toeplitz. The covariance structure that yielded the lowest corrected Akaike information criterion was used in the model (Littell et al., 1998). Cow was the experimental unit and considered as a random effect. Week was included in the model as a repeated measurement with cow as subject. All performance variables were analyzed as weekly averages. Least squares means were calculated and are presented with standard errors of means (SEM). Degrees of freedom were estimated by using the Kenward-Roger method in the model statement (Littell et al., 1998). Residual distribution was evaluated for normality and homoscedasticity.

Dry matter intake change from 3 wk before and after calving, milk yield change from calving to 3 wk, blood BHBA, plasma NEFA, serum and milk IgA, Ig M, IgG and serum haptoglobin concentrations were analyzed as continuous variables using the MIXED procedure. A multivariable logistic mixed models (GLIMMIX procedure) was used for the dichotomized variables (SCK, NEFA, RP, DA, CK, MET, MAST, FS, GA, and HAPTOGLOBIN). The

procedure used for each variable is also indicated in the results section for each outcome of interest. A log transformation was used for the variables NEFA, SCC, and haptoglobin for better homogeneity of the distribution of residuals. Means shown in tables and graphs for these variables are back-transformed. Statistical significant declared as P value lower than 0.05, and tendency declared as P value lower than 0.10.

RESULTS

The ingredient composition of the diets fed to cows is detailed in Table 3.1 and the analyzed chemical composition is shown in Table 3.2. Performance outcome variables results are shown in Table 3.3. Body weight, BCS, SCC, and MUN were not affected ($P > 0.19$) by treatment, and no treatment \times week interaction ($P > 0.23$) was observed (Figures 3.1, 3.2, 3.3, and 3.4).

Dry matter intake was not affected by the DFM supplementation during prepartum or postpartum periods, nonetheless we observed an overall treatment \times week tendency ($P = 0.10$; Figure 3.5).

Cows receiving DFM increased DMI by 33.9% from wk -3 to -1 relative to calving, whereas cows receiving CON decreased DMI by 7.85% during the same period. Even though there was a numerical difference, there was no statistical difference ($P > 0.22$) observed for the DMI change. Cows receiving DFMT increased DMI by 29.3% DMI from wk 1 to 3 relative to calving, whereas cows receiving CON increased DMI by 50.5%. There was a numerical difference but there was no statistical difference ($P > 0.44$) observed for the DMI change (Table 3.3).

Milk yield, milk fat production, FCM, and ECM were not affected by DFM supplementation (Figures 3.6, 3.7, 3.8, and 3.9). However, the treatment \times week interaction was statistically significant ($P < 0.01$) for all the aforementioned outcome variables. This interaction may have

happened due to the fact that cows receiving DFMt numerically produced more milk from weeks 1 to 4 and 9 to 10. Cows that received DFMt had similar (49.9% vs 53.0%, $P > 0.90$) milk yield increase (%) from wk 1 to 3 (Table 3.3). Additionally, cows receiving CON had higher DMI from wk 15 through wk 22 after calving than cows receiving DFMt (Figure 3.5).

Milk fat percentage was not affected by treatment (Figure 3.10). However, there was almost a tendency ($P < 0.12$) for a treatment \times week interaction for milk fat percentage. Cows that received DFMt produced milk with 0.07 percentage units less ($P < 0.07$) lactose content when compared to cows that received CON. On the other hand, milk lactose yield was not affected by DFM supplementation (Figure 3.11).

Milk production efficiencies (calculated based on FCM and ECM) were higher ($P < 0.07$) by 0.1 kg of milk per kilogram of DMI in cows that received DFMt when compared to cows that received CON. There was no treatment effect observed on FE based solely on milk yield. No treatment \times week interaction was observed on any FE outcome variable.

There was a day relative to calving effect for serum NEFA concentration as a continuous outcome variable but not a treatment effect (Table 3.4; Figure 3.12). At 14 DIM there was a tendency ($P = 0.07$) for cows receiving CON having greater odds (OR = 3.21) of being classified as HNEFA when compared to DFMt cows (Table 3.5). At 5 DIM there was a tendency ($P = 0.06$) for cows receiving CON having greater odds (OR = 3.85) of being classified as SCK when compared to DFMt cows (Table 3.5).

There were no differences ($P > 0.25$) in the concentration of IgG and IgM in milk or serum samples. Milk concentrations of IgA was higher ($P = 0.03$) during the first week from DFMt cows when compared to CON, however, there was no difference ($P = 0.42$) in serum IgA concentrations between treatments. At 14 DIM there was a tendency ($P = 0.09$) for cows

receiving CON having greater odds (OR = 3.55) of being classified as POSITIVE when compared to DFMT cows (Table 3.5). All the measured health occurrences are shown in Table 3.5. When analyzed for its frequency, cows receiving CON tended ($P = 0.08$) to have higher probability of digestive occurrence when compared to DFMT cows (Table 3.5).

DISCUSSION

The DMI interaction prepartum may be explained due to the fact that DMI of cows that received DFMT only decreased 0.15 kg/d whereas cows that received CON decreased 2.16 kg/d from wk - 2 to - 1 relative to calving. However, DMI increased faster for CON cows than for DFMT cows after calving (Table 3.3). Cows that received DFMT seemed to have higher DMI in the last week before calving than cows that received CON, which may have been pivotal for a sound transition period and consequently supported higher milk production during early lactation.

Lactose production was not different from DFMT to CON, even though the lactose percentage was tended to higher in CON than DFMT. Milk yield was numerically higher for cows receiving DFMT when compared to cows receiving CON; therefore, the percentage difference was neutralized by the difference in milk production.

Cows that received DFMT had higher feed conversion (FE) either using FCM or ECM as the variable of interest (Figures 3.13 and 3.14). This finding may indicate that cows that received DFMT more efficiently converted feed into milk components during the first 22 wk of lactation (Figures 3.13, 3.14, and 3.15). The extra milk output in relation to DMI (which did not differ between treatments) came with no penalty from the perspective of cow's body tissues mobilization as we assessed by the findings of no treatment differences for BW and BCS. Cows that received CON showed signs of excessive adipose tissue mobilization as indicated by higher

levels of BHBA and NEFA after calving when compared to DFMT cows (Table 3.5). During this experiment, we were not able to detect a milk fat percentage difference between treatments.

However, milk fat yield (kg) had an interesting treatment by week interaction. Cows receiving DFMT were able to produce considerable higher fat yield in the beginning of lactation (week 2) when compared to cows receiving CON (Figure 3.10).

The aforementioned findings partially agree with Ferguson et al. (2010) where the authors concluded that the *Bacillus* treatments (**BAC**; *Bacillus pumilus* 8G-134 offered at 5×10^9 and 1×10^{10} CFU/d) significantly increased milk volume and milk fat content when compared to the control treatment and milk volume compared to the *Lactobacillus* treatment (**LAC**). The authors also suggested that cows on BAC treatments could have mobilized more body tissue than the control cows to produce more milk and eat less than expected, but serum NEFA, glucose, and BHBA suggested that those cows were in similar energy status as control cows. Additionally, the authors found that BW and BCS were similar for the BAC groups relative to the control group and also suggested that cows receiving BAC did not mobilize more body tissue to produce the additional milk volume.

In contrast to the experiment of Ferguson et al. (2010), we did not find statistical treatment differences for milk yield. One of the reasons for this could be the fact that cows on that experiment produced (or had lower potential for milk production) less milk overall when compared to cows in our experiment (~35 kg vs ~41 kg). Secondly, the data in the present manuscript collectively support the conclusion that cows receiving DFMT did not have to mobilize more adipose tissue reserves when compared to cows receiving CON.

A relationship between occurrence of postpartum metabolic disorders and blood NEFA and BHBA concentration has been proposed (Drackley et al., 2001; Hegardt, 1999). Ospina et al.

(2010) collected blood samples from 1,672 cows from 60 different herds in the U.S. Samples were collected weekly starting from 2 weeks before calving until 2 weeks after calving. By measuring cows' serum concentrations of NEFA and BHBA postpartum, the authors were able to conclude that cows above the identified cut-off points (0.7 mEq/L for NEFA and 1.2 mmol/L for BHBA): 1) were +1.7% more at risk for disease occurrence (CK and DA); 2) had - 0.8% pregnancy rate; and 3) produced 332 kg less ME305 milk; when compared to herd mates below the cut-off points. The current experiment resulted relative higher NEFA and BHBA concentrations compared to the other experiments performed around peripartum (Janovick et al., 2011; Douglas et al., 2006), which might be a result from the high starch content in the diet for the pre-partum period in the present research.

Immunoglobulin G is the most abundant immunoglobulin in both serum and milk of dairy cows (Stelwegen et al., 2011). The majority of milk IgG is produced by plasma cells systemically and is secreted into milk through blood vessels in the mammary gland. In contrast to IgG, the majority of milk IgM and IgA is produced locally by plasma cells in mammary tissue (Hurley and Theil, 2011). These IgM and IgA producing cells originate in gut associated lymphoid tissue and migrate to mammary gland. This could explain why DFMt was able to increase milk concentrations of IgA, but not IgG. While there is direct contact between DFMt and mammary tissue, DFMt do interact with the mucosal immune system in the gastrointestinal tract and may have influenced the activity of plasma cells prior to their migration to the mammary gland.

Immunoglobulin A have protective effects that would aid in the defense against pathogens in the mammary gland. Immunoglobulin A is normally found at mucosal sites, binds to bacterial cells and prevents their adhesion to epithelial cells. The additional immune defense provide by DFMt could explain the difference in haptoglobin levels between DFMt and CON cows. In a

healthy cow, serum haptoglobin concentration is below 20 µg/ml. In response to disease such as mastitis or endometritis, haptoglobin serum concentrations can increase 100 fold or more (Eckersall and Bell, 2010). An increase in IgA in the mammary gland may provide greater protection against infection, reducing the incidence of disease and elevated haptoglobin concentrations. Administration of DFMT has the potential to improve animal health by boosting immunity and reducing the occurrence of pathogen related diseases.

The mechanisms to better explain the DFMT mode of action certainly encompass the rumen microbiome and dynamics. Ferguson et al. (2010) reported numerically higher proportion of acetate in rumen fluid in comparison to propionate, butyrate, lactate and isobutyrate for BAC when compared to control and LAC. Another interesting finding from that experiment was that the rumen fluid had numerically lower concentration of rumen ammonia (mg/dL) for BAC when compared to control and LAC.

If rumen ammonia concentration is being reduced by DFMT that can be a result of the reduction (by competition, inhibition, or phages) of hyper-ammonia producing ruminal bacteria (**HAB**) in the rumen. Co-culture experiments with HAB found that washed mixed-rumen bacteria (**MRB**) from cattle fed grain were able to decrease ammonia production from HAB, but MRB from cattle fed hay did not affect HAB (Rychlik and Russel, 2000). The authors also used autoclaved MRB and did not see the HAB inhibition. Grain diets are the ones commonly used in the U.S.

Some examples of HAB are *Clostridium spp.*, *Peptostreptococcus anaerobius*, *Clostridium aminophilum*, and *Fusobacterium necrophorum* (Attwood, et al., 1998). These bacteria can harm the dairy cow; *F. necrophorum* is the main agent that causes liver abscesses in cattle. Therefore, decreasing HAB population could lead to a better “immunological status” of the cow by reducing

the potential for cows being at risk.

Cattle enterotoxaemia is one of numerous pathologies caused by *Clostridium perfringens*. These anaerobic Gram-positive bacteria are naturally present in the intestinal flora of mammals, but their uncontrolled multiplication under certain conditions results in the overproduction of toxins in the intestinal tract.

Clostridial organisms are normal flora of dairy cattle and only become problematic with uncontrolled multiplication in situations of injury, changes in diet or management, stress, or parasitism that favor clostridial organism growth, which then may result in production of potent toxins (e.g., enterotoxaemia). Specifically, *Clostridium perfringens* have been incriminated in enteric syndromes affecting both calves and cows (Lebrun et al., 2010). Interestingly, cows that received DFMT had firmer (higher) FS when compared to cows that received CON.

Ionophores (e.g., monensin) can inhibit HAB population in the rumen. Ionophores were not included in our experimental diets. Diverging from this theory, we could also think that DFM had a direct effect in the rumen and hind-gut of cows (Seo et al., 2010).

CONCLUSION

Despite the fact that the exact mode of action of the DFMT remains unknown, the outcomes from the present study are complementary and synergize with the fact that cows receiving DFMT had a sound transition period and higher FE when compared to cows receiving CON. Cows receiving CON seemed to have higher DMI by the end of the experimental period, which contributed to the increased FE for cows receiving DFM. Cows that received DFMT tended to have higher fecal score and potential better immunity than CON cows.

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TABLES AND FIGURES

Table 3.1. Ingredient composition of prepartum and postpartum diets (DM basis) fed to cows receiving Control or DFMt treatments throughout the experimental period.

Ingredient, % DM	Prepartum	Postpartum
Alfalfa hay	1.81	3.11
Grass hay	7.01	6.84
Corn silage	32.7	31.25
Alfalfa silage	6.33	3.91
Wet brewers grain	5.11	8.52
Cottonseed	---	3.08
Soy hulls	---	3.55
Dry ground corn grain	---	30.4
Grain mix ¹²	29.5	12.5

¹ Prepartum grain mix was formulated for 15.0% CP, 38.1% NDF, 23.3% ADF, 3.1% lignin, 2.5% crude fat, and -23.0 mEq/100g DCAD, and contained: 52.3% ground corn, 11.5% soybean hull, 9.0% soybean meal, 6.4% Soy Chlor; 6.3% limestone, 3.9% magnesium sulfate, 2.9% ammonium chloride, 1.0% vitamin E, 1.8% blood meal, 1.2% molasses, 1.9% soy plus and <1% of each of the following; dicalcium phosphate vitamin A, vitamin D magnesium oxide, and trace minerals.

² Postpartum grain mix was formulated for 43.8% CP, 8.9% NDF, 5.1% ADF, 0.3% lignin, 12.2% crude fat, and 42.0 mEq/100g DCAD, and contained: 35.8% soybean meal, 20.2% Soy Plus; 12.6% Enertia, 10.0% limestone, 4.57% sodium bicarb, 4.7% urea, 4.6% sodium bicarbonate, 2.8% dicalcium phosphate, 2.0% magnesium sulfate, 2.0% white salt, 1.8% blood meal, 1.3% vitamin E, and <1% of each of the following; calcium sulfate dehydrate, magnesium oxide, vitamin A, vitamin D and trace minerals.

Table 3.2. Mean chemical composition and standard error of mean (SEM) for diets fed throughout the experimental period.

Component	Prepartum	Postpartum	SEM
DM, %	44.4	46.8	1.44
CP, % of DM	15.3	15.8	0.28
ADF, % of DM	24.2	24.6	1.16
NDF, % of DM	36.4	36.3	1.27
Lignin, % of DM	3.57	3.63	0.28
NFC, % of DM	37.6	37.8	1.09
Starch, % of DM	31.2	26.3	2.65
Crude fat, % of DM	4.03	4.61	0.46
Ash, % of DM	8.73	7.70	0.57
TDN, % of DM	69.0	70.9	1.43
NE _L , Mcal/kg of DM	0.73	0.75	0.02
Ca, % of DM	1.26	1.02	0.15
P, % of DM	0.31	0.32	0.02
Mg, % of DM	0.38	0.28	0.05
K, % of DM	1.14	1.11	0.03
Na, % of DM	0.11	0.23	0.06
S, % of DM	0.32	0.23	0.03
Cl % of DM	0.97	0.46	0.17
Fe, ppm	464	363	53.8
Zn, ppm	125	88.4	10.1
Cu, ppm	19.7	15.5	1.73
Mn, ppm	118	91.3	12.0
Mo, ppm	0.90	0.91	0.09

Table 3.3. Least squares means and associated SEM for DMI, BW, BCS and milk parameters response of Holstein cows top-dressed with *Bacillus pumilus* 8G-134 (DFMt) or placebo (CON) from wk 4 before calving through wk 22 after calving.

	Treatment			P-value		
	CON	DFM	SEM	Trt	Week	Week×Trt
Overall						
DMI, kg/d	20.6	20.0	0.49	0.35	<0.01	0.10
BW, kg	642	635	8.04	0.51	<0.01	0.75
BCS, (1 – 5)	2.93	2.92	0.02	0.70	<0.01	0.48
Prepartum						
DMI, kg/d	12.6	11.9	0.58	0.62	<0.06	0.13
DMI change from week – 3 to – 1, %	-7.85	33.9	23.8	0.22	---	---
BW, kg	722	712	5.51	0.19	<0.01	0.23
BCS, (1 – 5)	3.13	3.11	0.04	0.71	0.44	0.95
Postpartum						
DMI, kg/d	21.9	21.3	0.54	0.44	<0.01	0.09
DMI change from week 1 to 3, %	50.5	29.3%	10.5	0.16	---	---
BW, kg	628	620	8.65	0.53	<0.01	0.75
BCS, (1 – 5)	2.89	2.88	0.02	0.76	<0.01	0.48
Milk yield, kg/d	41.6	42.2	0.88	0.61	<0.01	<0.01
Milk yield changed from week 3 to 1, %	49.9	53.0	16.8	0.90	---	---
Feed conversion (ECM/DMI)	1.92	2.02	0.04	0.06	<0.01	0.87
Feed conversion (FCM/DMI)	1.98	2.09	0.05	0.07	<0.01	0.81
Feed conversion (Yield/DMI)	2.00	2.05	0.05	0.42	<0.01	0.32
3.5% FCM, kg/d	41.3	42.4	0.87	0.40	<0.01	<0.01
Energy corrected milk, kg/d	40.1	40.9	0.86	0.46	<0.01	<0.01
Fat, %	3.51	3.58	0.61	0.42	<0.01	0.12
Fat, kg/d	1.45	1.50	0.03	0.27	<0.01	<0.01
Protein, %	2.76	2.77	0.03	0.93	<0.01	0.32
Protein, kg/d	1.14	1.16	0.03	0.63	<0.01	<0.01
Lactose, %	4.78	4.71	0.03	0.07	<0.01	0.89
Lactose, kg/d	2.00	1.99	0.05	0.99	<0.01	0.02
Somatic cell cont, x 1,000/ml	42.5	60.9	1.28	0.30	<0.01	0.81
Milk urea nitrogen, mg/dL	16.8	16.4	0.50	0.57	<0.01	0.74

Table 3.4. Least squares means of blood and milk metabolites from Holstein cows top-dressed with *Bacillus pumilus* 8G-134 (DFM) or placebo (CON) from wk 4 before calving through wk 22 after calving at 5 and 14 d relative to calving.

