ABSTRACT

BAIRD, EMILY JEAN. Effect of Calcium Supplementation on Ruminal Soluble Calcium Concentrations and Ruminal Fermentation. (Under the direction of Jerry W. Spears).

Two experiments were conducted to determine the effect of Ca source and concentration on ruminal soluble Ca concentration and microbial fermentation. In the first study, experimental diets (14 g DM/d) were added to continuous flow culture fermentors in two equal portions at 0800 and 1700-h. Treatments consisted of control diet (0.18% Ca) or the control supplemented with 0.60% Ca from either 1) $CaCO_3$, 2) Ca propionate-prilled (CaP, Nutrocal®) or 3) Ca propionate-powder. Following a 2-d stabilization period, fermentors were sampled over a 4-d collection period. Each treatment was replicated five times. Calcium supplementation of the control diet increased ruminal soluble Ca concentrations. Ruminal soluble Ca concentrations were higher in cultures receiving Ca propionate compared to $CaCO_3$. Ruminal pH was lower in Ca supplemented diets compared to control, and $CaCO_3$ supplemented cultures yielded higher pH values than Ca propionate treatments. Digestibility of NDF was higher in fermentors receiving prilled Ca propionate compared to those supplemented with powdered Ca propionate. Total VFA production in ruminal cultures was increased by supplemental Ca. In response to Ca source, total VFA production was higher for Ca propionate than $CaCO_3$ and Ca propionate-prilled compared to Ca propionate-powder treatments. Propionate production and molar proportion were higher in Ca propionate than control or $CaCO_3$ treatments. Butyrate production was higher for Ca propionate-prilled and $CaCO_3$ treatments compared to control and Ca propionate-powder treatments.

Molar proportion of butyrate was higher for $CaCO_3$ than the other treatments. Production and molar proportion of isovalerate was lower for Ca propionate-powder compared to other treatments. Calcium supplementation did not affect C16:0, C18:0, C18:2, CLA *cis*9, *tran*11 or CLA *trans*10, *cis*12 isomers. However, the proportion of C18:1 *trans* isomers as well as the total proportion of C18:1 isomers increased as a result of supplementing Ca. Calcium carbonate increased C18:0 and proportions of C18:1 *cis*, and reduced C18:2 compared to Ca propionate addition.

The second experiment utilized sixteen Angus steers initially averaging 274 kg (trial 1) or 252 kg (trial 2). Animals were divided by weight and randomly allotted to three treatments. Eight steers were randomly assigned to one of the following treatments in each of two trials: 1) control (no supplemental Ca), 2) $CaCO_3$ and 3) Ca propionate (prilled). Each steer was supplemented with 1.36 kg of a corn-corn gluten feed supplement per day. The supplement provided 14.8 g of Ca per day for steers in treatments two and three. Chopped orchardgrass hay containing 0.2% Ca was also fed to all steers based on what each animal would consume in a 24-h period. Following a 14-d adjustment period, steers were placed in metabolism crates. Steers were acclimated to the metabolism crates for 5-d followed by a collection period of 5-d in order to obtain a total collection of urine and feces. Dry matter intake and digestibility during the digestion period were both similar across treatments. NDF intake and digestibility were also not affected by Ca level or source. Ca supplementation increased ruminal soluble Ca concentrations; however, concentrations were much higher in steers supplemented with Ca propionate compared to $CaCO_3$. Plasma Ca concentrations were not affected by Ca supplementation. Total VFA concentrations were not affected by Ca level or source.

However, the addition of Ca significantly decreased molar percentages of acetate. In fact, the proportion of acetate was lower with supplemented Ca propionate compared with $CaCO_3$. The addition of Ca significantly increased the molar percentages of propionate. Supplemental Ca propionate resulted in much greater proportions of propionate compared to $CaCO_3$ addition. Steers supplemented with Ca propionate had a lower acetate:propionate ratio than those supplemented with $CaCO_3$. Molar proportions of butyrate, valerate, and the isoacids, isobutyrate and isovalerate, were not affected by the addition of Ca.

These results indicate the importance of considering the difference in solubility of Ca found in feedstuffs and supplemental Ca sources. In the fermentor study, both Ca level and source affected ruminal soluble Ca concentrations and fermentation. Soluble Ca concentrations were greater in cultures supplemented with Ca propionate compared with those supplemented with $CaCO_3$. This may be due to the difference in solubility of the supplemental Ca sources. The amount of ruminal soluble Ca will depend on the solubility present in feedstuffs as well as supplemental sources of Ca. However, increased ruminal soluble Ca concentrations in the second experiment did not affect ruminal fermentation. The lack of a significant increase in digestion as a result of Ca supplementation may be explained in that adequate amounts of soluble Ca may have existed in the supplement and orchardgrass hay to meet the microbes' Ca requirement. This suggests that the Ca present in the hay was fairly soluble in the ruminal environment and contributed to ruminal soluble Ca concentrations. These results indicate that dietary Ca source affects ruminal soluble Ca concentrations and ruminal fermentation.

EFFECT OF CALCIUM SUPPLEMENTATION ON RUMINAL SOLBULE CALCIUM CONCENTRATIONS AND RUMINAL FERMENTATION

by

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DEDICATION

"If you raise your children to feel that they can accomplish

any goal or task they decide upon, you will have succeeded as a parent and you will have given your children the greatest of all blessings."