Variable	n ¹	Treatment			P-value		
		CON	DFM	SEM	Trt	DRC ²	DRC×Trt
Blood							
NEFA, mEq/L	42	0.78	0.69	0.06	0.60	0.07	0.67
BHBA, mmol/L	42	1.56	1.59	0.22	0.93	0.54	0.26
IgA, µg/ml	42	98.9	110	10.2	0.42	0.92	0.34
IgG, µg/ml	42	17159	15805	1335	0.46	0.74	0.31
IgM, µg/ml	42	89.8	108	11.4	0.25	0.39	0.94
Haptoglobin, µg/ml	79	1.87	0.72	0.57	0.15	0.04	0.46
Milk ³							
IgA, µg/ml	40	478	584	35.0	0.03	---	---
IgG, µg/ml	40	514	512	65.0	0.99	---	---
IgM, µg/ml	40	6.94	8.02	0.90	0.40	---	---

¹ Observations used from both CON and DFM treatments.

² Days relative to calving.

³ Sample collected on the first week of lactation.

Table 3.5. Multivariable logistic mixed models of blood metabolites and health occurrences from Holstein cows top-dressed with *Bacillus pumilus* 8G-134 (DFMt) or placebo (CON) from week 4 before calving through week 22 after calving.

Variable	DRC ¹	n ²	TRT diff. ³	Level	Coefficient	SEM	Odds Ratio	95% CI ⁴	P-value
Blood									
NEFA, mEq/L	5	37	CON – DFM	HNEFA	-0.6061	0.67	0.54	0.14 - 2.05	0.37
	14	42	CON – DFM	HNEFA	1.1675	0.64	3.21	0.91 - 11.4	0.07
BHBA, mmol/L	5	37	CON – DFM	SCK	1.3499	0.72	3.85	0.94 - 15.8	0.06
	14	42	CON – DFM	SCK	-0.1053	0.62	0.90	0.26 - 3.07	0.86
Haptoglobin, µg/ml	5	37	CON – DFM	Positive	0.5523	0.57	1.73	0.55 - 5.53	0.34
	14	42	CON – DFM	Positive	1.2692	0.75	3.55	0.78 - 16.19	0.09
Health occurrences									
RP ⁵		43	CON – DFM	Yes	10.8448	148.8	>99	---	0.94
DA ⁶		43	CON – DFM	Yes	0.05407	0.88	1.05	0.19 - 5.92	0.95
CK ⁷		43	CON – DFM	Yes	0.05407	0.88	1.05	0.18 - 5.91	0.95
MAST ⁸		43	CON – DFM	---	---	---	---	---	---
MET ⁹		43	CON – DFM	---	---	---	---	---	---
FS ¹⁰		5945	CON – DFM	FS ≤ 2	0.02971	0.0175	1.03	0.99 – 1.07	0.08
GA ¹¹		5945	CON – DFM	Altered	-0.00063	0.03	0.99	0.95 – 1.06	0.98

¹ Days relative to calving.

² Observations used from both CON and DFM treatments.

³ Differences of treatment least squares means.

⁴ 95 % confidence interval.

⁵ RP: Retained placenta; CON (n = 21; YES = 2, and NO = 19) and DFM (n = 22; YES = 0, and NO = 22).

⁶ DA: Displacement of abomasum; CON (n = 21; YES = 3, and NO = 18) and DFM (n = 22; YES = 3, and NO = 19).

⁷ CK: Clinical Ketosis; CON (n = 21; YES = 3, and NO = 18) and DFM (n = 22; YES = 3, and NO = 19).

⁸ MAST: Mastitis; CON (n = 21; YES = 0, and NO = 21) and DFM (n = 22; YES = 0, and NO = 22).

⁹ MET: Metritis; CON (n = 21; YES = 0, and NO = 21) and DFM (n = 22; YES = 0, and NO = 22).

¹⁰ FS: Fecal score: 1 = runny; 2 = loose; 3 = soft; 4 = dry; CON (n = 2920; “FS ≤ 2” = 123, and HEALTHY = 2797) and DFM (n = 3024;

“FS ≤ 2”, and HEALTHY = 2951). No time ($P = 1.00$) or treatment by time ($P = 1.00$) effect observed.

Table 3.5. Continued

¹¹ GA: General appearance: 1: Bright and alert; 2: Depressed; 3: Reluctant to rise. CON (n = 2920; Altered = 27, and HEALTHY = 2893) and DFM (n = 3024; Altered = 27, and HEALTHY = 2997). No time ($P = 1.00$) or treatment by time ($P = 1.00$) effect observed. NEFA classes based on: serum concentrations ≤ 0.7 mEq/L (LNEFA; referent); and high > 0.7 mEq/L (HNEFA). BHBA classes based on: blood concentrations ≤ 1.2 mmol/L (SCK = NO; referent); and high > 1.2 mmol/L (SCK = YES). Haptoglobin classes based on: serum concentrations ≤ 150 $\mu\text{g/ml}$ (Haptoglobin = NEGATIVE, referent); and positive > 150 $\mu\text{g/ml}$ (Haptoglobin = POSITIVE).

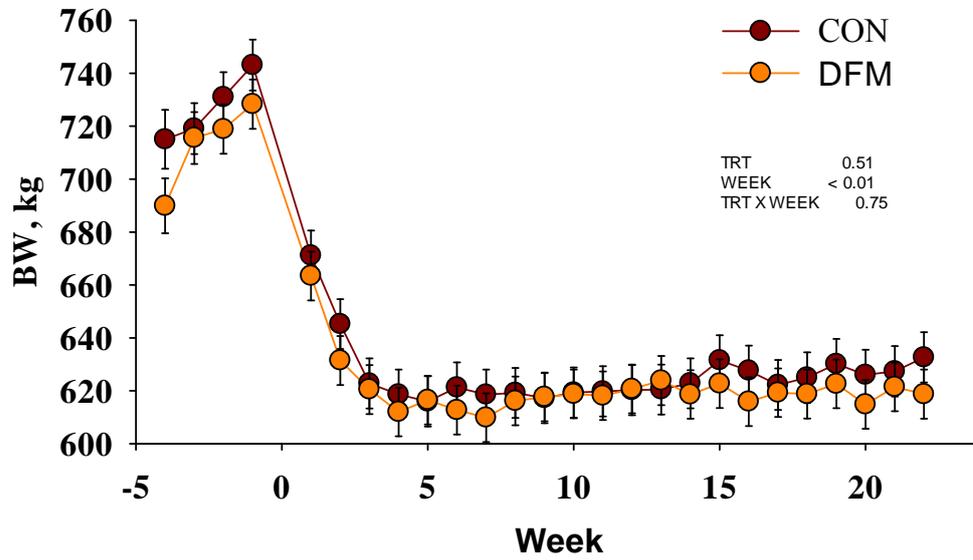


Figure 3.1. Body weight least square means and SEM for cows receiving control (CON; n = 22) or direct-fed microbial (DFM; n = 21) from wk - 4 to 22 relative to calving.

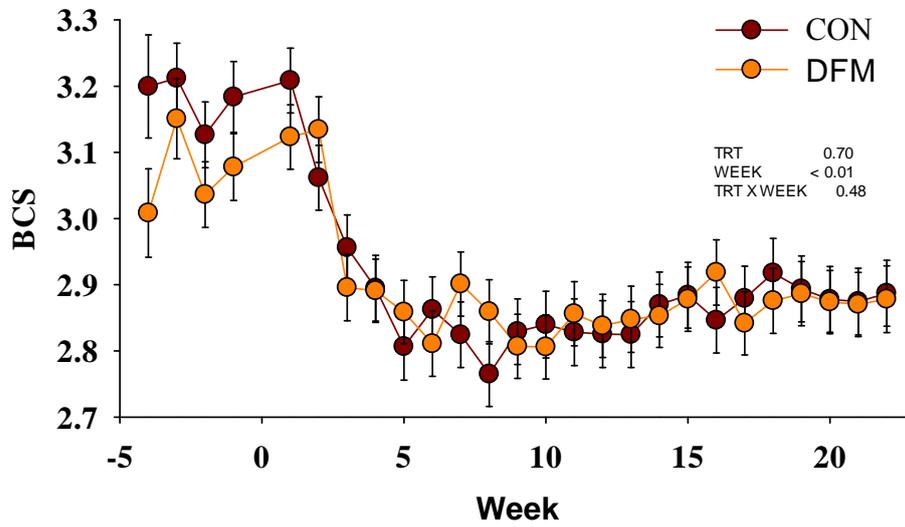


Figure 3.2. Body condition score least square means and SEM for cows receiving control (CON; n = 22) or direct-fed microbial (DFM; n = 21) from wk – 4 to 22 relative to calving.

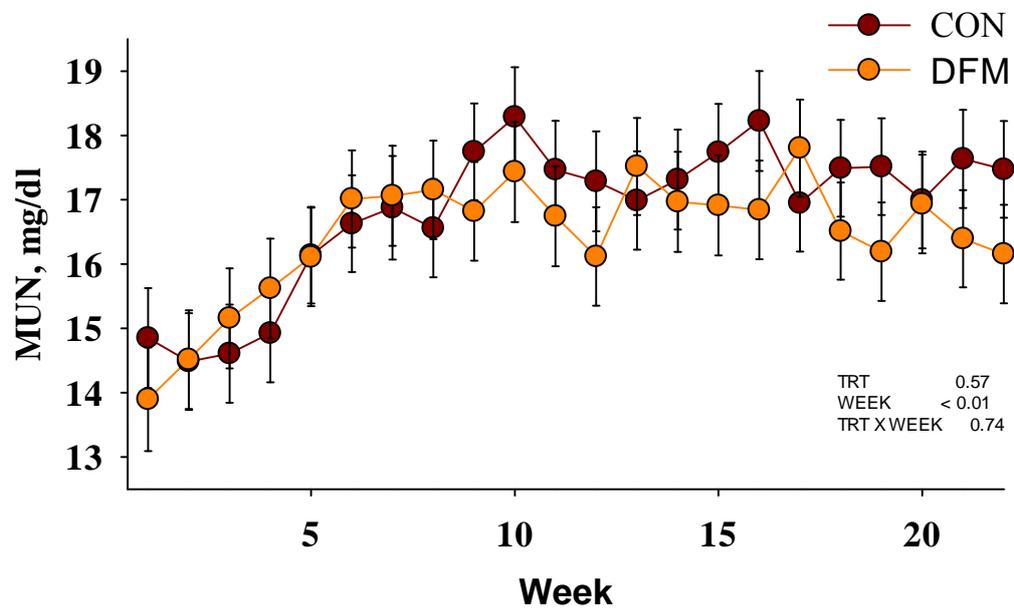


Figure 3.3. Milk urea nitrogen (mg/dL) least square means and SEM for cows receiving control (CON; n = 22) or direct-fed microbial (DFM; n = 21) from wk - 4 to 22 relative to calving.

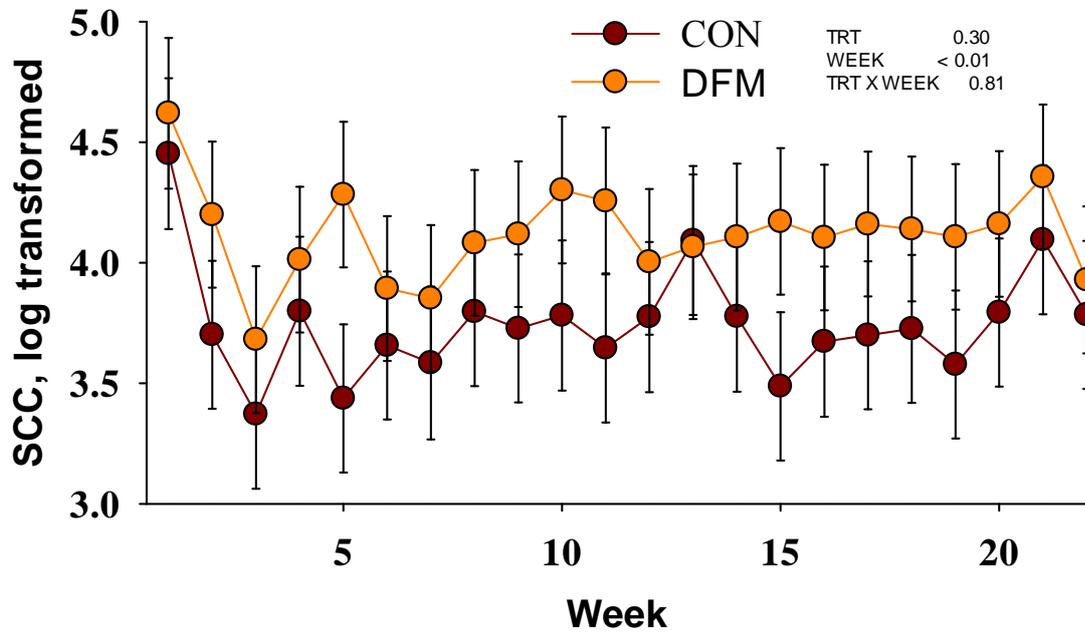


Figure 3.4. Milk somatic cell count least square means and SEM for cows receiving control (CON; n = 22) or direct-fed microbial (DFM; n = 21) from wk - 4 to 22 relative to calving.

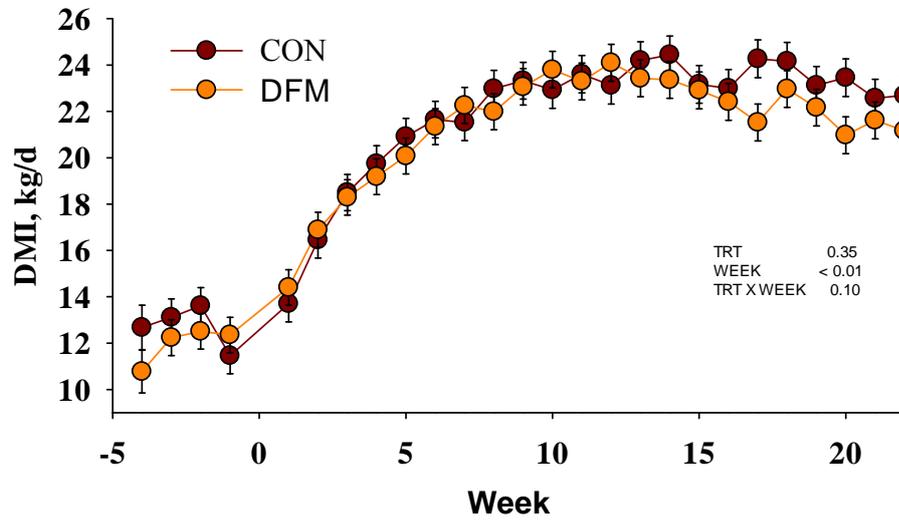


Figure 3.5. Dry matter intake least square means and SEM for cows receiving control (CON; n = 22) or direct-fed microbial (DFM; n = 21) from wk - 4 to 22 relative to calving.

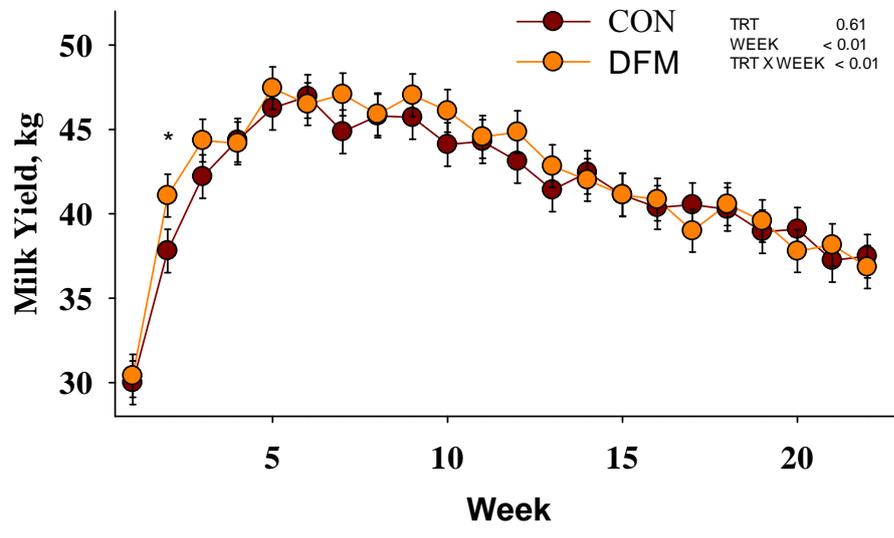


Figure 3.6. Milk yield (kg) least square means and SEM for cows receiving control (CON; n = 22) or direct-fed microbial (DFMt; n = 21) from wk - 4 to 22 relative to calving.