–Brian Tracy

The author would like to dedicate this work to her parents, Bill and Harriett Baird. They both provided me with a very early love and reverence for all living things, and that untimely knowledge has influenced my entire life. Without their unending support, love and encouragement, I know I never would have achieved so many of my goals.

BIOGRAPHY

Emily Jean Baird was born in Hickory, North Carolina on October 28, 1979 to Harriett M. and William E. Baird. She was raised in rural Mountain View, North Carolina, along with her younger brother, Charlie. Emily graduated from Fred T. Foard High School in May of 1998 and enrolled in the College of Agriculture and Life Sciences at North Carolina State University in Raleigh, North Carolina in August of 1998. She received a Bachelor of Science degree with a major in Zoology from North Carolina State University in May of 2002. In January of 2003, she entered North Carolina State University's Animal Science program in pursuit of a Master of Science degree under Dr. Jerry W. Spears. In August of 2004, she entered Auburn University College of Veterinary Medicine in Auburn, Alabama in pursuit of a veterinary medical degree.

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Introduction

In 1808, British scientist, Sir Humphrey Davey discovered Ca as a white, silvery alkaline earth metal. Derived from the Latin, *calx*, Ca is the fifth most abundant element in the earth's crust and the most abundant cation in the animal body making up one to two percent of total body weight (NRC, 1980). At the time of Davey's discovery, bone was discovered to contain a specific Ca compound, Ca phosphate. Therefore, this information became clinically important in the prevention and treatment of rickets, a debilitating childhood disorder concerning abnormal bone development (Underwood and Suttle, 1999). Similarly, food animals were being fed energy-rich, Ca deficient grain-based diets thereby encouraging the incidence of bone disorders in livestock populations as well (Underwood and Suttle, 1999). In addition to these problems, animal breeders maintained an interest in Ca nutrition by selecting traits with high requirements for Ca such as increased growth rate, milk yield, litter size and egg production (Underwood and Suttle, 1999). Thus, the essentiality of proper nutrition and Ca integration became more apparent as these problems persisted and solutions were recommended.

Calcium Function

The majority of uni- and multicellular organisms require Ca for normal growth and function. Bone provides support and structure to the body, but it is also a vital physiological tissue serving to provide a readily available source of Ca for maintenance of homeostasis in the body. In fact, most of the Ca found in the body is specifically used to form and maintain bone and teeth. This is supported by the fact that ninety-eight percent of Ca found in the body is contained in bone and another one percent located in teeth as Ca phosphate and carbonate combined with collagen (AMA, 1995). This is not the only structural role of Ca in

tissues, however. Calcium bound to phospholipids, proteins and nucleic acids also plays a role in maintaining the structure and function of all eukaryotic cells and tissues (Campbell, 1983). The remaining one percent of Ca in the body circulates in blood and performs a number of essential jobs. Despite the vital role of Ca in maintaining the composition of teeth and bone, this essential nutrient is required to regulate individual cell membranes in moving nutrients, hormones and other substances into and out of cells (Bronner, 1964). Ionized Ca is also important during muscle contraction in the transmission of electrical and chemical messages between nerve cells and along nerve axon fibers as well as manufacturing and maintaining the function of many hormones and enzymes important to normal body function. For example, several extracellular degradative enzymes in both eukaryotes and prokaryotes require Ca for stability or maximal activity including proteases and certain enzymes involved in blood clotting (Campbell, 1983). Another important function of Ca includes its role as an intracellular or second messenger. Normally, intracellular ionized Ca concentrations are maintained at very low levels due to Ca binding proteins in the cell that bind Ca or due to the limited entry of the element into the cell due to the potential to overexcite and possibly kill the cell (Campbell, 1983). However, hormones such as adrenaline and insulin both affect the membrane potential of cells, as well as stimulating glucose transport in cells such as those of adipose tissue and muscle. Therefore, in order to convey the effect these hormones have on metabolism within the cell, a second messenger such as Ca is required resulting in increased intracellular Ca concentrations (Campbell, 1983).

Calcium Metabolism

In order for Ca levels in the body to be maintained, the amount of Ca that is absorbed is balanced with the amount of Ca that is lost. Thus, Ca metabolism is an important array of

chemical activities regulated by certain hormones to achieve and support this Ca balance. Specifically, three hormones are needed to maintain the level of Ca found in the blood at approximately 2.5 mmol/L (Bronner and Stein, 1995). Parathyroid hormone (PTH), secreted by the parathyroid glands, causes the body to release Ca from bone and increases absorption of Ca from the intestine and kidney as a result of hypocalcemia. The parathyroid gland is very sensitive to small deviations in the ionic Ca concentrations in extracellular fluids, and when concentrations decline, PTH is normally secreted and activates 1,25-dihydroxycholecalciferol (Omadahl and DeLuca 1973; Borle 1974). 1,25-dihydroxycholecalciferol is hydroxylated to 25-hydroxy-D3 in the liver and further in the kidney to two compounds, 24,25-$(OH)_2$D3 or 1,25-$(OH)_2$D3. In the intestinal mucosa, 1,25-$(OH)_2$D3 opens up Ca channels and facilitates intestinal Ca uptake and bone Ca mobilization (Hurwitz *et al*., 1995). However, calcitonin is also responsible for maintaining Ca homeostasis, but this hormone actually opposes the action of PTH and 1,25-$(OH)_2$D3. Therefore, it is often secreted when blood Ca levels are too high. During such hypercalcemic events, calcitonin prevents release of Ca from bone into the blood (Underwood, 1981). Thus, the combination of these three hormones is vital to maintaining Ca metabolism in the body.