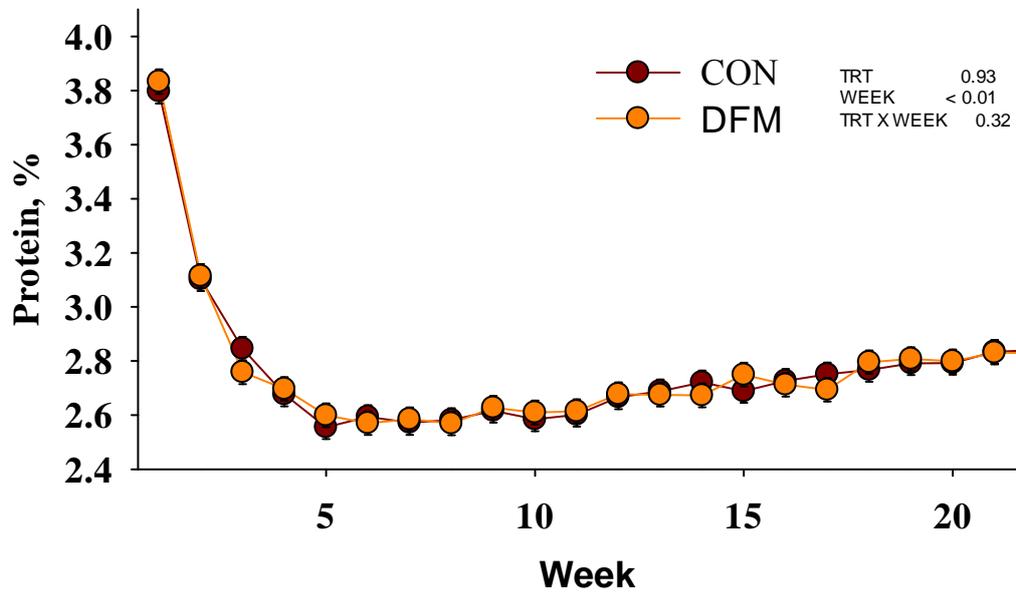


Figure 3.7. Milk protein (%) least square means and SEM for cows receiving control (CON; n = 22) or direct-fed microbial (DFM; n = 21) from wk - 4 to 22 relative to calving.

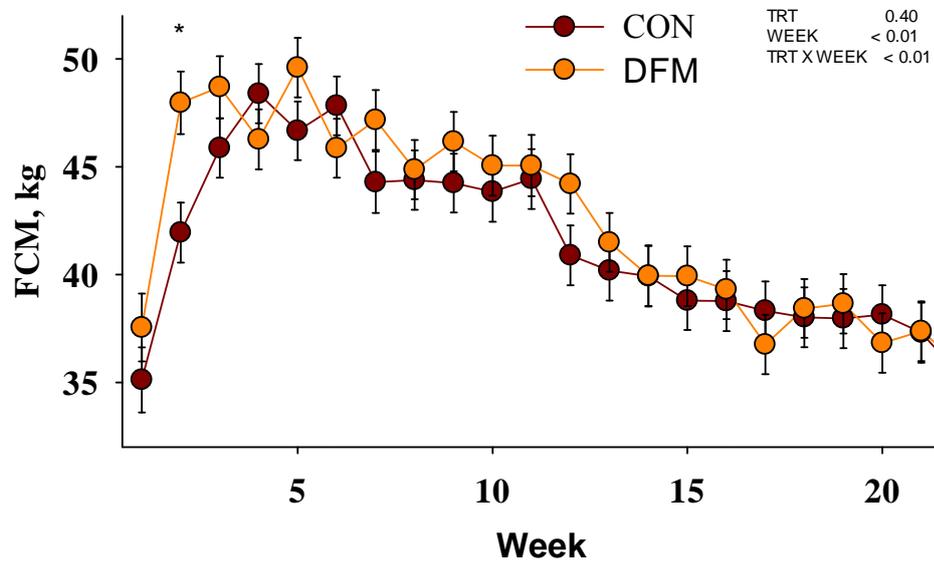


Figure 3.8. Fat-corrected milk (kg) least square means and SEM for cows receiving control (CON; n = 22) or direct-fed microbial (DFM; n = 21) from wk - 4 to 22 relative to calving.

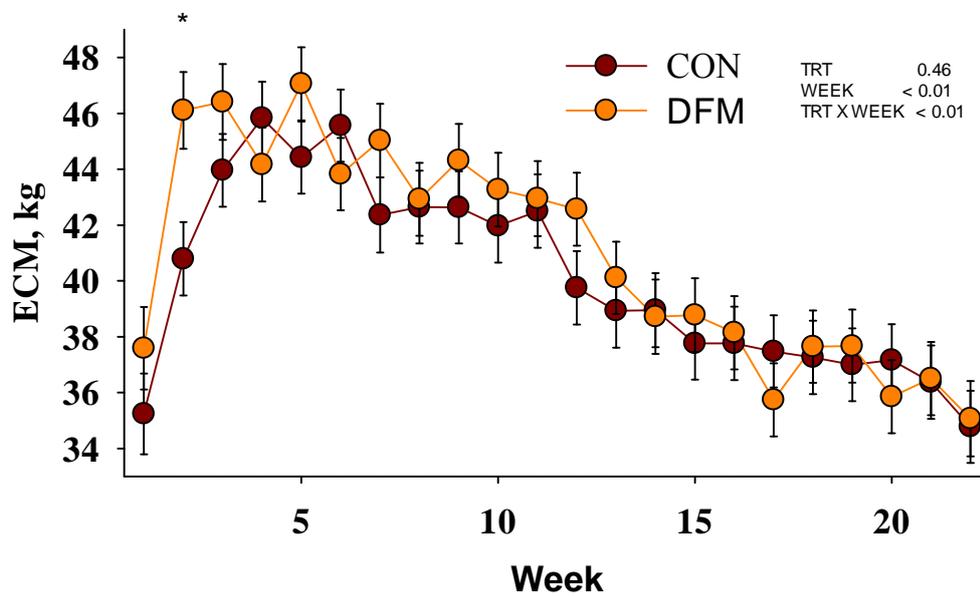


Figure 3.9. Energy-corrected milk (kg) least square means and SEM for cows receiving control (CON; n = 22) or direct-fed microbial (DFM; n = 21) from wk - 4 to 22 relative to calving. Treatment DFM resulted higher (P < 0.01) ECM on second week of lactation than CON.

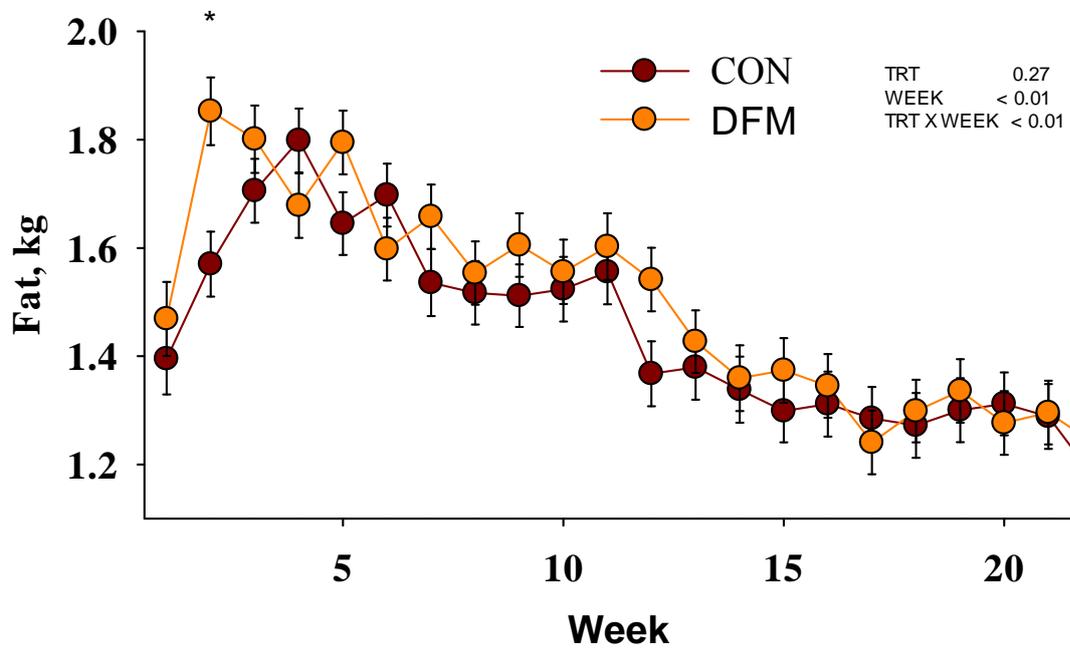


Figure 3.10. Milk fat yield (kg) least square means and SEM for cows receiving control (CON; n = 22) or directed-fed microbial (DFM; n = 21) from wk - 4 to 22 relative to calving. Treatment DFM resulted higher (P < 0.01) milk fat production on second week of lactation than CON.

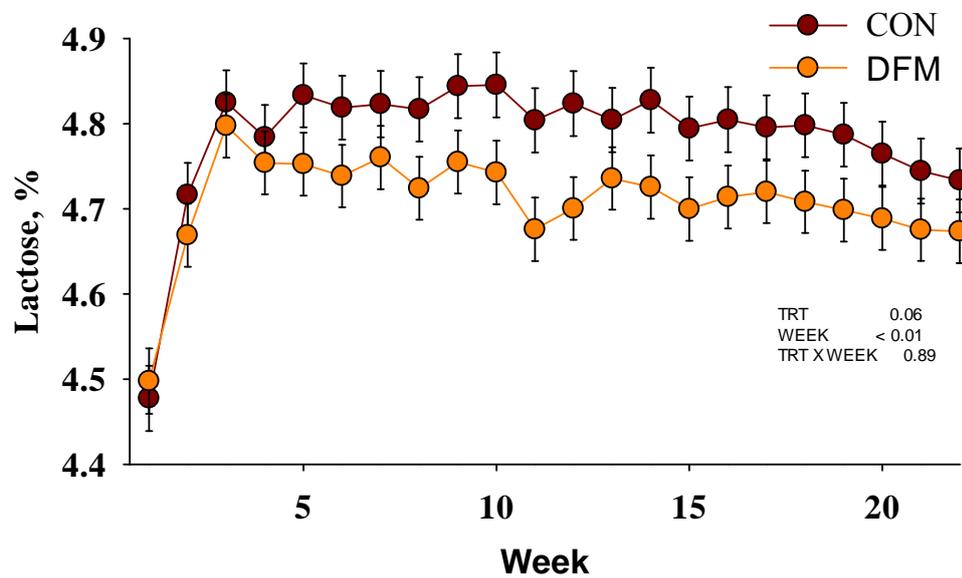


Figure 3.11. Milk lactose (%) least square means and SEM for cows receiving control (CON; n = 22) or direct-fed microbial (DFMt; n = 21) from wk - 4 to 22 relative to calving.

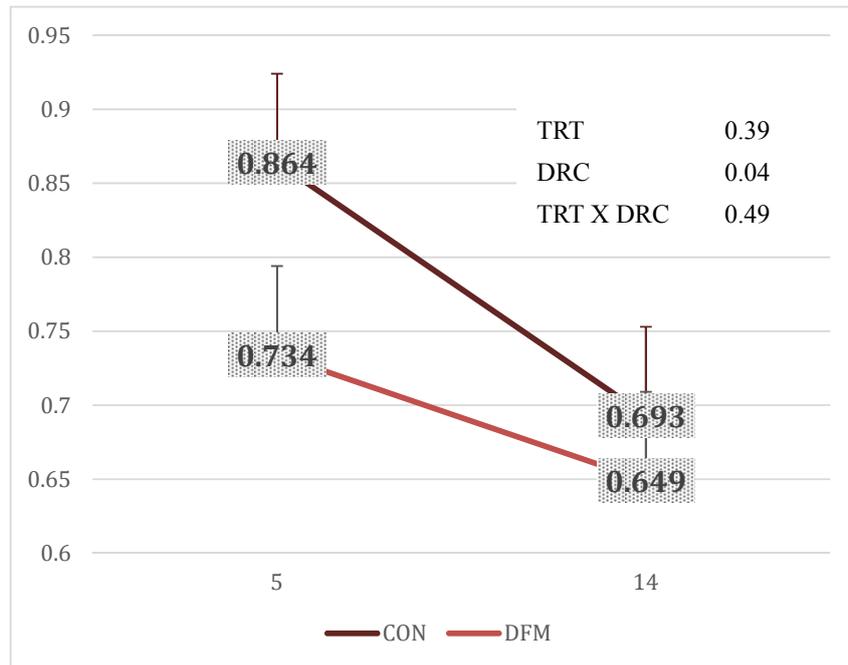


Figure 3.12. Least squares means for serum concentrations of non-esterified fatty acids (NEFA) of cows (n=42) at days 5 and 14 relative to calving receiving control (CON) or direct-fed microbial (DFMt) from wk - 4 to 22 relative to calving.

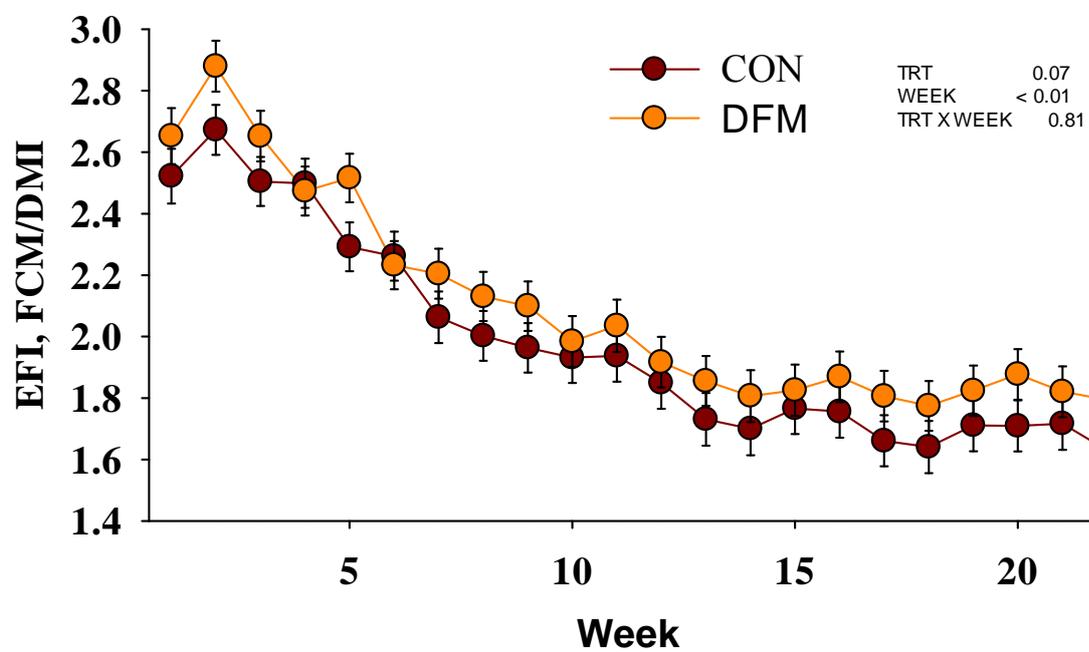


Figure 3.13. Feed Efficiency [Fat-corrected milk (kg)/DMI (kg)] least square means and SEM for cows receiving control (CON; n = 22) or direct-fed microbial (DFMt; n = 21) from wk – 4 to 22 relative to calving.

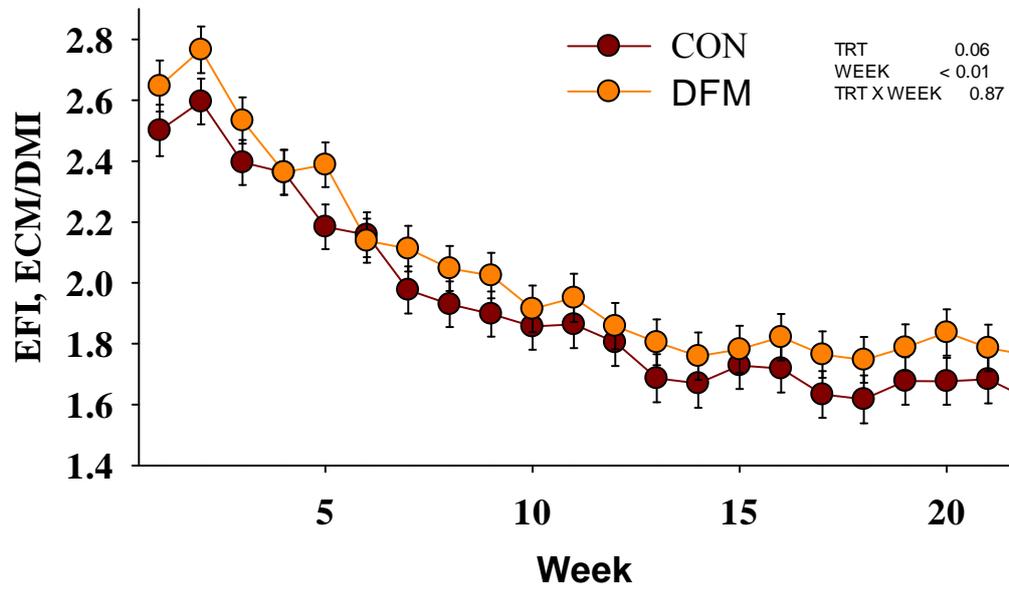


Figure 3.14. Feed Efficiency [Energy-corrected milk (kg)/DMI (kg)] least square means and SEM for cows receiving control (CON; n = 22) or direct-fed microbial (DFMt; n = 21) from wk - 4 to 22 relative to calving.