The path of Ca in the body involves ingestion, digestion, intestinal transit during which a portion of the Ca is absorbed transepithelially. However, Ca absorption through the intestinal wall proceeds via two mechanisms, an active transcellular process that takes place in the duodenum and a passive paracellular process throughout the small intestine (Bronner, 1998).

Transcellular absorption occurs as an active process that allows a particular amount of Ca to be absorbed, but as the system becomes saturated it will decline further Ca absorption.

It is essentially localized to the upper duodenum and is totally dependent on vitamin D (Pansu *et al*., 1983). When Ca intake is low, active transcellular Ca transport in the duodenum is upregulated and a larger proportion of Ca is absorbed by the active process than by the passive paracellular process that prevails in the jejunum and ileum (Bronner and Pansu, 1998).

This active absorptive process involves three steps: entry across the cell wall, diffusion through the cytoplasm and exit at the basolateral cell pole. The entry of Ca into the intestinal cell across the microvillar or brush border membrane requires no metabolic energy, as Ca moves down a steep electrochemical gradient (Fullmer, 1992). The concentration of Ca in the lumen is variable, but often in the millimole-per-liter range. Although total cellular Ca is relatively high (mmol/L), the intracellular free Ca level is maintained at 10^{-7} to 10^{-6} mole/L (Fullmer, 1992).

The rate-limiting step in transcellular Ca movement is the diffusion of the Ca ions across the cytoplasm. This may appear surprising, but the self-diffusion rate of the Ca ion in an aqueous medium at 37˚C is only approximately 1/70 of the experimentally determined transport rate (Bronner *et al*., 1986). On the basis of available data, self diffusion of the Ca ion in the cytoplasm would likely be slower than the experimentally determined transport rate by about two orders of magnitude if the duodenal cell did not contain calbindin D_{9K}, the vitamin D-dependent enterocytic Ca-binding protein (Bronner *et al*., 1986; Wasserman and Fullmer, 1995; Wasserman *et al*., 1968). Calbindin D_{9K} is found in duodenal cells and is absent from ileal cells and from all intestinal cells in vitamin D-deficient animals (Pansu *et al*., 1983). Calbindin D_{9K} functions by raising the Ca ion concentration, thereby amplifying the Ca flux rate (Stein and Hoshen, 1996; Wyman, 1966). In a vitamin D-deficient intestinal

cell, Ca ion moves across the plasma membrane down its chemical gradient and accumulates along the inner aspect of the plasma membrane with very little Ca found in the cytoplasm. However, in an intestinal cell from a vitamin D-replete animal, Ca ions occur throughout the cytoplasm (Wasserman and Fullmer, 1995). Therefore, calbindin D_{9K} acts to positively influence the intracellular diffusion rate of the Ca ion into the cell.

Extrusion occurs against an electrochemical gradient and thus constitutes the energy-requiring step of transcellular transport. The extrusion step is modulated by vitamin D, but does not appear to be rate-limiting. Calcium that arrives at the basolateral pole becomes bound to a site at the cytoplasmic aspect of the CaATPase that spans the basolateral membrane. A phosphorylation-induced change in the conformation of the CaATPase occurs, and the Ca ion is extruded through the channel formed by the enzyme transmembrane elements (Bronner and Pansu, 1998).

Passive Ca transport or, paracellular diffusion down a chemical gradient, occurs throughout the length of the small intestine and is the transport mechanism that accounts for most Ca absorption when Ca intake is high. Specifically, nearly ninety percent of the time spent by the chyme in the small intestine of the rat is spent in the lower third of the jejunum and ileum, with the ileum being the site in the small intestine where most Ca is absorbed (Marcus and Lengemann, 1962). This process of paracellular diffusion occurs due to the fact that high Ca intake leads to downregulation of active transport (Buckley and Bronner, 1980). In rats on low Ca intakes, active transport in the duodenum can account for about fifty percent of the total absorbed, but the portion accounted for by active transport diminishes rapidly as Ca intake increases (Pansu *et al*., 1993).

The amount of Ca absorbed by the paracellular route is directly related to soluble Ca concentrations in the intestinal lumen (Bronner, 1998). Soluble sources such as Ca propionate may result in higher rates of paracellular absorption of Ca than insoluble sources of Ca such as $CaCO_3$. In fact, Goff and Horst (1993) found that oral administration of large doses of Ca from Ca propionate increased plasma Ca concentrations in dairy cows while $CaCO_3$ did not. Spears *et al.* (2002) also observed that Ca from Ca propionate was absorbed and retained to a greater extent than $CaCO_3$ when provided at low dietary concentrations.

In terms of the large intestine, Ca absorption occurs in the cecum and ascending colon, but not in the transverse colon (Petith and Schedl, 1976; Escoffier, 1996). It is also interesting to note that some fifteen percent of the total soluble Ca found in the entire intestinal tract is located in the colon (Duflos, 1994). If we assume that the rate of paracellular movement is similar in the small and large intestines, then approximately eleven percent of Ca absorbed by the paracellular route throughout the entire tract is absorbed in the large intestine (Bronner and Pansu, 1998). Active Ca transport also takes place in the colon. Some seven percent of the total intestinal calbindin is found in the rat large intestine (Escoffier, 1996). Therefore, due to the proportional relationship between active Ca transport and calbindin D_{9K}, the cecum and colon would account for about seven percent of the total active Ca transport.

In relation to ruminants, experimental studies have been conducted in order to illustrate that the reticulo-rumen plays a crucial role in Ca absorption. In fact, studies with the application of radioactive Ca and arteriovenous difference experiments have shown that the rumen wall of sheep is actually permeable to Ca in both directions (Jones and Mackie, 1959; Yano *et al*., 1978). Considering the electrical potential at the luminal membrane and

the low concentration of free Ca inside the cell, Ca entry into the cell should be passive, following an electrical and a chemical gradient, whereas Ca extrusion across the basolateral membrane is against both gradients and therefore requires metabolic energy (Bronner, 1987).

Approximately eighty-five percent of the Ca ions absorbed from the intestine into the blood are found for a period of time in bone (Comar *et al.,* 1953). Thus, practically all the Ca ions which are absorbed from the tract will enter the bone and replace similar Ca ions, some of which will be excreted as a portion of endogenous Ca into the intestine. In other words, the endogenous Ca ions have for the most part been involved in bone exchange prior to being secreted into the gastrointestinal tract (Comar *et al*., 1953). Fecal Ca is composed of this endogenous, intestinal Ca secretion as well as unabsorbed dietary Ca. In domestic species, the endogenous fecal Ca excretion is little affected by the amount of dietary Ca or by the age or Ca status of the individual. The amount of endogenous fecal Ca is small, relative to total fecal output and varies little among the domestic herbivores. Some reported values are 15-20 mg of endogenous fecal Ca per kg of body weight for sheep and cattle (Braithwaite and Riazuddin, 1971; Comar *et al*., 1953). Thus, changes in total fecal Ca output are largely due to changes in dietary Ca intake and Ca absorption. In fact, sheep and cattle have been noted to absorb Ca from their gut according to need and can alter the efficiency of absorption to meet a change in requirement. For example, Braithwhite and Riazuddin (1971) have shown that young sheep with a high Ca requirement absorb Ca at a higher rate and with greater efficiency than mature animals with a low requirement. An increase in absorption and an increased efficiency of absorption also occurs in mature sheep when their requirement for Ca is increased through pregnancy, lactation or following a period of Ca deficiency (Braithwhite, 1974). Ramberg *et al*. (1970) obtained similar results concerning cattle in that

the efficiency of absorption of Ca in the small intestine of the dairy cow increased in response to a reduction in dietary Ca intake and to the onset of lactation.

In addition to Ca deficiency, the age of an animal is a very important factor in how well the individual regulates Ca levels. In fact, an inverse relationship exists between age and the body's ability to efficiently absorb Ca. Thus, due to the requirement for larger amounts of Ca in developing teeth and bones, children and adolescents were shown to absorb approximately seventy-five percent of total dietary Ca whereas only thirty to forty percent Ca absorption was noted in healthy human adults (AMA, 1995).

In contrast to fecal Ca excretion, urinary Ca excretion is generally very small and not typically influenced by Ca status in domestic ruminants (Braithwhite and Riazuddin, 1971). However, Ca excretion resembles sodium excretion in that it is responsive to the plasma level and is apparently not characterized by a maximal rate of absorption. In fact, it has been generally assumed that the concentration of Ca in plasma ultrafiltrate is an accurate measure of its concentration in the glomerular filtrate (Comar and Bronner, 1960).

Studies on the effects of various agents on Ca excretion are somewhat complicated by the fact that the output of Ca tends to vary with urine flow (Wolf and Ball, 1949; 1950). Thus, the rate of Ca reabsorption from the kidney, in common with the reabsorption of several other ions, may depend on its concentration in the tubular fluid. The rate of excretion of unreabsorbable anions may also influence Ca reabsorption. Phosphate infusion may produce increments in Ca excretion, exceeding fifteen percent of filtered Ca (Jahan and Pitts, 1948; Chen and Neuman, 1955). The presence of increased quantities of unreabsorbed anion in the filtrate may account for the rise in Ca excretion following infusions of sodium

pyruvate, lactate, hippurate, acetate and malate during urea diuresis (Brull and Bernimolin, 1956).

Calcium Bioavailability

The value of a feed as a source of Ca depends not only upon the Ca content, but also on the amount that the animal can extract and retain for its own use. Theoretically, one of the factors limiting mineral Ca absorption includes the solubility of the Ca from the mineral source. Calcium chloride represents a source of highly soluble Ca. Hansard *et al*. (1954) noted more than 95 percent efficiency with the use of $^{45}CaCl_2$ as a source of radioactive tracer for Ca absorption in young calves. Thus, $CaCl_2$ was assigned an efficiency of absorption coefficient of 95 percent, but $CaCO_3$ was found to have an absorption coefficient of only 75 percent (Hansard *et al*., 1957). However, Ca from limestone generally is slightly less available than from pure $CaCO_3$ and has been assigned an efficiency of absorption coefficient of 70 percent (Hansard *et al*., 1957).

The amount of available Ca that will actually be absorbed will vary with the age of the animal as well. Hansard *et al*. (1954) and Horst *et al*. (1978, 1990) reported that the efficiency of absorption of Ca decreases as animals age due to a decline in vitamin D receptors in the intestinal tract. This is significant in that it reduces the animal's ability to respond to 1,25-dihydroxycholecalciferol, the hormonal form of vitamin D_3. Typical of other steroid hormones, the action of 1,25-dihydroxycholecalciferol is mediated by an intracellular receptor protein (VDR) in target tissues. The VDR/hormone complex located within the nucleus regulates gene expression and, therefore, controls the synthesis of specific proteins that alter cell function. Consequently, target cell responsiveness to 1,25-dihydroxycholecalciferol is impaired when receptor numbers are reduced or lacking.