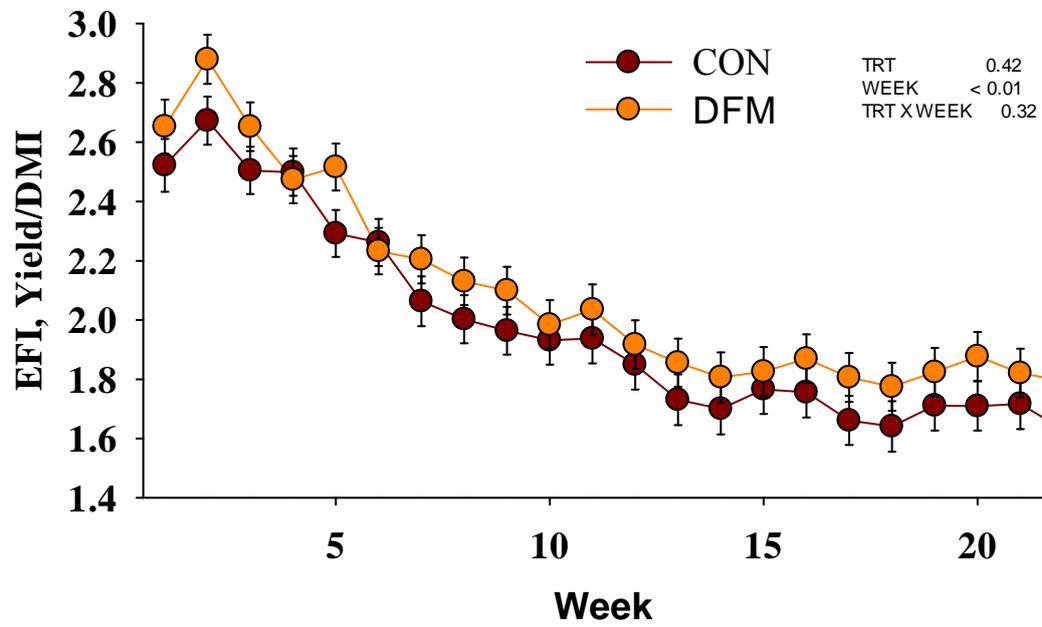


Figure 3.15. Feed Efficiency [Milk yield (kg)/DMI (kg)] least square means and SEM for cows receiving control (CON; n = 22) or direct-fed microbial (DFM; n = 21) from wk – 4 to 22 relative to calving.

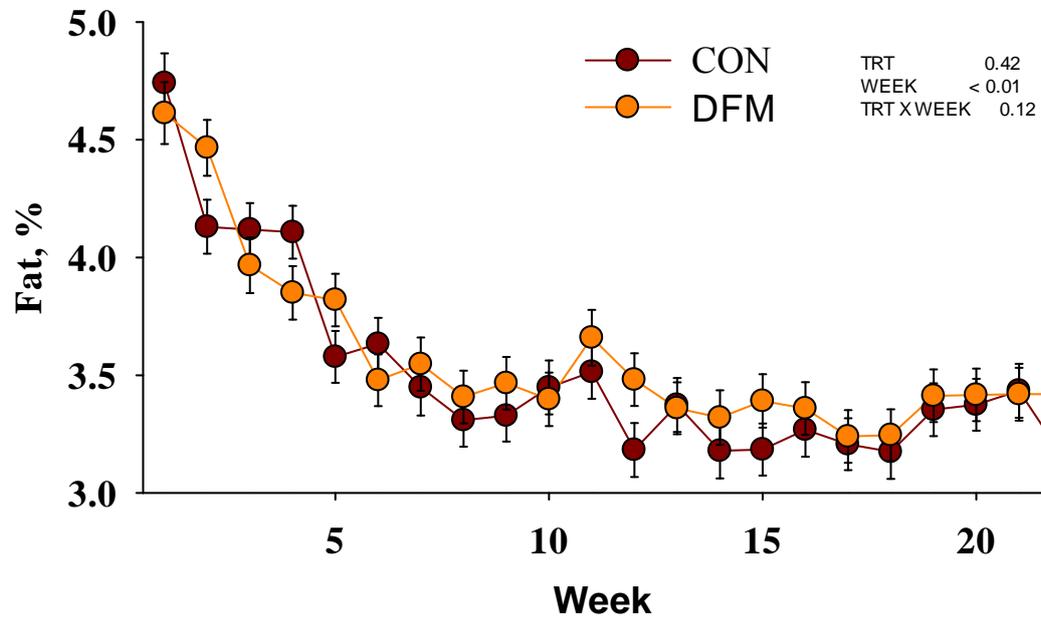


Figure 3.16. Milk fat (%) least square means and SEM for cows receiving control (CON; n = 22) or direct-fed microbial (DFM; n = 21) from wk - 4 to 22 relative to calving.

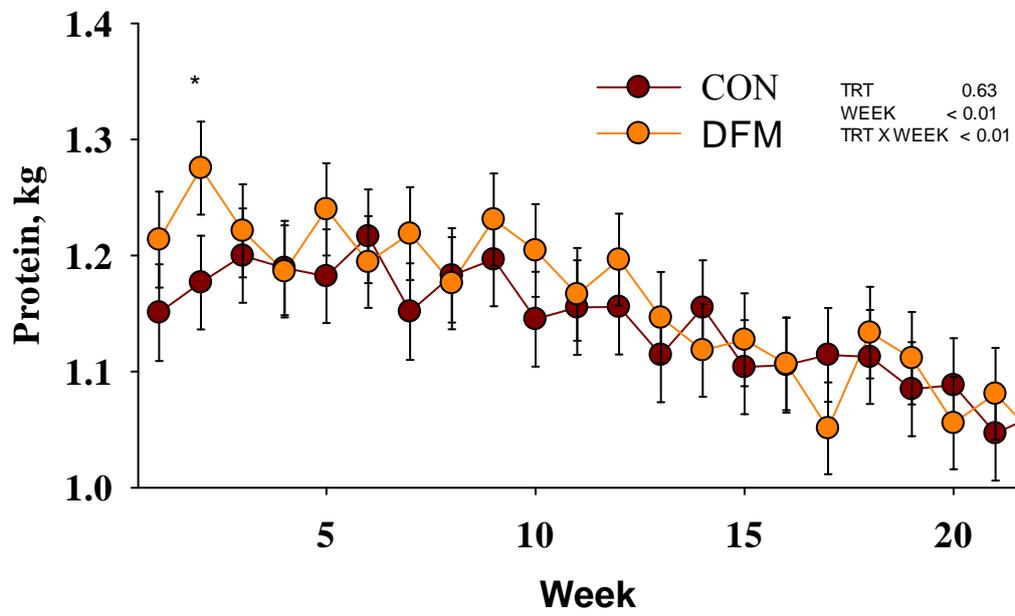


Figure 3.17. Milk protein yield (kg) least square means and SEM for cows receiving control (CON; n = 22) or direct-fed microbial (DFM; n = 21) from wk - 4 to 22 relative to calving.

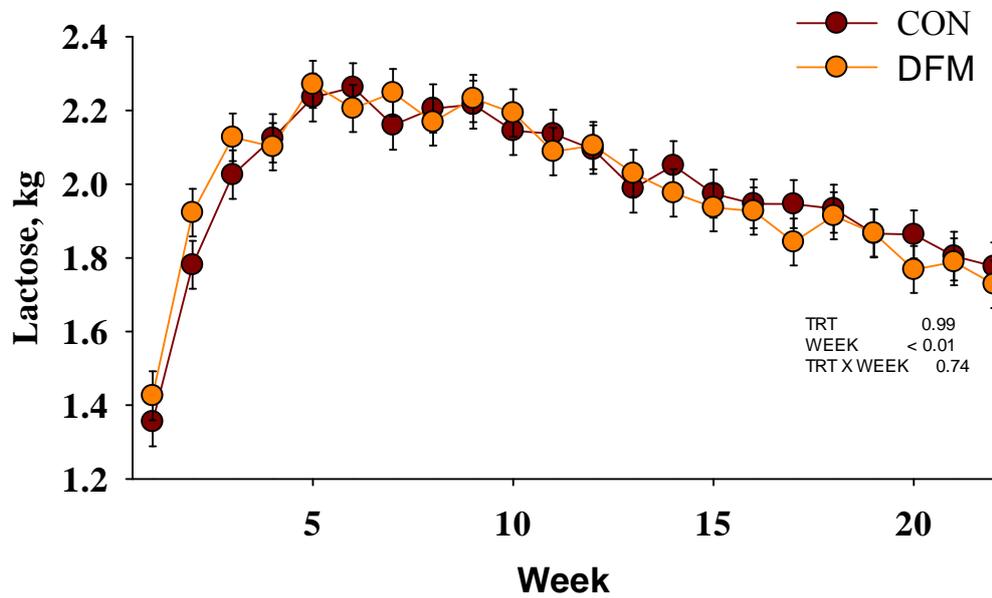


Figure 3.18. Milk lactose yield (kg) least square means and SEM for cows receiving control (CON; n = 22) or direct-fed microbial (DFM; n = 21) from wk - 4 to 22 relative to calving.

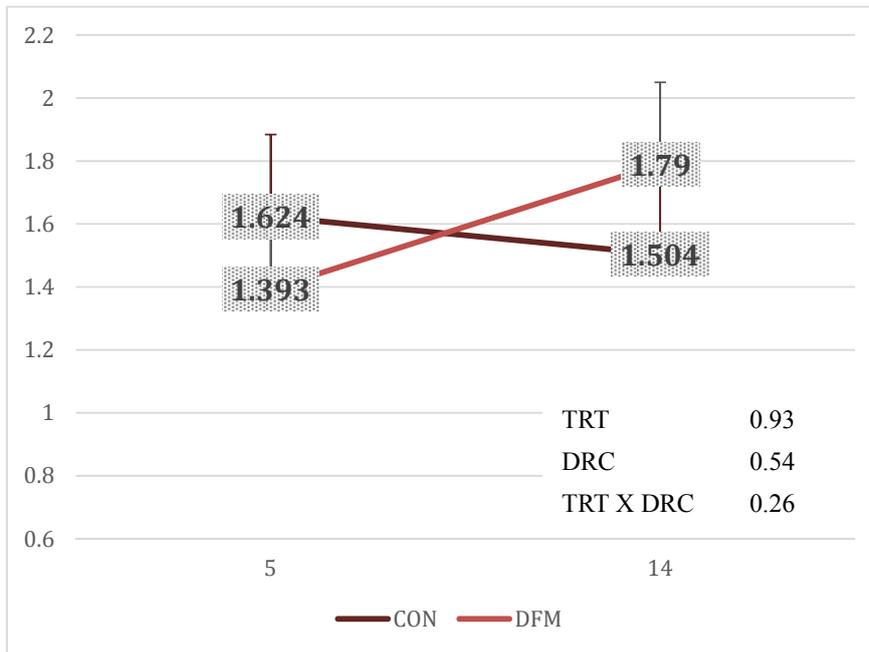


Figure 3.19. Least squares means for blood concentrations of Beta-hydroxybutyrate (BHBA) of cows (n=42) at days 5 and 14 relative to calving receiving control (CON) or direct-fed microbial (DFMt) from wk – 4 to 22 relative to calving.

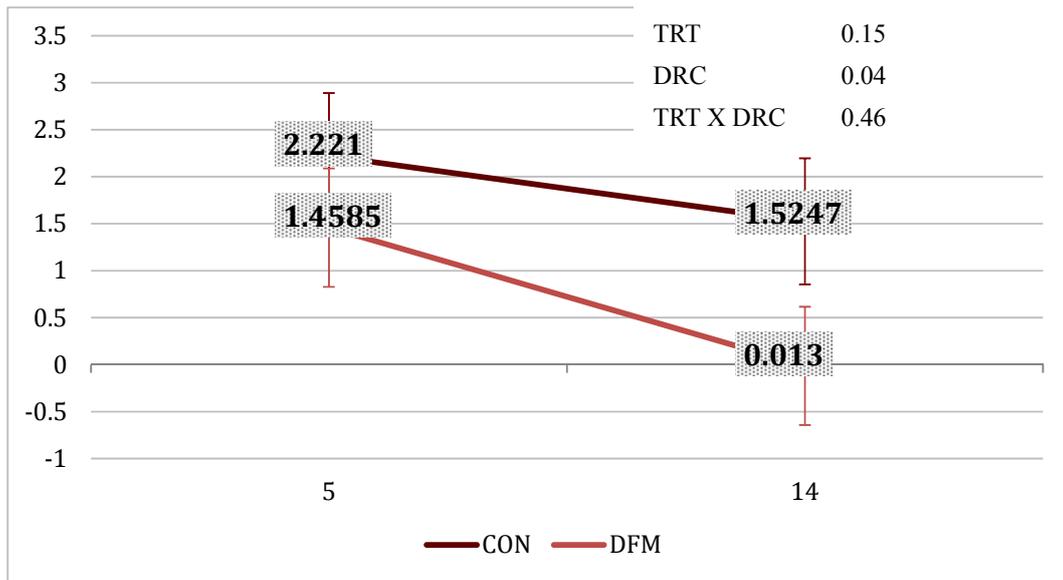


Figure 3.20. Least squares means for blood concentrations of haptoglobin of cows (n=42) at days 5 and 14 relative to calving receiving control (CON) or direct-fed microbial (DFMt) from wk - 4 to 22 relative to calving.

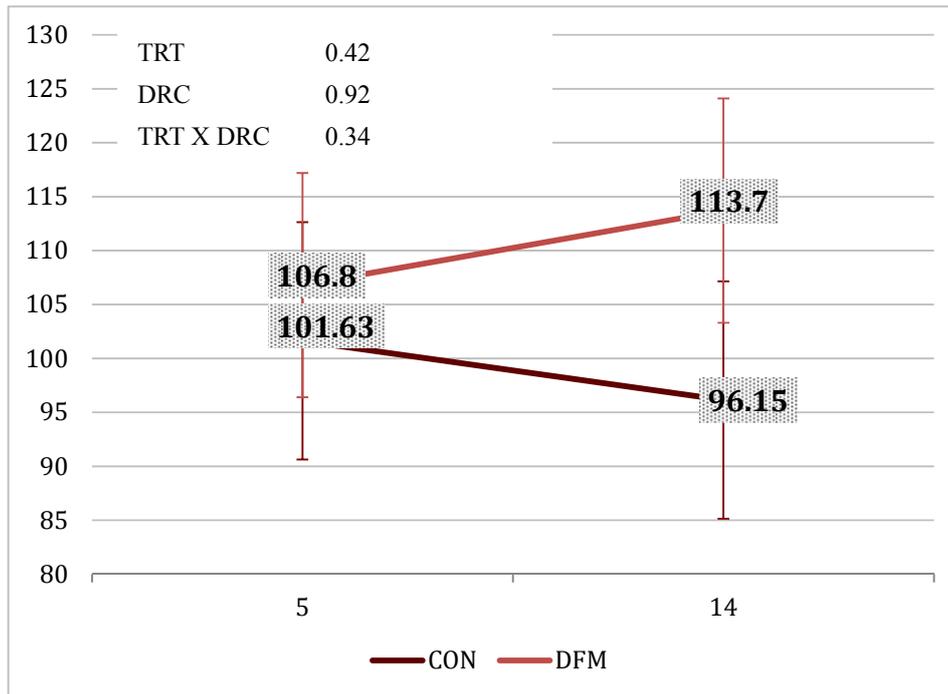


Figure 3.21. Least squares means for blood concentrations of IgA of cows (n=42) at days 5 and 14 relative to calving receiving control (CON) or direct-fed microbial (DFMt) from wk - 4 to 22 relative to calving.

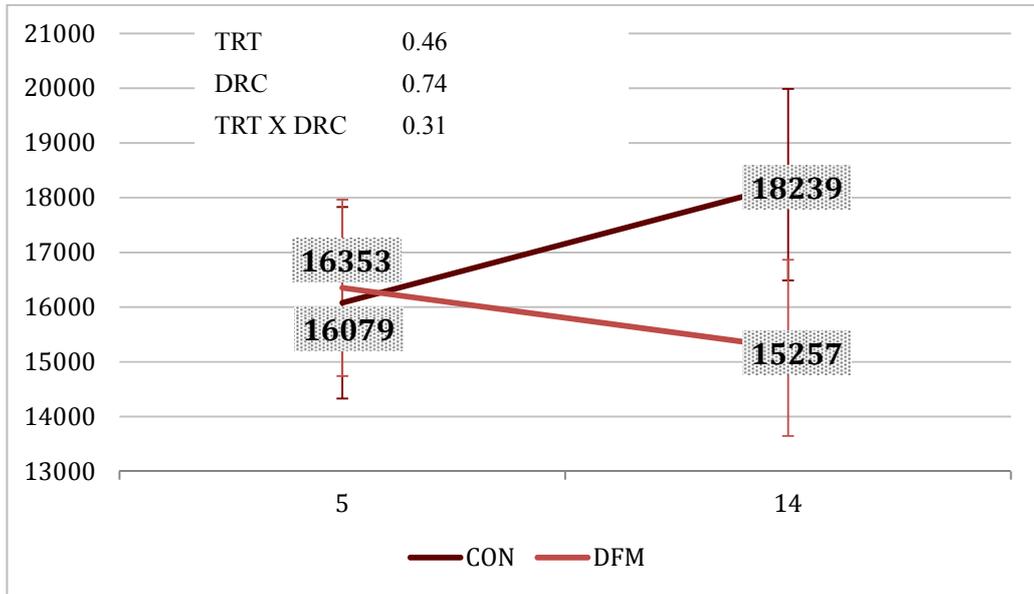


Figure 3.22. Least squares means for blood concentrations of IgG of cows (n=42) at days 5 and 14 relative to calving receiving control (CON) or direct-fed microbial (DFMt) from wk - 4 to 22 relative to calving.