Therefore, changes in Ca metabolism with age may reflect alterations in the quantity or physiochemical properties of VDR in target tissues.

In addition to the effect of age on Ca availability, pregnancy and lactation each affect the regulation of intestinal Ca absorption. In fact, Ramberg (1974) reported that the rate of entry of Ca into the extracellular fluid pool from the intestine increased about 1.55-fold from the day before parturition until 10 days in milk. Braithwaite *et al.* (1970) also noted an increase in the rate of absorption of Ca from the intestine during pregnancy, but it was insufficient to meet the full requirements of late pregnancy and early lactation. Thus, the extra Ca was supplied by increased removal of Ca from bone (Braithwaite, *et al*., 1970; Ramberg, *et al*., 1970).

Calcium and Rumen Fermentation

R.E. Hungate (1950), after extensively reviewing the literature pertaining to rumen cellulose digesting bacteria, concluded that: 'The first step in ruminant nutrition is microbial nutrition.' The feeding of cattle and other ruminant animals often includes the feeding of grains and protein-rich feeds in combination with roughage. The possibility was presented that these grain and protein-rich feeds when fed with roughage supply not only cellulose, but also nutritional factors such as Ca needed by rumen microorganisms in the digestion of the roughage fraction of the ration (Burroughs *et al*., 1950c). Even though it is not certain whether all bacteria require Ca for growth, the cation is necessary for enzyme activities responsible for such crucial processes as nitrogen fixation. According to Silver (1977), all microbial Ca functions are located at the cellular membrane or external to the membrane, thus providing no intracellular role for Ca in bacterial cells. Therefore, all Ca-requiring

enzymes are thought to be extracellular and include many hydrolytic enzymes such as proteases, nucleases, lipases, α-amylases and cellulases (Silver, 1977).

Experiments with cattle and sheep have shown that roughage digestion can be favorably altered by Ca supplementation of ruminant diets (Burroughs *et al*., 1950d; Swift *et al*., 1950). However, research has indicated that other divalent cations such as cobalt and copper also exert positive influences upon ruminal digestion of cellulose and cellulose-containing roughages. Specifically, Saxena and Ranjhan (1977) noted the supplementation of cobalt and copper increased the *in situ* digestibility of cotton-thread from 58.6 to 68.7 percent in fistulated Hariana calves. The half life of cotton was also calculated, and the amount of time taken for fifty percent digestion of cotton was lowest with the supplementation of cobalt and copper. Since the relationship between the rate of cellulose digestion and half life is an inverse one, cobalt and copper supplementation favored the growth of rumen microorganisms resulting in increased digestibility of cellulose (Saxena and Ranjhan, 1977).

Hubbert *et al*. (1958) also evaluated specific inorganic mineral requirements of rumen microorganisms for maximum cellulose digestion by determining the optimum and excessive concentrations of various elements in the *in vitro* fermentation medium. Consequently, addition of 50 to 300 μg of dietary Ca (from $CaCl_2$) per milliliter of fermentation medium appeared to result in a consistent increase of approximately ten percentage units in cellulose digestion (Hubbert *et al*., 1958). It is also interesting to note that the addition of 450 μg or more of Ca significantly depressed cellulose digestion below the response noted within the previously mentioned optimum range (Hubbert *et al*., 1958). However, with the addition of 1000 μg of Ca per milliliter, cellulose digestion was not depressed. Thus, it seems that

rumen microorganisms exhibit considerable tolerance to excess Ca levels in the fermentation medium.

Similarly, experiments were conducted with the addition of alfalfa ash, and its effect on cellulose digestion was noted to be related to the amount of Ca in the alfalfa ash (White *et al*., 1958). Improvements were noted in crude fiber digestion of 5.9 and ten percentage units with the addition of alfalfa ash by Burroughs *et al*. (1950b) and Swift *et al*. (1950), respectively. Digestion trials were used in measuring the influence of alfalfa hay and fractions of alfalfa hay upon corncob crude fiber digestion (Burroughs *et al*., 1950b). Corncob digestion was improved by 17.9 percentage units with the addition of alfalfa hay, 14.5 percentage units with the addition of a water extract of dehydrated alfalfa meal and 13.5 percentage units due to the ash of the alfalfa meal fed at the rate equivalent to four pounds meal daily per steer (Burroughs *et al*., 1950b).

Therefore, due to the importance of Ca in improving cellulose digestibility of low quality forages, the mechanisms behind these events have been examined. It has been postulated that divalent cations may function as bridges between bacteria and plant cell walls, both of which tend to be negatively charged. In other words, when a negatively charged bacterium has difficulty attaching to similarly charged particles, a divalent cation such as Ca, possessing two positive charges, could serve as a link between the two negatively charged surfaces (Lopez-Guisa and Satter, 1992). Somers (1973) noted that cell walls prepared from onion bulbs were found to exhibit an affinity for Ca as a result of 25 percent of the total pectic carboxylic groups in the cell wall occupied by Ca. Thus, it was determined that Ca exchange capacity of plant tissues was positively related to pectic carboxylic groups in that

the cation was known to form stable salts with pectin and related polymers of galacturonic acid (Jansen *et al.*, 1960; Knight *et al.*, 1961).

Calcium may also improve fiber digestibility by alleviating the adverse effects of fatty acids on fiber digestibility via formation of Ca soaps. Supplemental fat is often used to increase the energy density of ruminant diets, but the addition of this lipid can also lead to the depression of fiber digestibility. Some researchers (Brooks *et al.*, 1954; Rhodes *et al.*, 1956; Ward *et al.*, 1957; Summers *et al.*, 1957) have reported a reduction in digestion of dry matter by sheep fed low-quality roughage diets containing three percent supplemental fat.

It has been proposed that the adverse effect of fat on fiber digestibility is due to fat coating fibrous particles and preventing microbial attack (Brooks *et al.*, 1954; Ward *et al.*, 1957; Pfander *et al.*, 1957). White *et al.* (1958) noted a progressive depression of cellulose digestion rather than an initial depression with no further decrease. A purely physical effect on cellulose digestion would be alleviated by passage of the fat-coated cellulose from the rumen. However, recovery from the decreased cellulose digestibility did not occur until after 17 days. These data suggest that supplemental fat decreases certain microbial metabolic activity and/or modifies the rumen microbial population concerned with cellulose digestion (White *et al.*, 1958). Specifically, growth of cellulolytic bacteria in the rumen is often inhibited by long-chain fatty acids; however, various researchers noted that Ca supplementation would reverse the depression of cellulose digestion caused by added dietary fat. The results of White *et al.* (1958) and Davison and Woods (1961) clearly indicate that $CaCO_3$ will alleviate the depressing effect of corn oil on diet digestibility. Supplemented corn oil decreased digestibility of dry matter and organic matter, but the addition of $CaCl_2$ or $CaCO_3$ alleviated the effects of corn oil on organic matter digestibility (Davison and Woods,

1961). However, Jenkins and Palmquist (1982) noted that supplemented dicalcium phosphate reacted only slightly with fatty acids to increase the insoluble soap content after 24 hours of incubation, but an equivalent amount of Ca as $CaCl_2$ increased both insoluble soaps and digestibility more rapidly and extensively.

White et al. (1958) suggested that it may be possible to explain the Ca-fat relationship due to the formation of Ca soaps. Galbraith *et al.* (1961) also noted the formation of insoluble Ca soaps as the primary role of metal cations in reducing the antibacterial activity of long-chain fatty acids. Neutral fat has been shown to be hydrolyzed in the rumen to fatty acids and glycerol (Garton *et al.*, 1958). Glycerol is fermented to propionate and the fatty acids may act as antimetabolites to the rumen bacteria, but in the presence of Ca the fatty acids were precipitated as Ca soaps permitting the remainder of the ingesta to be fermented normally (Johns, 1953; Camien and Dunn, 1957). The results of Davison and Woods (1961) showed an increase in digestible energy when Ca was added to rations containing corn oil. For this to occur, it would be necessary for the fatty acids and Ca to disassociate and permit the fatty acid to be absorbed. Garton (1951) has shown the pH of the abomasal and duodenal contents of sheep to be approximately three and that the solubility of Ca increases as it passes from the rumen into the abomasum. Therefore, it is suggested that the Ca and fatty acids pass into the small intestine in the disassociated state and either the Ca is absorbed before the pH becomes neutral or alkaline and then the fatty acids are absorbed in the presence of bile, or the fatty acids are absorbed immediately upon passing from the abomasum. Some soaps may undoubtedly escape in the feces as well.

It is apparent that Ca is important for ruminal fermentation. Galbraith *et al.* (1961) noted the role of Ca in formation of Ca soaps in order to enhance ruminal fermentation by

reducing the antimicrobial activity of fatty acids. It has also been postulated that Ca may enhance microbial digestion by functioning as a bridge between negatively charged bacteria and plant cell walls (Lopez-Guisa and Satter, 1992). However, in order to meet the Ca requirement of ruminal microorganisms, Ca probably needs to be present in a soluble form in the ruminal environment. The amount of ruminal soluble Ca will depend on the solubility of the Ca present in feedstuffs as well as supplemental sources of Ca. Addition of Ca as $CaCO_3$ has not been shown to affect ruminal soluble Ca to the extent that Ca propionate does. In fact, Spears *et al.* (2002) noted that when supplemental Ca was supplied from Ca propionate, ruminal soluble Ca increased greatly. However, increasing dietary Ca from $CaCO_3$ had little effect on ruminal soluble Ca concentrations. Therefore, the current studies were conducted to determine the effect of dietary Ca level and source on ruminal fermentation.

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RUNNING HEAD: Calcium Effects on Ruminal Soluble Calcium and Ruminal Fermentation

Effect of Calcium Source and Form on Ruminal Soluble Calcium and Ruminal Fermentation in Continous Culture Fermentors[1]

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Introduction

Calcium is necessary for ruminal microbial digestion of the fiber portion of the diet. Studies with sheep and cattle have shown that fiber digestion is positively affected by Ca supplementation (Burroughs *et al*., 1950d; Swift *et al*., 1950). In fact, Hubbert *et al.* (1958) noted the addition of 50 to 300 µg of dietary Ca (from $CaCl_2$) per milliliter of fermentation medium appeared to result in a consistent increase of approximately ten percentage units in cellulose digestion. It has been hypothesized that Ca may improve ruminal fiber digestion by serving as a bridge between bacteria and plant cell walls, both of which tend to be negatively charged (Lopez-Guisa and Satter, 1992). Calcium may also improve fiber digestibility by alleviating the antimicrobial effects of fatty acids on ruminal fermentation via formation of Ca soaps (Galbraith *et al.*, 1971).