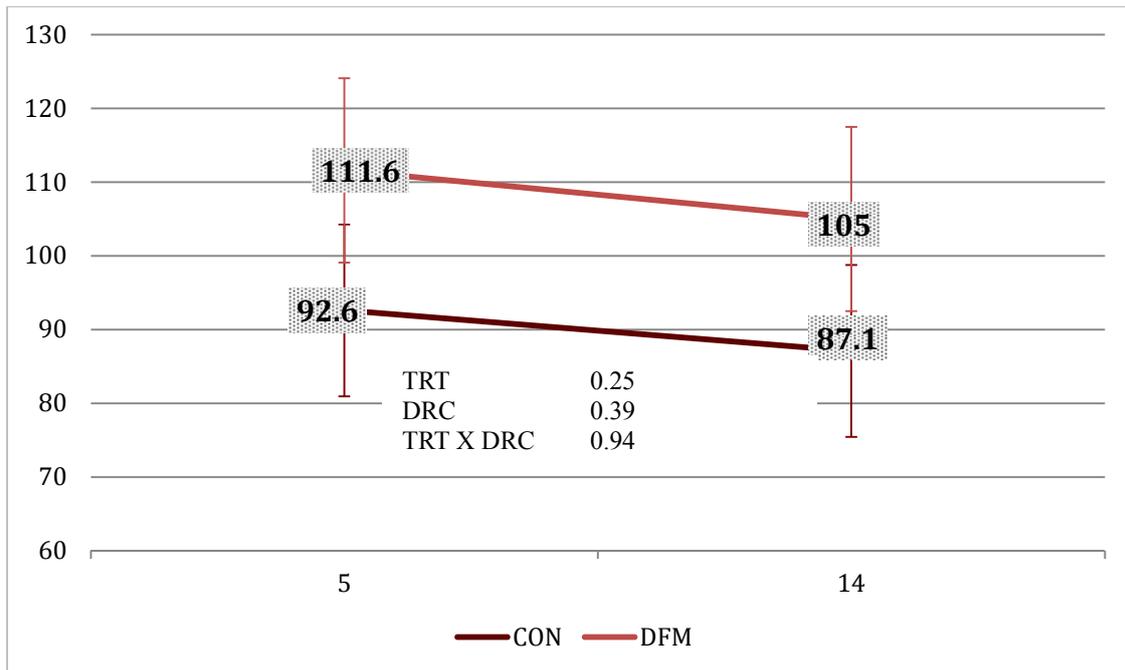


Figure 3.23. Least squares means for blood concentrations of IgM of cows (n=42) at days 5 and 14 relative to calving receiving control (CON) or direct-fed microbial (DFMt) from wk - 4 to 22 relative to calving.

CHAPTER 4

Overall Summary, Conclusions, and Perspectives

The overall objective of this dissertation was to evaluate rumen environment in dairy cows and its association with nutritional challenge and opportunity. One hypothesis related to the ability to detect SARA through monitoring urine or fecal pH. The other hypotheses were that the supplementation of a direct-fed microbial (DFM, *Bacillus pumilus* 8G-134) to dairy cows would improve production by stabilizing the rumen environment.

In chapter 2, we evaluated different aspects related to the rumen pH: effects of induced sub-acute ruminal acidosis (SARA) on ruminal pH, urine pH, fecal pH, starch digestibility, and milk composition of lactating cows; and potential differences in responses to SARA by Holstein and Jersey lactating cows. Furthermore, we evaluated health response to the SARA induction. We concluded that induction of SARA decreased urine pH in Holstein cows. Rumen pH can be predicted by urine pH within 2 h after feeding [Rumen pH = (Urine pH – 6.2916)/0.2946]. Fecal pH can be used to predict rumen pH depression as early as 7 and 15 h after feeding for JER and HOL cows, respectively. In addition, Holstein cows' DMI and health were not affected when rumen pH was depressed to between 5.6 and 5.8, for less than 4.3 h.

In chapter 3, we evaluated the effect of *B. pumilus* 8G-134 (NRRL B-50174) as a DFM supplement on DMI, milk production and composition, and feed efficiency from pre-partum to mid-lactation. In addition, we evaluated the effect of the DFM on prevalence of metabolic disorders during the transition period. In this chapter, we concluded that cows received DFM had a more sound transition period and higher FE when compared to cows received CON, meaning

that cows received *Bacillus pumilus* as DFM might produce more milk without consuming more DMI and had a reduced potential risk for health disorders.

In the present work, the potential relationship between rumen pH and fecal pH has been proposed. It would be extremely beneficial to producers to be able to predict the rumen pH accurately onsite. Higher frequency of sampling during the day might provide more precise time points relate to the depression of ruminal pH.

Another area worth exploring would be the effect of restriction feeding on SARA induction. Previous researches showed that DMI is related to severity of SARA; therefore, an additional treatment, in which there was no restriction feeding before SARA challenge, might be beneficial to detect the effect of induced SARA on milk production, milk composition, and health parameters.

Jersey cows had different responses to SARA challenge than Holstein cows; however, the reasons for those differences are not clear. Additional research might investigate potential breed effects related to rumen pH, rumen VFA production, and microbial balance and passage rate on Jersey cows. It would be beneficial to Jersey producers, who can gain more knowledge on the difference on rumen fermentation production differences, and its relationship with milk production and milk composition compared to Holstein cows.

Higher FE by supplementation of DFM is an improved economic benefit. Feed additives that can increase “dairy efficiency” are highly desirable by dairy farmers and the dairy industry at large. However the knowledge about the mode of action in the rumen and lower digest tract has not been understood. Further research might investigate the growth rate of the DFM. Furthermore, the rumen VFA production and pattern might also help to explain the mode of action.

APPENDIX

Effects of Different Amounts of Dietary Protected and Unprotected Niacin on Responses of Blood Metabolites and Production Parameters and to an Epinephrine Challenge in Holstein Cows

ABSTRACT

Niacin (nicotinic acid and nicotinamide) is an essential organic compound for ruminants. Nonetheless, no niacin requirement has been proposed (NRC, 2001). Niacin can be obtained from both diet and rumen microbial synthesis (Weiss and Ferreira, 2006). Our objectives were to evaluate the effect of unprotected and different amounts of protected niacin on an epinephrine challenge, blood metabolites, and production parameters. Six multiparous rumen-cannulated Holstein cows were assigned to 1 of 6 dietary niacin treatments in a completely randomized 6 × 6 Latin square with an extra period to measure carryover effects. Periods consisted of a 7-d adaptation period followed by a 7-d measurement period. Treatments were: CON, no niacin; INF, abomasal infusion of 12 g UN; N12, 12 g UN; BN3, 3 g PN; BN6, 6 g PN; and BN12, 12 g PN. Treatments N12, BN3, BN6, and BN12 were top-dressed on the TMR twice daily. Treatment INF was divided in 5 equal portions and infused every 4 h. Milk yield was recorded and DMI measured daily. Milk samples were obtained from d 8 to 12 for composition analysis. Blood samples were collected on d 10 (0, 3, and 6 h after feeding), d 13 (0, 3, 6, 9, 12, and 18 h after the morning feeding), on d 14 (0 and 6 h after feeding). On d 12, cows received an i.v. infusion of EPI (1.4 µg/kg of BW), and blood was sampled at -45, -30, -20, -10, -5, 2.5, 5, 10, 15, 20, 30, 45, 60, 90, and 120 min after epinephrine infusion. Liver and adipose samples were obtained on d 13 from INF and CON cows. Whole blood was collected on d 10 for neutrophil function test. INF resulted in higher plasma niacin concentration than all other treatments with niacin

supplement. Cows that received CON had lower niacin concentration than all other treatments with niacin supplementation. Niacin concentration had a linear response among treatments BN3, BN6, and BN12 with niacin supplementation. A quadratic effect existed among treatments BN3, BN6, and BN12 for non-esterified fatty acids (NEFA) AUC following the EPI challenge. Time to peak NEFA concentration tended to be greater for N12 (22.1 ± 3.2 min) than for BN12 (14.8 ± 3 min). For glucose, INF resulted in greater AUC than N12, and BN12 tended to have greater AUC than N12. Glucose AUC displayed a quadratic response among treatments BN3, BN6, and BN12. Time to peak and peak concentration of glucose, as well as NEFA peak concentration, did not differ among treatments ($P > 0.1$). Increasing protected niacin supplementation was associated with a linear decrease of DMI, and linear increases of FE and ECM. Lipolysis was not prevented with niacin supplementation during an EPI challenge. Plasma niacin concentration exhibited rebound effect after niacin supplementation stopped. Expression of *PDHAI* was up-regulated in the liver, which may indicate that niacin affects glucose metabolism in liver.

INTRODUCTION

Niacin (nicotinic acid and nicotinamide) is an essential organic compound for ruminants. Nonetheless, no niacin requirement has been proposed (NRC, 2001). Niacin can be obtained from both diet and rumen microbial synthesis (Weiss and Ferreira, 2006). Niacin in the blood stream can be converted to nicotinamide adenine dinucleotide (**NAD**) and nicotinamide adenine dinucleotide phosphate (**NADP**). Both components play important roles in oxidation-reduction reaction in cells (Bender, 1992; Niehoff et al., 2009; Pires and Grummer, 2007).

Niacin has been proven to regulate activity of prostaglandin D synthase in Langerhans cells (Benyó et al., 2006; Meyers et al., 2007; Maciejewski-Lenoir et al., 2006), which can increase

sweating rate and skin flushing (Beny et al., 2005; Zimbelman et al., 2010). Niacin also can affect activity of HM74A (PUMA-G/GPR109A), which is a G-protein-coupled membrane receptor in adipose tissue. This membrane receptor has been proven to be regulated by blood niacin concentrations (Wise et al., 2003; Karpe and Frayn; 2004, Carlson, 2005), which inhibit lipolysis. Niacin is involved within carbohydrate, lipid, and amino acid metabolism (NRC 2001). However, specific enzymes and metabolic pathways in liver and adipose tissues with niacin effects have not been examined in depth in dairy cows.

A negative relationship has been proposed between blood NEFA concentration and peripheral blood mononuclear leukocyte function on transition dairy cows (Hammon et al., 2006). Other researchers reported that periparturient dairy cows have impairment of peripheral blood mononuclear leukocyte phagocytosis and oxidative burst activity (Kehrl Jr et al., 1989; Da Silva et al., 1998; Hoeben et al., 2000). Niacin has been described as a potential modulator of lipolytic responses in adipose tissue when administered in pharmacological doses (Carlson, 2005). Researchers (Fronk and Schultz 1979; Dufva et al., 1983; Waterman and Schultz, 1972; Pires and Grummer, 2007; Pescara et al., 2010; Pires et al., 2007) reported that blood NEFA concentration had been depressed with niacin supplementation in dairy cows.

Niacin is known to be highly degradable in the rumen so that oral administration leads to unknown quantities absorbed (Campbell et al., 1994). Unprotected niacin has 80 to 98.5% ruminal disappearance rate (Riddell et al., 1985, Zinn et al., 1987, Santschi et al., 2005). Oral supplementation of niacin has been reported to increase milk yield in dairy cows (Cervantes et al., 1996; Christensen et al., 1996; Drackley et al., 1998). However, its effects on milk components, milk yield, blood NEFA, glucose and BHBA have been variable among studies (Martinez et al., 1991; Erickson et al., 1990; Drackley et al., 1998).

Protection of niacin against degradation by rumen microbiota might help achieve consistent response to niacin. Our objectives were to determine 1) the effects of 3 levels of rumen-protected niacin in comparison with unprotected niacin either in the diet or infused into the abomasum on blood metabolites and performance; 2) the effect of an epinephrine challenge on blood metabolites; 3) the effect of abomasum niacin infusion on gene expression in liver and adipose tissue; and 4) the effect of niacin supplementation on peripheral blood mononuclear leukocyte function.

MATERIALS AND METHODS

Animals and housing

All experimental procedures were conducted according to protocols approved by the University of Illinois (Urbana) Institutional Animal Care and Use Committee. Cows were housed in tie stalls with sand bedding and ad libitum water and feed access. Cows were allowed to exercise daily for 3 h in an outside lot. Cows were fed according to NRC (2001) recommendations and milked 3 times daily within 8 h interval.

Treatments

Multiparous rumen-cannulated Holstein cows (BW = 656 ± 50 kg, BCS = 3.0 ± 0.2) after peak lactation (128 ± 23 DIM) were assigned to 1 of 6 treatments in a balanced 6 × 6 Latin Square design, with an extra period to measure carryover effects. Periods consisted of a 7 d (d 1 to 7) adaptation followed by 7 d (d 8 to 14) of measurement. Treatments were no niacin supplement (control, **CON**); abomasal infusion of 12 g/d of unprotected niacin (**INF**); 12 g/d of unprotected niacin in the diet (**N12**); 3 g/d of protected niacin in the diet (**BN3**); 6 g/d of protected niacin in the diet (**BN6**); and 12 g/d of protected niacin in the diet (**BN12**). Treatments

were provided during the first 12 d of each period. Cows were fed individually throughout the experiment. For treatments N12, BN3, BN6, and BN12, niacin was top-dressed on the TMR twice daily. The daily dose of treatment INF was divided into 5 equal portions and infused every 4 h during the day. Cows that received treatments other than INF were abomasally infused with the same volume of water at the same time points.

Blood samples

Blood samples were collected from a tail vein or artery on d 10 of each period at 0, 3, and 6 h after morning feeding (6 AM). On d 11, a catheter (16-gauge, Abbocath-TAL catheter; Abbott Laboratories, North Chicago, IL) was placed in the jugular vein of each animal. On d 12, cows received an i.v. infusion of epinephrine (**EPI**; 1.4 µg/kg of BW). Blood samples were collected at -45, -30, -20, -10, and -5 min before EPI infusion and 2.5, 5, 10, 15, 20, 30, 45, 60, 90, and 120 min after. On d 13, blood samples were collected via the jugular catheter at 0, 3, 6, 9, 12, and 18 h after the morning feeding; On d 14, blood samples were collected at 0 and 6 h post feeding. Blood samples were collected into tubes (BD Vacutainer; BD and Co., Franklin Lakes, NJ) containing either clot activator or lithium heparin for serum and plasma. Serum and plasma were obtained by centrifugation at $1,300 \times g$ for 15 min and stored at -20°C until analyzed. Analysis for NEFA was performed using the Wako NEFA-HR(2) Microtiter Procedure kit (Wako Diagnostics, Richmond, VA). Serum glucose and blood urea nitrogen (**BUN**) were measured at the University of Illinois Veterinary Medicine pathobiology laboratory using automated methods according to Stein (1965). Blood urea nitrogen was not measured on d 10, 13 and 14. Analysis for lactate was performed using Sigma Lactate Assay Kit (Sigma, St Louis, MO). Serum lactate concentration measured on 0 and 6 h after feeding on d 10, 0 and 120 min after feeding on d 12, and 0 and 6 h after feeding on both d 13 and 14 from BN12, CON, and INF treatments only.

Plasma niacin concentration was determined by using the VitaFast (R-Biopharm, Dharmstadt, Germany) niacin microbiological assay as described by Zimbelman et al. (2010).

Blood samples from all cows on d 10 and 12 were characterized as with niacin supplementation (**WN**), vs d 13 and 14 as without niacin supplementation (**WON**). Plasma samples were pooled within individual cow across time points in each period to provide a single plasma sample for analysis for niacin concentration as pooled with niacin supplementation (**Pooled WN**) and pooled without niacin supplementation (**Pooled WON**). Blood glucose, NEFA, BUN and lactate concentrations were analyzed on different time points at d 10 as WN, d 13 and 14 as WON. To compare glucose and NEFA response (e.g. rebound) with and without niacin supplementation, samples from 0 and 6 h after feeding on d 10, 13 and 14; 0 and 120 min after feeding on d 12 from INF, BN12 and CON treatment were chosen. Blood urea nitrogen concentration was analyzed on samples on d 10, 13, and 14, samples from d 10 considered as with niacin supplementation, vs d 13 and 14 as without niacin supplementation. Area under the curve (**AUC**) responses of plasma NEFA and glucose concentration on d 12 before and after EPI challenge were calculated with the positive incremental trapezoidal rule (Cardoso et al., 2011). Base value was calculated by averaging all time point values before the challenge (e.g. -45, -30, -20, -10, and -5 min).

Measurement and sample collection

Feed ingredients and TMR samples were obtained weekly and analyzed for dry matter (**DM**) content (AOAC, 1995) by drying for 24 h in a forced-air oven at 110°C. Dietary DM was adjusted weekly for changes in DM content. Total mixed ration samples were taken weekly, and stored at -20°C until submitted for analysis. One composite sample per period was analyzed for contents of DM, crude protein (CP), acid-detergent fiber (ADF), neutral-detergent fiber (NDF),

lignin, starch, fat, ash, Ca, P, Mg, K, Na, Fe, Zn, Cu, Mn, Mo, and S using wet chemistry methods (Dairy One, Ithaca, NY). Intake from each cow was measured and DMI was recorded daily. Feed conversion (**FE**) was calculated as the ratio of energy-corrected milk over DMI.

Milk weights were recorded daily and samples were obtained from the 3 consecutive milkings during d 8 to d 12 of each period. Consecutive samples were composited in proportion to milk yield at each sampling and preserved (800 Broad Spectrum Microtabs II; D&F Control Systems, Inc., San Ramon, CA). Composite milk samples were analyzed for fat, protein, lactose, urea nitrogen (**MUN**), total solid and somatic cell count (**SCC**) using mid-infrared procedures (AOAC, 1995) at a commercial laboratory (Dairy Lab Services, Dubuque, IA). Energy-corrected milk (ECM) and 3.5% fat-corrected milk (FCM) were calculated according to NRC (2001) guidelines.

Body weight was measured and BCS was assigned in quarter unit increments (Ferguson et al., 1994) for each cow on d 3, 8, and 10 in each period. Three individuals assigned BCS independently at each time of scoring throughout the experiment and the median value was used.

Rumen pH was measured on d 3, 10, and 14 of each period at 2 h after the noon milking. Representative samples of rumen fluid (40 ml) were collected and composited from multiple locations through the rumen cannula using a syphon. The pH of the fluid was measured with portable pH meter (AP110 Fisher Scientific, Pittsburgh, PA) immediately after collection from the rumen.