Calcium probably needs to be present in a soluble form in the ruminal environment in order to meet the Ca requirement of ruminal microorganisms. The amount of ruminal soluble Ca will depend on solubility of Ca present in feedstuffs as well as supplemental sources of Ca. However, little is known regarding the effect of different sources of Ca on ruminal fermentation and ruminal soluble Ca levels. Spears *et al.* (2002) noted that when supplemental Ca was supplied from Ca propionate, ruminal soluble Ca increased greatly with increased dietary Ca in steers fed a corn-cottonseed hull-based diet. However, increasing dietary Ca from $CaCO_3$ had little effect on ruminal soluble Ca concentrations (Spears *et al.*, 2002). Kegley *et al.* (1991) noted that ruminal soluble Ca concentrations only increased in steers by 11% when dietary Ca was increased from 0.35 to 0.71% as a result of adding limestone to a corn silage diet. Therefore, Ca sources such as $CaCO_3$ that are poorly soluble at lower ruminal pH may be less effective in meeting the microbial Ca requirement. Thus,

the present study was conducted to determine the effect of Ca level and source (Ca propionate vs $CaCO_3$) and form of Ca propionate (powder vs prilled) on ruminal fermentation in continuous cultures of ruminal microorganisms.

Materials and Methods

Fermentor Conditions

Whole ruminal contents were obtained from a nonlactating, ruminally-fistulated Holstein cow fed a predominantly forage diet. Animal handling procedures and surgery protocol for the cannulated cow were approved by The North Carolina State University Institutional Animal Care and Use Committee (Approval No. 02-045). Approximately 4.0 L of whole rumen contents were collected 2-h postprandially and transported to the laboratory in preheated vacuum containers. Prior to inoculation of fermentors, the ruminal contents were strained through double-layered cheesecloth. Approximately 700 mL of the filtered ruminal fluid was placed into each of four 1 L culture vessels designed to allow independent flow of liquid and particulate matter (Teather and Sauer, 1988). Anaerobic conditions in the cultures were maintained by providing a constant flow of CO_2 (20 mL/min) in order to preserve positive internal pressure. Artificial saliva was prepared as described by Slyter *et al*. (1966) and delivered to culture vessels via a precision pump at a flow rate of 0.73 mL/min. During a 24 h period, 1.1 L of artificial saliva was delivered to each vessel to yield a liquid fractional dilution rate of 6.3%/h. The temperature of each fermentor was maintained at 39˚C by a circulating water bath, and ruminal contents were continually stirred throughout the experiment at 10 to 15 rpm with the assistance of a central paddle (Fellner *et al*., 1995).

Dietary Treatments

Dietary treatments consisted of: 1) control (no supplemental Ca); 2) 0.60% supplemental Ca from $CaCO_3$; 3) 0.60% supplemental Ca from prilled Ca propionate (Nutrocal, Kemin Americas) and 4) 0.6% supplemental Ca from Ca propionate powder. Ingredient composition of the diets is shown in Table 1. A vitamin-premix was also incorporated into each diet at 0.12% Ca (DM basis). The control diet was formulated to meet or exceed requirements for lactating dairy cattle as determined by NRC (2001) with the exception of Ca, which contained approximately half of the suggested Ca requirement. All three Ca sources replaced cottonseed hulls in amounts to result in 0.60% of added Ca. Total dietary crude protein was formulated to be 16.7%.

Corn silage was processed in a food chopper (approximately 1 cm in length; model FC 19, G.S. Blakeslee & Co., Cicero, IL). All dietary ingredients were weighed and gradually added to the chopper. Before their gradual addition to the chopper, the vitamin-mineral premix and Ca sources were pre-weighed and mixed with the concentrate. All dietary components were allowed to mix thoroughly for 15-min before being placed in bags and stored in the refrigerator for immediate use in a freezer or at (-20˚C) for use during later replications. The first three replications took place from May 14 through 22, 2003 using fresh corn silage stored in the refrigerator. However, the last two runs were not conducted until August 13 through 21, 2003, therefore, corn silage was stored at (-20˚C) until needed.

Dietary treatments were fed for 48-h during the adaptation period followed by a 4-d period of data collection. Experimental diets (14 g DM basis) were added to ruminal fermentation vessels in two equal portions at 0800 and 1700-h.

The procedure was repeated five times in order to incorporate four continuous culture fermentors on five separate 6-d runs (2-d adjustment period preceding a 4-d collection period) in the experimental design. The first three replications provided information concerning Ca effects on ruminal fermentation and the last two runs were to examine rate of solubility of the different Ca sources.

Sample Collection and Analytical Procedures

During the sampling periods of runs one through five, approximately 5 mL of thoroughly mixed culture contents were collected 2-h after the first feeding of the day, centrifuged and the supernatant was analyzed for VFA by GLC (model CP-3380; Varian, Walnut Creek, CA). Five to ten milliliters of thoroughly mixed culture contents were collected at this time, centrifuged at 28,000 *g* x 30-min, and analyzed for soluble Ca concentration. Measurements for pH were made throughout the day using pH probes (model 912600; Orion Research, Inc., Beverly, MA). Ten microliters of headspace gas samples from the fermentor were drawn into a gas tight syringe (Hamilton Co., Reno, NV) throughout the day and analyzed for methane (CH_4) using GLC. Daily methane output (nmol/ml) was calculated as reported earlier (Jenkins *et al.*, 2002) using the following equation: methane concentration in fermentor headspace (mmol/mL) x CO_2 gas flow through the fermentor headspace (20 mL/min) x 60-min x 24-h. Separate 5 mL samples of the mixed culture contents were taken on the last day of each period at 2-h after the morning feeding and frozen (-74˚C) for long chain fatty acids (LCFA) analysis. The frozen samples were thawed, methylated, and then analyzed for LCFA by GLC (Kramer *et al.*, 1997). Feed and fermentor samples were prepared for mineral analysis using a microwave digestion (Mars 5™, CEM Corp., Matthews, NC) procedure described by Gengelbach *et al.* (1994). Soluble Ca content