Liver samples were collected by puncture biopsy (Dann et al., 2006) and adipose tissue was collected by blunt dissection (Ji et al., 2012) from cows under local anesthesia before feeding on d 12 only if the cows received CON or INF treatment. Liver and adipose tissue were frozen immediately in liquid nitrogen and stored at -80°C until RNA extraction.

RNA extraction and qPCR analysis

The extraction and qPCR analysis were performed in the laboratory of Dr. J.J. Loor using established protocols (Khan et al., 2013; Khan et al., 2014; Moyes et al., 2014). The final data were normalized using the geometric mean of ACTB, RPS9, and UXT, which were validated as suitable internal control genes in bovine liver and adipose tissue. Data reported were on log₂ scale.

Neutrophil function assessment

Heparinized whole blood was collected on d 10 of each period from cows receiving CON or INF treatments for measuring immune response to pathogens in blood granulocytes. Phagocytotic capacity and oxidative burst were examined using the Phagotest and Phagoburst kit (Orpegen Pharma GmbH, Heidelberg, Germany) respectively as specified by the manufacturer.

Phagocytosis. 20 µL of fluorescein isothiocyanate (FITC) labeled *Escherichia coli* was added to 2 separate test tubes containing 100 µL heparinized whole blood and incubated on ice or a 37°C horizontal shaking water bath for 10 min. 100 µL of coomassie brilliant blue was then added to quench fluorescence of bacteria attached only to the cell surface. After two washes, the cells were lysed and fixed at room temperature and resuspended in 200 µL of DNA staining solution containing propidium iodide until analyzed by flow cytometer.

Oxidative burst. 20 µL of FITC non-labeled *Escherichia coli* was added to 1 of 4 test tubes containing 100 µL heparinized whole blood and incubated in a 37°C horizontal shaking water bath for 10 min. Twenty (20) µL of wash solution, N-formyl-Met-Leu-Phe (fMLP) or phorbol 12-myristat 13-acetate (PMA), were added respectively to the rest of the tubes before incubation in the water bath as negative, low, or high control. All tubes then received 20 µL of dihydrorhodamine (DHR), the fluorogenic substrate, and incubated for another 10 min at 37°C in

water bath. The cells were lysed and fixed at room temperature and resuspended in 200 μ L of DNA staining solution containing propidium iodide until analyzed by flow cytometer.

Statistical Analysis

Statistical analysis was performed using the MIXED procedure of SAS (version 9.2; SAS Institute Inc., Cary, NC) with the following models:

$$Y_{ijkl} = \mu + T_i + C_j + P_k + T_{p_l} + T \times T_{p_{il}} + e_{ijkl}$$

where Y_{ijkl} = an observation from the i th treatment, j th cow, in the k th period; μ = the grand mean; T_i = the fixed effect of the i th treatment; C_j = the random effect of the j th cow; P_k = the random effect of the k th period; T_{p_l} is the effect of time points, $T \times T_{p_{il}}$ is the interaction of treatment and time point, and the interaction was removed if $p > 0.3$, e_{ijkl} = the random effect of the experimental error from the i th treatment, j th cow, at l th time point in the k th period. Data were subjected to ANOVA using the MIXED procedure of SAS (Littell et al., 1996). The estimation method was restrictive maximum likelihood (REML) and the degrees of freedom method was Kenward-Rogers (Littell et al., 2002). Repeated measurement conducted by compound symmetric co-variance structure. Least squares means were separated using the Tukey adjustment statement in SAS. Orthogonal contrasts were conducted to compare unprotected niacin and without niacin supplement (Comparison 1: N12 vs CON), unprotected niacin and niacin infusion (comparison 2: N12 vs INF), unprotected niacin and protected niacin (comparison 3: N12 vs BN12), niacin supplementation or no niacin supplementation (comparison 4: $\frac{INF+BN12}{2}$ vs CON), niacin infusion and rumen-protected niacin (comparison 5: INF vs BN12). Treatment linear and quadratic effects were tested among CON, BN3, BN6 and BN12 treatments.

RESULTS

The ingredient composition of the diets is detailed in Table A.1. Diet chemical composition analyses are shown in Table A.2. There were no chemical composition differences ($P > 0.28$) detected over periods. Performance parameters are shown in Table A.3. Blood parameters are shown in Table A.4. Carry-over effect was tested and was not present for any outcome variable of interest ($P > 0.30$). Cows were healthy throughout the experiment and no sick animals were reported with clinical ketosis, pneumonia, mastitis, or depression of DMI.

Performance parameters

Body weight and BCS were not different ($P > 0.21$). The DMI was lower ($P = 0.02$) for BN12 (21.5 ± 1 kg) than for N12 (24.3 ± 1 kg). There was a linear ($P = 0.04$) effect among BN3, BN6, and BN12 for DMI. However, there was no overall treatment effect ($P > 0.15$) was detected for DMI, and no treatment by period interaction was observed.

There was no overall treatment effect on milk yield or milk composition ($P > 0.09$). However, milk yield tended ($P = 0.06$) to be greater for N12 (37.1 ± 2.3 kg) than for BN12 (33.4 ± 2.3 kg). Feed conversion was greater ($P = 0.04$) for BN12 (1.7 ± 0.1) than for N12 (1.5 ± 0.1). Furthermore, there was a linear ($P = 0.03$) effect among BN3, BN6, and BN12 for FE. The milk fat/protein ratio (**F/P**) was higher ($P = 0.03$) for BN12 (1.28 ± 0.09) than for N12 (1.15 ± 0.09). There was a linear ($P < 0.01$) effect among BN3, BN6, and BN12 for F/P. The ECM was higher ($P < 0.03$) for BN6 (37.86 ± 2.25) than for BN3 (36.03 ± 2.26), BN12 (35.37 ± 2.25) N12 (36.11 ± 2.25). Furthermore, the linear and quadratic effects among BN3, BN6 and BN12 were present ($P < 0.05$). Fat corrected milk had ($P = 0.02$) quadratic effect among BN3, BN6 and BN12. Fat percentage had ($P = 0.05$) linear effect among BN3, BN6 and BN12.

There was no rumen pH differences ($P = 0.70$) observed among BN3 (6.10 ± 0.10), BN6 (6.00

± 0.10), BN12 (6.10 ± 0.09), CON (6.13 ± 0.09), INF (6.08 ± 0.010), and N12 (6.02 ± 0.10). Rumen pH on d 14 (5.97 ± 0.08) was lower ($P < 0.01$) while rumen pH on d 10 (6.05 ± 0.08) tended to be lower ($P = 0.10$) than rumen pH on d 3 (6.18 ± 0.08).

Epinephrine challenge

There was no difference ($P > 0.29$) on glucose base value, time to peak or peak concentration among treatments (Table A.4). However, glucose AUC displayed a quadratic ($P = 0.03$) effect among treatments BN3, BN6, and BN12. There was no overall treatment effect ($P > 0.28$) observed on glucose AUC. However, INF resulted in greater ($P = 0.04$) AUC than N12 and BN12 tended ($P = 0.07$) to have greater AUC than N12.

There were no differences ($P > 0.10$) on NEFA base value, time to peak or peak concentration among treatments. However, NEFA concentration time to peak tended ($P = 0.08$) to be greater for N12 (22.1 ± 3.2 min) than for BN12 (14.8 ± 3 min). There was an overall treatment effect tendency ($P = 0.10$) observed on NEFA AUC. A quadratic ($P = 0.01$) effect among treatments BN3, BN6, and BN12 for NEFA AUC was observed.

Glucose and NEFA concentration increased ($P < 0.01$) after EPI challenge. Plasma NEFA concentration decreased to the same ($P < 0.13$) concentration as before the challenge 30 min after EPI administration. However, glucose concentration decreased to the pre-challenge concentration on 120 min after EPI administration. Treatment N12 tended ($P < 0.10$) to have lower glucose concentration than BN12 and BN6.

Blood parameters response to niacin supplementation

Niacin

Pooled WN plasma niacin concentration was higher ($P < 0.04$) for INF when compared with

BN3, BN6, BN12, N12 and CON. Treatment CON resulted in lower ($P = 0.02$) pooled WN plasma niacin concentration when compared with BN12 (Table A.4). Pooled WN plasma niacin concentration displayed a linear ($P = 0.02$) response among treatments BN3, BN6, and BN12. Pooled WON plasma niacin concentration was continuously higher ($P < 0.01$) in INF when compared with all other treatments. Treatments (BN3, BN6, BN12, INF, N12 and CON) had similar ($P > 0.67$) plasma niacin concentrations in pooled WON samples. Treatment BN12 and INF tended to be higher ($P < 0.09$) for plasma niacin concentration in pooled WN than pooled WON samples, whereas, treatments BN3, BN6, BN12, N12 and CON had similar ($P > 0.98$) plasma niacin concentration in pooled WN and pooled WON samples.

NEFA

On d 10 and d 12, BN12 had higher ($P < 0.03$) NEFA concentration than BN3 and BN6. Treatment BN3, BN6, CON, INF and N12 resulted in similar ($P > 0.13$) NEFA concentration (Figure A.1). On d 13 and d 14, BN6 resulted in higher ($P = 0.04$) NEFA concentration than BN3. Treatments BN3, BN6, and BN12 displayed a linear ($P = 0.01$) and quadratic ($P < 0.01$) effects on plasma NEFA concentration.

Glucose

On d 10, there were no differences ($P = 0.58$) detected for plasma glucose concentration among treatments (Figure A.2). Glucose concentration declined ($P = 0.05$) from feeding (66.6 ± 2.26) to 6 h after feeding (62.7 ± 2.28) on all treatments. On d 13 and d 14, BN12 tended to have higher glucose concentration than INF and N12, and higher glucose concentration than BN3 and CON.

Blood urea nitrogen

On d 10, BN12 resulted higher ($P = 0.02$) BUN concentration than CON (Figure A.3).

However, other treatments had similar ($P > 0.44$) BUN response to BN12 and CON. On d 13 and d 14, BN12 resulted in lower ($P < 0.05$) BUN concentration than treatments BN3, BN6, INF, N12 and CON. Furthermore, there were linear ($P < 0.01$) and quadratic ($P < 0.01$) effects observed among BN3, BN6 and BN12 without niacin supplementation.

Period with no niacin supplementation (Rebound)

There were no NEFA concentration differences ($P > 0.37$) observed between WN and WON (Figure A.4). The contrast BN12 and INF resulted in higher ($P = 0.01$) glucose concentration in WON when compared to WN (Figure A.5). However, CON resulted in similar ($P = 0.18$) glucose concentration between WN and WON. BN12 and INF tended to have higher ($P = 0.06$) BUN in WN than WON, whereas CON had no BUN difference ($P = 0.49$) between WN and WON (Figure A.6).

Lactate

On d 13 and d 14, there were no differences ($P = 0.55$) observed among treatments on serum lactate concentration (Figure A.7). All treatments had similar lactate concentrations at feeding. However, for the lactate increment at 6 h after feeding, CON was higher ($P = 0.02$) than the average of INF and BN12 (Figure A.7). Furthermore, CON had higher ($P = 0.04$) serum lactate concentration than average of BN12 and INF at 6 h after feeding. On d 13 and d 14, there were no differences ($P > 0.68$) observed among BN12, CON and INF treatment. Serum lactate concentration was higher ($P < 0.01$) for INF and BN12 in WN than WON. Treatment CON had higher ($P = 0.03$) serum lactate concentration in WN than WON.

Immune function

There was no difference ($P = 0.66$) between CON (2395 ± 329) and INF (2190 ± 339) in the percentage of granulocytes able to undergo phagocytosis. There was no difference ($P = 0.54$)

between CON (218 ± 112) and INF (315 ± 112) in the percentage of granulocytes able to undergo oxidative burst.

Liver and adipose tissue

All measurement of qPCR performance for Gene Symbol, primer sequences and primer sequencing results can be found in Tables 5 and 6. Results indicated that *PDHAI* was up-regulated ($P < 0.01$) in INF than CON treatment.

DISCUSSION

Body weight and BCS suggested that throughout the experiment cows were in positive energy balance. Supplementing unprotected or different amounts of protected niacin to dairy cows did not influence DMI, milk yield, FE, FCM, ECM, F/P, or any of the milk components compared to those values in CON. The results agreed with previous experiments (Erickson 1990; Campbell 1994; Ottou 1995), which had experimental animals in middle lactation. However, other experiment showed significant increase in milk yield and protein production (Cervantes et al 1996). The reason for the contradictory results remains unclear. There was no rumen pH change detected with niacin supplementation, which agreed with Campbell et al, (1994). This indicated minor effect on rumen pH of niacin supplementation.

Supplementation of 3, 6, and 12 g/d of protected niacin resulted in a linear decrease of DMI, and linear increase of FE and ECM with niacin supplementation. This might have indicated that an increase of satiety with increased niacin supplementation occurred due to ATP generation from fuels derived from hepatic oxidation, which sends signals to brain, then terminate meals (Allen et al., 2009).

Niacin

Cows that received INF had higher plasma niacin concentration than all other treatments in both WO and WON. For CON cows, plasma niacin was unchanged between WO and WON as expected. Supplementation of 12 g/d of unprotected niacin to the TMR resulted in a 40% increase in plasma niacin, whereas abomasal infusion of 12 g/d of niacin led to a plasma concentration during administration nearly 3 times greater than CON (193% increase). With 80% bio-availability niacin in the protected form, the supplementation of protected niacin 3, 6, or 12 g/d provided 2.4, 4.6, and 9.2 g/d of bioavailable niacin, which resulted in increases in plasma niacin of 22%, 31%, and 75%, respectively. This indicated that increased protected niacin dose elevated blood niacin concentration, which is agreed with Rungruang et al (2014). However, the INF resulted 167% higher plasma niacin concentration than BN12 in the WN sample. This difference might be because the INF group were given niacin directly into the abomasum, whereas the protected niacin fed with feed has to pass through rumen, then passed to lower tract with much lower concentration. Furthermore, the protection layer might decrease the absorption rate of bio-available niacin.

When administration was stopped, the plasma niacin concentration in the WON samples declined. However, cows fed BN3, BN6, BN12, and N12 had numerically higher plasma niacin concentration than CON, and INF had higher niacin concentration than other treatments in WON. Those might indicated a niacin rebound effect, since excess niacin supply will be stored in the liver as NAD, which is converted to nicotinamide then transported to extra-hepatic tissues as blood niacin concentration dropped (Gropper et al., 2009). However, no plasma NEFA affect was detected with the niacin rebound. Furthermore, INF appeared stronger plasma niacin rebound effect than other treatments. This might because the INF delivered higher amount of bio-

available (e.g. 12 g/d vs. 2.4, 4.6 and 9.2 g/d) niacin to the animal compared to the other treatments. Researchers reported decreased blood or plasma NEFA after stopping niacin supplementation suggesting a NEFA rebound effect, however, all the studies did not report blood niacin concentration (Pires and Grummer, 2007, Pires et al., 2007, Pescara et al., 2010). The relationship between blood niacin and NEFA concentration might be an area to investigate in the further.

Research (Zinn et al., 1987, Santschi et al., 2005) showed that 80 to 98.5% unprotected niacin is expected to be degraded in the rumen, however, the N12 resulted in similar plasma niacin concentrations as BN3, BN6 and BN12, and numerically higher than CON. This indicated that 12 g/d unprotected niacin supplied absorbable bio-available niacin, which is in agreement with Driver et al. (1990) and Cervantes et al. (1996), but is contrary to others (Martinez et al., 1991; Lanham et al., 1992; Campbell et al., 1994). This might be because of the blood fraction analyzed or the vitamer or analytical methods chosen (Niehoff et al., 2009). Additionally, rumen absorption might occur. Ruminal niacin concentration was not evaluated but may not be only elevated with niacin supplementation, but also elevated when high grain diets are fed to cattle (Niehoff et al., 2009). The diet fed to the experimental cows had 51.8% concentrate, which would be consistent with a high rumen microbial niacin synthesis. This might lead to high base amount of niacin supply, which decrease the differences among with and without supplementation.

NEFA

On d 10, in response to the niacin supplementation, BN12 resulted in higher plasma NEFA concentration than BN3 and BN6, but similar with other treatments (Figure A.1). However, the differences among treatments were numerically small, and, furthermore, there was no plasma

NEFA concentration difference between CON and INF, which is not expected if niacin suppresses lipolysis. Results from previous studies are contradictory (Niehoff et al., 2009). Martinez et al. (1991) reported that plasma NEFA concentration increased with niacin supplementation (Martinez et al., 1991). However, other researchers (Carvantes et al., 1996; Drackley et al., 1998) reported no plasma NEFA concentration change after niacin supplementation. Furthermore, a plasma NEFA concentration decline was detected by other researchers (Morey et al., 2011; Yuan et al., 2012). The contradictory results are all from studies that supplemented less than or equal to 12 g niacin per day per head. The ineffectiveness or variable results of niacin supplementation might have been due to the low dosage provided since researchers reported that abomasal infusion of approximately 25 and 50 g niacin per day significantly decrease plasma NEFA on feed restricted dairy cows. However, lower dosage (less than 8 g niacin per d) did not depress plasma NEFA concentration (Pires and Grummer, 2007; Pires et al., 2007; Pescara et al., 2010).

The aforementioned results most likely indicate that the excess niacin supply undergoes storage or oxidation rather than binding to receptor in adipocyte. Another reason for the contradictory NEFA response might be the activity of the niacin receptor. Niacin depresses lipolysis by stimulation of a G protein coupled membrane receptor, which inhibits hormone sensitive lipase (Karpe and Frayn, 2004; Carlson, 2005). However, the adipose mobilization is lower under positive energy balance than negative energy balance (e.g. feed restriction or postpartum), therefore, the hormone sensitive lipase is in a low activity state. In this experiment, the BW, BCS, and DMI data showed that the experimental cows were in positive energy balance, which would indicate a low hormone sensitive lipase activity when niacin supplementation was given. This might have caused less niacin binding sensitivity in adipose tissue. Therefore, the

effect of NEFA with niacin supplementation under positive energy balance would be subtle.

Glucose and BUN

On d 10, serum glucose and BUN concentrations were unaffected by niacin supplementation. This suggested that experimental cows were under energy homeostasis with niacin supplementation. Furthermore, pyruvate oxidative decarboxylation might have been affected by niacin supplementation, since niacin is a precursor of NAD, which works as a cofactor of the reaction of pyruvate to acetyl CoA. Glycogen synthesis might hide the changes in plasma glucose concentration, however we do not have data to support this assumption. The similar level of serum BUN and milk protein production indicated that 12 g/d of unprotected niacin supplementation might not have significantly affected rumen microbial production.

Treatment BN12 had higher serum glucose concentration in WN than WON, whereas there was no difference for CON and INF. There was no apparent explanation for this observation, because INF was expected to have higher glucose concentration difference if the increment was a response from stopping niacin supplementation. Treatments INF and BN12 induced lower BUN WN than WON. However, INF and BN12 were not different from CON on both WN and WON. Pires and Grummer (2007) reported that serum urea nitrogen was not affected by niacin supplementation. Therefore, the differences might not be a response from stopping niacin supplementation.

Lactate

Serum lactate concentration was depressed after niacin administration. This was not expected, because increased niacin supplementation would increase NAD supply, which is a cofactor of pyruvate to lactate pathway, which is the initiation of Cori cycle activity (alternate pathway under low oxygen status). However, pyruvate to lactate pathway only occurred under anaerobic

or low oxygen availability, therefore, the reduced lactate concentration might result from increased oxygen supply to tissue cells, but we don't have data to support this assumption. More investigation is needed for better understanding of this occurrence.

Gene expression

Niacin has been reported as an anti-lipolytic agent in humans, however the mechanism is still not clear. Liver and adipose tissue are the major tissues involved in energy metabolism. We examined genes involved in fatty acid synthesis (*FASN*, *IRS1*, and *SLC2A4*) and lipolysis (*ABHD5*, *ADRB2*, *IDH1*, *LIPE*, and *PNPLA2*) in the adipose tissue; and genes involved with glycolysis (*PDHA1*, *LDHA* and *PDK4*), gluconeogenesis (*PCK1*) and beta-oxidation (*ACADVL* and *ACOX1*) pathways in liver. We detected that *PDHA1* was up-regulated by niacin supplementation. The *PDHA1* gene encodes alpha 1 subunit in pyruvate dehydrogenase (PDH), which is a key enzyme to connect glycolysis and the tricarboxylic acid (TCA) cycle (Gropper et al., 2009). This gene was up-regulated in cows that received niacin supplementation, which may have indicated that niacin could have increased glucose elimination from the blood. However, we did not observe serum glucose concentration difference among treatments. Furthermore, results for glucose response to niacin supplementation were contradictory (Niehoff et al., 2009). DiCostanzo et al, (1997) reported an increase in glucose concentration after niacin supplementation, whereas other researchers (Drackley et al., 1998; Yuan et al., 2012) reported no glucose concentration change.

Blood glucose concentration is a tightly controlled homeostatic system, which is regulated by animal energy status. In this experiment, we detected the up regulation of a gene that promote glucose elimination in the liver; additionally, there were less lactate supply that would also decrease precursor for gluconeogenesis. However, the serum glucose concentration remained

unchanged, which might have indicated other mechanisms that enhanced gluconeogenesis stimulated by niacin supplementation in positive energy status. The relationship between niacin supplementation and blood glucose concentration remains unclear.

Rebound effect

In human, it was often observed that after the effect of niacin decays there is a major rebound of plasma NEFA concentration (Chamberlain and LaCount, 2006). Researchers reported that large dosage of niacin supplementation provided repeatable NEFA rebound effect (Björntorp, 1965; Froesch, 1967; Nye and Buchanan, 1969; Pires and Grummer, 2007; Pires et al., 2007; Pescara et al., 2010), however small dosage reflected contradictory results (Niehoff et al., 2009). The current experiment provided 12 g/d/head as niacin supplementation, but there was no NEFA rebound effect detected. The mechanism affected by that dosage is unknown. Affinity of niacin ligand in adipocyte might be an important factor involved in this mechanism.

EPI response

The niacin supplementation did not affect the glucose and NEFA peak time or change the incremental AUC after the EPI challenge. However, the INF (0.44 ± 0.04) resulted in a numerically higher NEFA concentration than BN3 (0.33 ± 0.04), BN6 (0.33 ± 0.04), BN12 (0.34 ± 0.04), CON (0.31 ± 0.04) and N12 (0.25 ± 0.04) at 10 min after EPI challenge. Peter et al (1990) showed that peak plasma NEFA concentration was higher on niacin supplementation (approximately 0.40 ± 0.05) than control (approximately 0.30 ± 0.02) cows at 15 min after challenge. The results indicated that the lipolysis was possibly enhanced by combination of niacin supplementation and EPI challenge. However, we did not observe a difference for the glucose peak concentration, which increased by approximately 50 mg/dL in the experiment performed by Erickson et al (1990). This might have been because of the high amount of EPI

used (i.e., 30 µg/kg of BW; in the present experiment 1.4 µg/kg of BW was injected). Therefore, niacin might be a ligand that bound different receptors, which are regulated by multiple hormones. This might suggest that energy status is an important factor, which would influence activity of niacin receptors. Further investigation is needed to better understand this mechanism.

Negative relationship has been reported between plasma NEFA concentration and chemiluminescence response of polymorphonuclear leucocytes activity (Hoeben et al., 2000). In the present experiment, there was no immune capacity change detected, which might indicate that the capacitive of phagocytosis oxidative burst are un-regulated with niacin supplementation. Furthermore, the lack of NEFA response from niacin supplementation or small number of experimental units used might other reasons for failed detection.

CONCLUSION

Increasing protected niacin supplementation (3, 6, or 12 g/d) was associated with a linear decrease of DMI, and a linear increases in FE and ECM. Lipolysis was not prevented with niacin supplementation and EPI challenge. Serum niacin concentration exhibited a rebound effect after niacin supplementation stopped. Expression of *PDHAI* was up-regulated in the liver.

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TABLES AND FIGURES

Table A.1. Ingredient composition of the lactation diet in DM basis fed to cows throughout the experiment

Ingredient,	% DM
Alfalfa hay	5.86
Corn silage	31.1
Alfalfa silage	11.2
Cottonseed	4.86
Wet Brewers grain	12.7
Dry ground corn grain	17.9
Soybean meal	6.90
Soy hulls	2.74
Sodium Bicarbonate	1.35
Limestone	1.02
Dicalcium phosphate	1.00
Energy Booster 100	0.91
Biotin	0.67
Blood meal 85%	0.59
Potassium chloride	0.52
Magnesium oxide	0.26
Salt (plain)	0.24
Mineral and Vitamin mix ¹	0.22

¹ Mineral and Vitamin mix was formulated with 5% Mg, 10% S, 7.5% K, 2.0% Fe, 3.0% Zn, 3.0% Mn, 5,000 mg/kg of Cu, 250 mg/kg of I, 40 mg/kg of Co, 150 mg/kg of Se, 2,200 kIU/kg of vitamin A, 660 kIU/kg of vitamin D₃, and 7,700 IU/kg of vitamin E.

Table A.2. Mean nutrition composition and standard error of mean (SEM) of diet fed throughout the experiment

Nutrient composition	%	SEM
DM, %	49.3	1.70
CP, % of DM	17.8	0.92
Available Protein, % of DM	16.9	0.96
ADICP, % of DM	0.97	0.29
Adjusted Crude Protein, % of DM	17.8	0.92
ADF, % of DM	21.9	2.23
NDF, % of DM	30.0	2.57
Lignin, % of DM	4.13	0.12
NFC, % of DM	39.2	0.60
Crude fat, % of DM	5.13	0.42
Ash, % of DM	7.91	1.17
TDN, % of DM	72.0	1.00
NE _L , Mcal/kg of DM	0.77	0.01
NE _M , Mcal/kg of DM	0.78	0.02
NE _G , Mcal/kg of DM	0.51	0.01
Ca, % of DM	1.06	0.30
P, % of DM	0.45	0.05
Mg, % of DM	0.32	0.01
K, % of DM	1.41	0.03
Na, % of DM	0.35	0.12
S, % of DM	0.22	0.02
Fe, ppm	437	79.5
Zn, ppm	102	14.1
Cu, ppm	20.3	1.53
Mn, ppm	107	11.5
Mo, ppm	1.7	0.35

Table A.3. Least squares means and associated SE for DMI and milk parameters response of cows in no niacin (CON); abomasal infusion of 12 g/d of unprotected niacin (INF), 12 g/d of unprotected niacin (N12), 3 g/d of protected niacin (BN3), 6 g/d of protected niacin (BN6) and 12 g/d of protected niacin (BN12) treatments.

	Treatment							P-value ¹			
	BN3	BN6	BN12	CON	INF	N12	SEM	Treatment	Period	Treatment	
										Linear ²	Quad ³
DMI, kg/d	24.0	23.9	21.5	22.2	23.8	24.3	1.07	0.15	0.48	0.04	0.51
Body weight, kg	656	670	647	655	663	661	10.6	0.62	NS	0.36	0.16
BCS	2.54	2.62	2.62	2.65	2.63	2.76	0.10	0.21	0.12	0.45	0.46
Milk yield, kg/d	36.1	36.4	33.4	34.8	36.6	37.1	2.44	0.43	0.07	0.15	0.51
3.5% FCM, kg/d	35.8	37.9	35.9	37.5	37.1	36.4	2.37	0.16	0.05	0.80	0.02
Feed conversion	1.53	1.58	1.70	1.64	1.63	1.54	0.11	0.24	0.23	0.03	0.93
Fat, %	3.67	3.72	4.05	3.75	3.74	3.66	0.36	0.31	0.16	0.05	0.70
ECM, kg/d	36.0	37.9	35.4	37.1	36.5	36.1	2.27	0.06	0.05	0.05	< 0.01
Protein, %	3.29	3.21	3.18	3.14	3.12	3.19	0.12	0.45	0.07	0.22	0.56
Fat/Protein	1.15	1.19	1.28	1.19	1.21	1.15	0.10	0.13	0.38	<0.01	0.66
Lactose, %	4.70	4.70	4.71	4.76	4.75	4.75	0.09	0.57	0.09	0.79	0.84
Total solid, kg/d	12.3	12.4	12.7	12.4	12.3	12.3	0.44	0.47	0.12	0.11	0.62
MUN, mg/dL	14.6	14.5	14.4	15.2	14.4	14.7	0.87	0.30	0.06	0.53	0.79
SNF, %	8.67	8.60	8.57	8.57	8.55	8.62	0.12	0.62	0.07	0.21	0.52
SCC, x 1,000/ml	4.86	5.02	4.55	4.19	4.36	4.73	0.62	0.53	NS	0.45	0.56

¹ ^{abcd} Means within a row with different superscripts are different ($P < 0.05$).

² Linear effect was tested among BN3, BN6 and BN12 treatments.

³ Quadratic effect was tested among BN3, BN6 and BN12 treatments.

Table A.4. Least squares means and associated SE on glucose, NEFA and niacin responses of cows in no niacin (**CON**); abomasal infusion of 12 g/d of unprotected niacin (**INF**), 12 g/d of unprotected niacin (**N12**), 3 g/d of protected niacin (**BN3**), 6 g/d of protected niacin (**BN6**) and 12 g/d of protected niacin (**BN12**) treatments after EPI challenge.

	Treatments						SEM	P-value			
	BN3	BN6	BN12	CON	INF	N12		Treatment	Period	Linear ³	Quad ⁴
Glucose											
Time to peak, min	13.6	16.7	10.0	14.0	12.5	8.14	2.77	0.29	0.17	0.43	0.13
Peak concentration, mg/dL	112	105	107	112	109	106	3.96	0.51	0.22	0.42	0.21
Base, mg/dL	61.6	66.0	63.4	61.6	62.8	62.2	1.05	0.80	0.11	0.75	0.24
AUC ¹ , mEq/L/120 min	2524	1885	1932	2118	2452	1746	321	0.28	0.13	0.86	0.03
Rebond,mg/dL	66.1	67.8	68.8	65.9	66.6	65.8	2.13	0.05	0.06	0.02	0.89
NEFA											
Time to peak, min	14.3	17.7	14.8	16.6	15.1	22.1	3.55	0.44	0.15	0.93	0.52
Peak concentration, mEq/L	0.36	0.41	0.35	0.34	0.37	0.38	0.04	0.34	NS	0.61	0.10
Base, mEq/L	0.17	0.15	0.18	0.17	0.17	0.15	0.01	0.10	0.18	0.24	0.03
AUC, mg/dL/120 min	1.26	4.69	1.68	1.60	2.14	3.16	1.35	0.10	0.31	0.92	0.11
Rebound, mEq/L	0.16 ^a	0.19 ^{ab}	0.20 ^b	0.17 ^{ab}	0.17 ^{ab}	0.18 ^{ab}	0.01	<0.01	0.07	<0.01	0.86
Niacin, µg/mL											
With supplement ⁵	2.03 ^{ac}	2.19 ^{ac}	2.94 ^c	1.67 ^a	4.91 ^b	2.34 ^{ac}	0.41	<0.01	0.13	0.02	0.64
Without supplement ⁶	1.88 ^a	2.07 ^a	1.91 ^a	1.62 ^a	3.72 ^b	1.86 ^a	0.41	<0.01	NS	0.97	0.54

¹ Area under curve calculated by Incremental area method: 120 minutes after epinephrine injection among different niacin treatments **CON**, no niacin; **INF**, abomasal infusion of 12 g/d unprotected niacin (**UN**); **N12**, 12 g/d UN; **BN3**, 3 g/d protected niacin (**PN**); **BN6**, 6 g/d PN; and **BN12**, 12 g/d PN.

² abc Means within a row with different superscripts are different ($P < 0.05$).

³ Linear effect was tested among BN3, BN6 and BN12 treatments.

⁴ Quadratic effect was tested among BN3, BN6 and BN12 treatments.

^{5,6} Plasma samples were pooled within individual cow across time points in each period to provide a single plasma sample for analysis for niacin concentration as pooled with niacin supplementation and pooled without niacin supplementation.

Table A.5. Gene symbol, hybridization position, sequence, and amplicon size of primers used to analyze gene expression in qPCR.

Gene	Primers ¹	Sequence (5'-3') ²	Size ³
Adipose			
<i>ABHD5</i>	F: 1141	CTGCAGATGATGTGGGAAAGC	100
	R: 1240	GACTGCCTGGTTCTCGTGTC	
<i>FASN</i>	F: 6383	ACCTCGTGAAGGCTGTGACTCA	92
	R: 6474	TGAGTCGAGGCCAAGGTCTGAA	
<i>IDH1</i>	F: 1061	ATGGCTCTCTTGGCATGATGA	100
	R: 1161	CATTCGGTAGTGACGGGTTACA	
<i>IRS1</i>	F: 73	TGTTGACTGAACTGCACGTTCT	112
	R: 184	CATGTGGCCAGCTAAGTCCTT	
<i>LIPE</i>	F: 1674	TCAGTGTCCAAGACAGAGCCAAT	106
	R: 1779	CATGCAGCTTCAGGCTTTTG	
<i>PDE3B</i>	F: 2235	CGTCTACATGCTGTTTGGTATCT	100
	R: 2334	AGGGTTAATTGCTGTTTCATTCC	
<i>PNPLA2</i>	F: 765	CACCAGCATCCAGTTCAACCT	102
	R: 866	CTGTAGCCCTGTTTGCACATCT	
<i>SLC2A4</i>	F: 79	CCTTGGTCCTTGCCGTATTC	102
	R: 180	TGTAGCTCTGTTCAATCACCTTCTG	
Liver			
<i>ACADVL</i>	F: 707	CCAGCCCCTGTGGAAAATACTA	62
	R: 768	GCCCCGTTACTGATCCAA	
<i>ACOX1</i>	F: 180	ACCCAGACTTCCAGCATGAGA	100
	R: 279	TTCCTCATCTTCTGCACCATGA	
<i>LDHA</i>	F: 661	CTGCTTCCGTTTCTAGGTA	104
	R: 763	CATGCTCCCCCAAGGATT	
<i>PCK1</i>	F: 601	AAGATTGGCATCGAGCTGACA	120
	R: 720	GTGGAGGCACTTGACGAACTC	
<i>PDHA1</i>	F: 999	GAGTGAGTTACCGTACCCGAGAA	101
	R: 1099	ACTGGCGAGATTGCTGTTCA	
<i>PDK4</i>	F: 2484	ACAAATAGCCTGCGGGTAAGAG	102
	R: 2585	CGTACGCTTTACGGCTTTCAT	

¹ F: forward primer; R: reverse primer; number: start of hybridization position

² Exon-exon junctions are underlined

³ Amplicon size expressed as base pair (bp).

Table A.6. Least squares means and associated SEM on expression profile of nucleotide esterases/hydrolases in adipose or liver tissue of cows in no niacin (**CON**) or abomasal infusion of 12 g/d of unprotected niacin (**INF**).

Gene Symbol	Treatment ¹		SEM	P-value
	CON	INF		Trt
Adipose				
<i>ABHD5</i>	-0.04 ¹	-0.62	0.56	0.45
<i>FASN</i>	0.28	-0.14	0.96	0.76
<i>IDH1</i>	-0.19	-1.64	-0.83	0.21
<i>IRS1</i>	1.01	0.88	0.31	0.76
<i>LIPE</i>	0.19	-0.11	0.74	0.77
<i>PDE3B</i>	0.01	-0.46	0.49	0.52
<i>PNPLA2</i>	-0.05	-0.67	0.92	0.65
<i>SLC2A4</i>	0.17	0.21	0.72	0.96
Liver				
<i>ACADVL</i>	-0.45	-0.48	0.13	0.84
<i>ACOX1</i>	-1.08	-0.33	0.31	0.11
<i>LDHA</i>	0.92	0.10	0.39	0.13
<i>PCK1</i>	-1.71	-2.01	0.48	0.66
<i>PDHA1</i>	-1.86	-0.71	0.20	<0.01
<i>PDK4</i>	-0.83	0.30	0.60	0.18

¹ All gene expression data are reported as log-2 scale transformation.

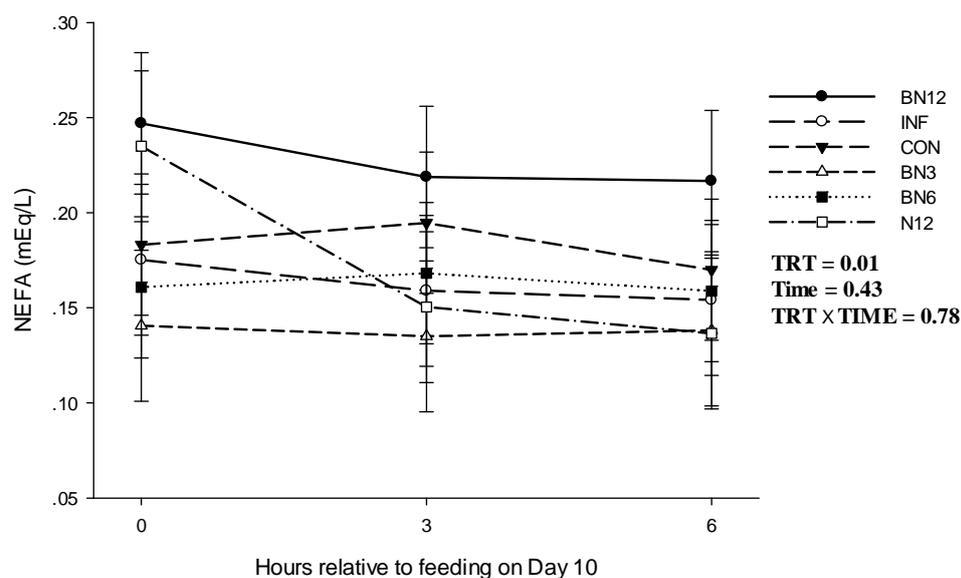


Figure A.1. Plasma NEFA concentration least square means and SEM on d 10 for Holstein cows received abomasal infusion of 12 g/d of unprotected niacin (INF), 12 g/d of unprotected niacin (N12), 3 g/d of protected niacin (BN3), 6 g/d of protected niacin (BN6); 12 g/d of protected niacin (BN12) and without niacin supplement (CON) from 0 to 6 hours after feeding. The results indicated none of the comparisons was different ($P > 0.13$). Treatment had linear ($P = 0.01$) and quadratic ($P < 0.01$) effect among BN3, BN6 and BN12.

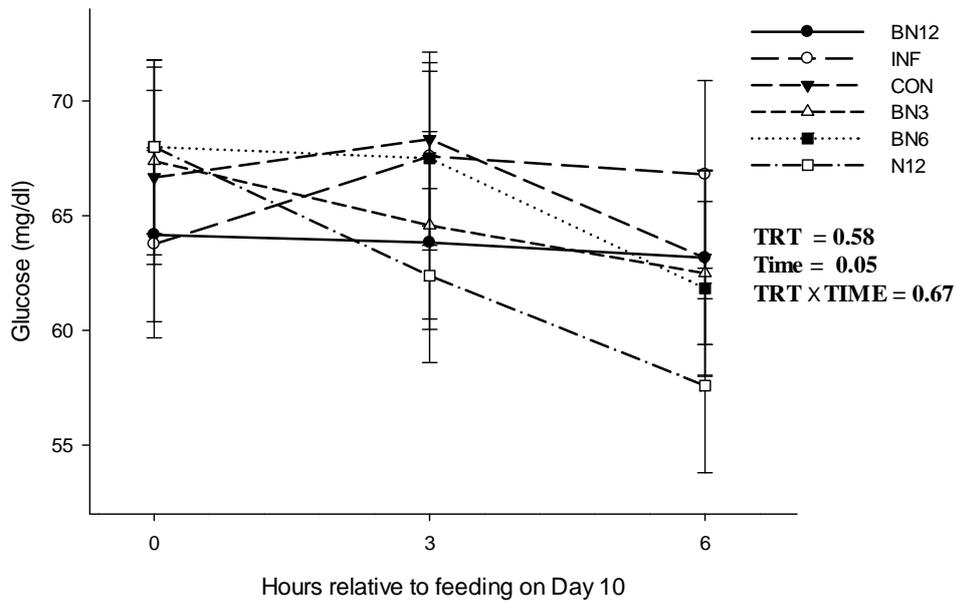


Figure A.2. Plasma glucose concentration least square means and SEM on d 10 for Holstein cows received abomasal infusion of 12 g/d of unprotected niacin (INF), 12 g/d of unprotected niacin (N12), 3 g/d of protected niacin (BN3), 6 g/d of protected niacin (BN6); 12 g/d of protected niacin (BN12) and without niacin supplement (CON) from 0 to 6 hours after feeding. The results indicated none of the comparison was different ($P > 0.13$). No linear or quadratic relationship detected ($P > 0.39$).

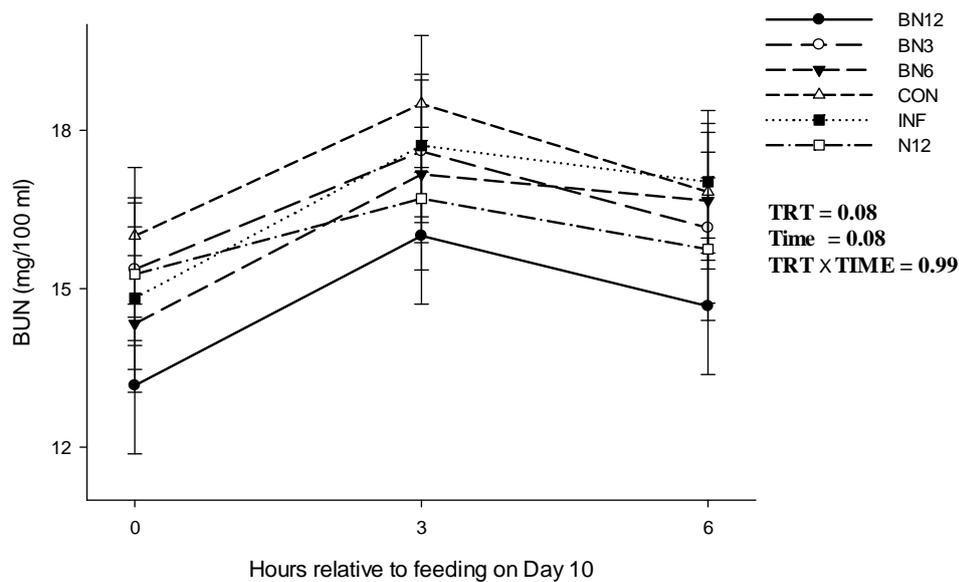


Figure A.3. Plasma BUN concentration least square means and SEM on d 10 for Holstein cows received abomasal infusion of 12 g/d of unprotected niacin (INF), 12 g/d of unprotected niacin (N12), 3 g/d of protected niacin (BN3), 6 g/d of protected niacin (BN6); 12 g/d of protected niacin (BN12) and without niacin supplement (CON) from 0 to 6 hours after feeding. The results indicated none of the comparisons was different ($P > 0.17$). No linear or quadratic relationship detected ($P > 0.11$).

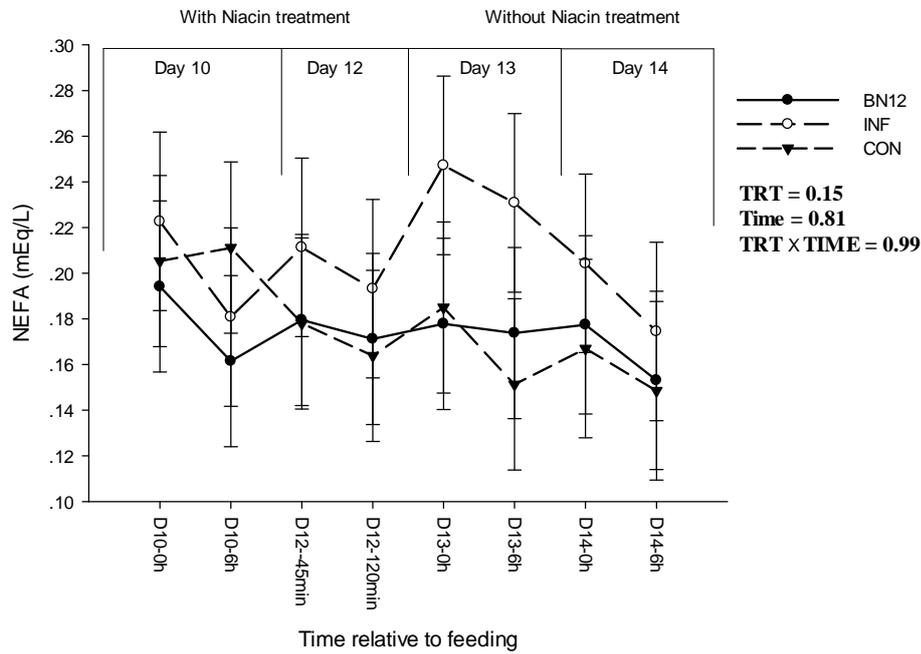


Figure A.4. Plasma NEFA concentration least square means and SEM for Holstein cows received abomasal infusion of 12 g/d of unprotected niacin (INF); 12 g/d of protected niacin (BN12) and without niacin supplement (CON) on d 10, 12, 13 and 14. There was no difference detected on any treatment between with niacin supplement and without niacin supplement ($P > 0.37$). There was no ($P > 0.43$) NEFA concentration increment detected between before and after feeding on BN12, CON and INF.

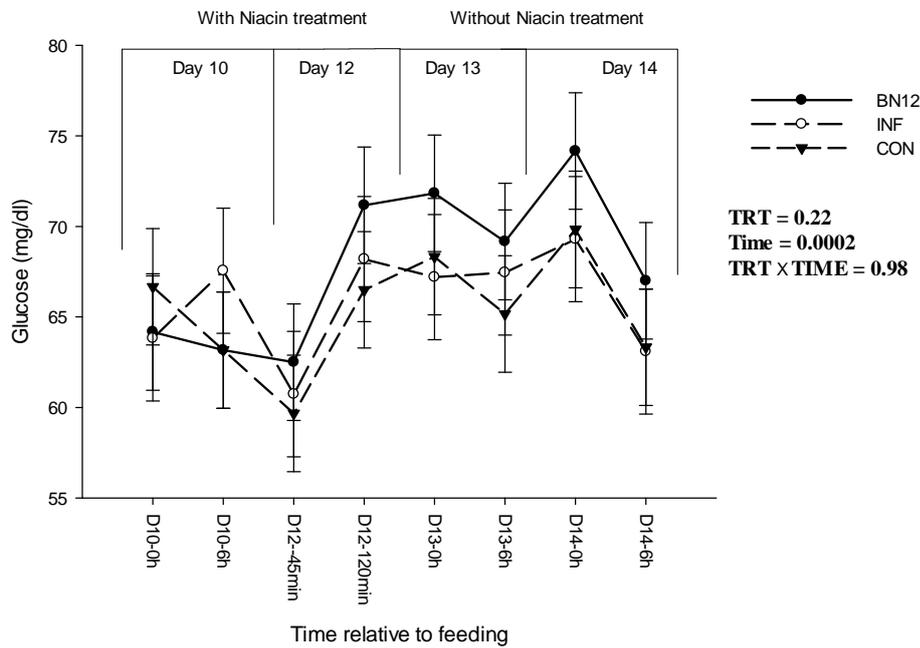


Figure A.5. Plasma glucose concentration least square means and SEM for Holstein cows received abomasal infusion of 12 g/d of unprotected niacin (INF); 12 g/d of protected niacin (BN12) and without niacin supplement (CON) on d 10, 12, 13 and 14. There was no difference ($P > 0.32$) detected among treatment on any individual time point. All treatment had no difference ($P > 0.42$) on glucose concentration increment between before and after feeding. BN12 had lower ($P < 0.01$) glucose concentration with niacin supplement (65.3 ± 2.12) than without niacin supplement (70.5 ± 2.12). However, there was no difference ($P > 0.18$) detected on CON and INF between with niacin supplement (64.0 ± 2.12 and 65.1 ± 2.22 respectively) and without niacin supplement (66.7 ± 2.12 and 66.7 ± 2.22 respectively).

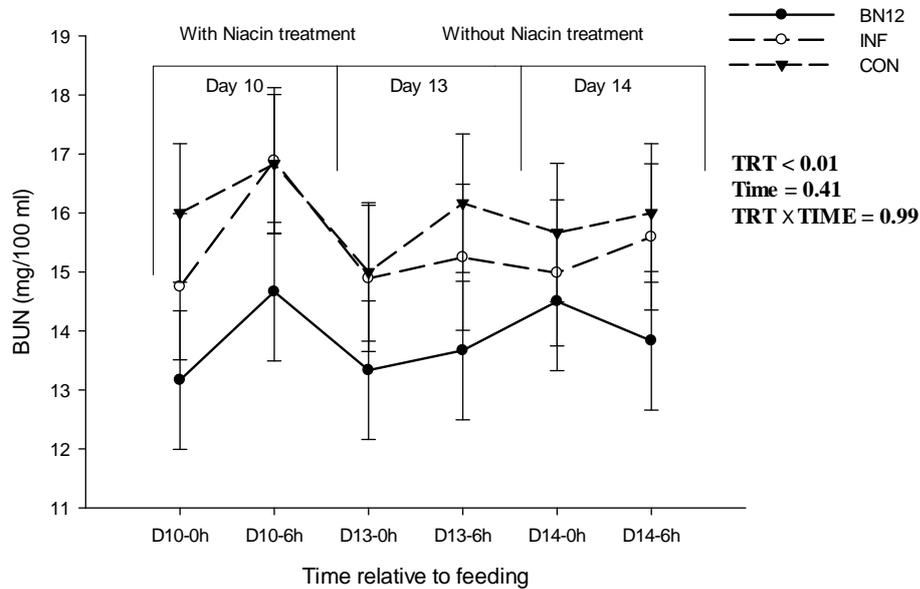


Figure A.6. Blood urea nitrogen concentration least square means and SEM for Holstein cows received abomasal infusion of 12 g/d of unprotected niacin (INF); 12 g/d of protected niacin (BN12) and without niacin supplement (CON) on d10, 13 and 4. BN12 (13.9 ± 0.89) has lower ($P < 0.01$) BUN than INF (15.9 ± 0.91) and CON (15.4 ± 0.89). CON had lower ($P = 0.04$) BUN than BN12 and INF at 0 h after feeding on d 10. However, there was no difference ($P = 0.28$) among BN12, CON and INF at 6 h after feeding on d 10. BN12 and INF tended to have higher ($P = 0.06$) BUN with niacin supplement than without niacin supplement. However, CON had no difference ($P = 0.49$) between supplement and no supplement.

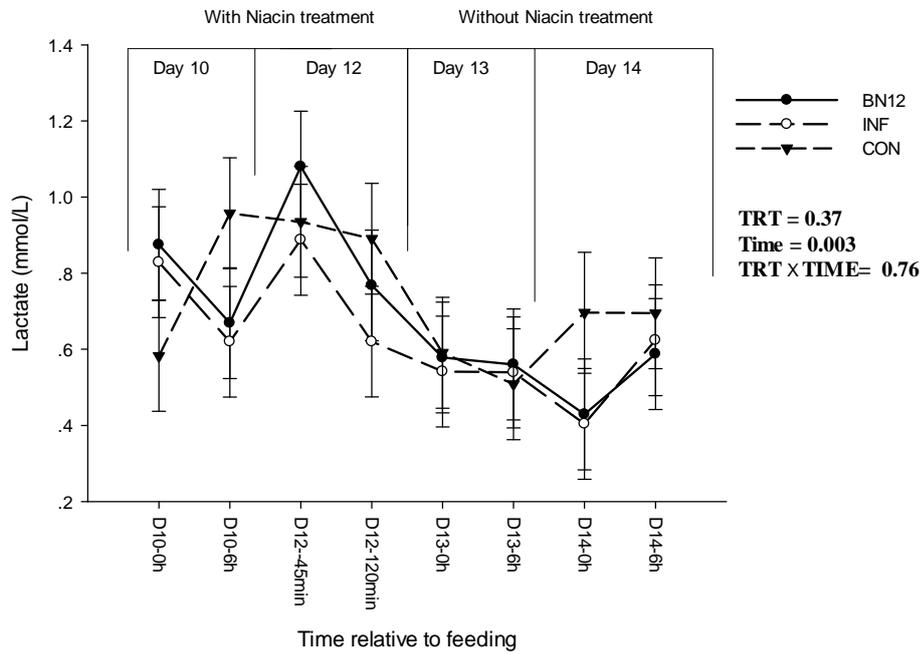


Figure A.7. Serum lactate concentration least square means and SEM for Holstein cows received abomasal infusion of 12 g/d of unprotected niacin (INF); 12 g/d of protected niacin (BN12) and without niacin supplement (CON) on d10, 12, 13 and 14. BN12 and INF resulted lower ($P < 0.04$) lactate concentration than CON at 6 hr after feeding with niacin supplement. Furthermore, BN12 and INF resulted less ($P < 0.02$) lactate concentration increment than CON between before and after feeding with niacin supplement. All three treatments (BN12, INF and CON) had higher ($P < 0.03$) lactate concentration with niacin supplement than without niacin supplementation.