in feed and ruminal contents was determined by atomic absorption spectrophotometry (AA-6701F, Shimadzu Scientific Instruments, Kyoto, Japan).

Throughout the experiment, effluent containers were emptied daily. On the last day of each period, effluent containers were drained and approximately 50 mL of the particulate matter were obtained and frozen for subsequent analysis. Frozen samples were thawed, centrifuged at 1,300 x *g* for 10-min, and analyzed for DM and NDF. Samples were analyzed for DM as described by AOAC (1999). Concentrations of NDF in ruminal culture samples were determined using an Ankom 200 fiber extractor (Ankom Technologies, Fairport, NY) according to the method of Van Soest *et al.* (1991).

During the last two runs, rate of solubility of Ca was measured. During the second to last day of the sampling period, approximately 10 mL samples were taken from fermentor contents at 0 hr (prior to morning feeding), 0.5 hr, 1 hr, 2 hrs, 4 hrs, and 6 hrs (prior to evening feeding). Thoroughly mixed culture contents were collected, centrifuged at 28,000 g x 30-min, and analyzed for soluble Ca concentration. Fermentor samples were prepared for mineral analysis using a microwave digestion (Mars 5™, CEM Corp., Matthews, NC) procedure described by Gengelbach *et al.* (1994). Soluble Ca content of ruminal contents was determined by atomic absorption spectrophotometry (AA-6701F, Shimadzu Scientific Instruments, Kyoto, Japan).

Statistical Analysis

Data were analyzed according to a randomized complete block design with repeated measures using the PROC GLM procedures of SAS (SAS Inst., Inc., Cary, NC, 1999-2001). The model for soluble Ca, NDF digestibility and VFA included the effect of treatment and the random effect of run. Preplanned comparisons were assessed in order to detect

differences among treatments. Contrasts evaluated included 1) control vs Ca carbonate, 2) control vs Ca, and 3) Ca propionate (prilled) vs Ca propionate (powder).

Results and Discussion

Based on actual Ca analysis of individual ingredients, total Ca content in the control diet was 0.18% of DM. Calcium concentration of $CaCO_3$, Ca propionate prilled, and Ca propionate powder was 38%, 21% and 20% (DM basis), respectively. Based on 14 g of total DM fed daily, the amounts of $CaCO_3$ and Ca propionate (prilled and powder) added daily to respective cultures were 0.22 g, 0.41 g, and 0.42 g, respectively. This maintained the amount of added Ca at 0.61% and total dietary Ca at 0.79% of DM.

As expected, supplemental dietary Ca resulted in greater ($P < 0.01$) soluble Ca concentrations in ruminal cultures compared to control (Table 2). Control cultures averaged 9.8 mg/L soluble Ca which was lower than 16.0 mg/L and 19.1 mg/L for $CaCO_3$ and Ca propionate treatments, respectively. Soluble Ca concentrations were greater ($P = 0.02$) in cultures supplemented with Ca propionate compared with those supplemented with $CaCO_3$. Spears *et al.* (2002) observed that when supplemental Ca was supplied from Ca propionate, ruminal soluble Ca increased greatly with increased dietary Ca. However, increasing dietary Ca from 0.25 to 0.75% as $CaCO_3$ resulted in minor effects on ruminal soluble Ca concentrations (Spears *et al.*, 2002). Kegley *et al.* (1991) also noted that ruminal soluble Ca concentrations in steers only increased by 11% when dietary Ca was increased from 0.35 to 0.71% by the addition of limestone to a corn silage diet. This may be due to the solubility of the supplemental Ca source. The amount of ruminal soluble Ca will depend on the solubility of Ca present in feedstuffs as well as supplemental sources of Ca. In order to meet the Ca requirement of ruminal microorganisms, Ca probably needs to be present in a soluble form in

the ruminal environment. Therefore, Ca sources such as $CaCO_3$ that are poorly soluble at ruminal pH may be less effective in meeting the microbial Ca requirement. Ruminal soluble Ca concentrations were greater ($P = 0.04$) for the prilled form than the powdered (20.8 mg/L and 17.4 mg/L, respectively) form of Ca propionate. According to preliminary data, this phenomenon may be related to a potential difference in rate of solubility of the two physical forms of Ca propionate (Fig. 1). For instance, the powdered form of Ca propionate may actually becoming soluble at a faster rate than the prilled form allowing it to exit the cultures into the overflow more quickly.

Ruminal pH ranged between 5.64 and 5.87 and was lower in cultures that received supplemental Ca ($P < 0.01$; Table 2). This could be due to increased rate of fermentation as a result of Ca supplementation. In fact, experiments with cattle and sheep have shown that cellulose digestion may be increased by the addition of Ca to ruminant diets (Burroughs *et al.*, 1950d). Burroughs *et al.* (1950d) did not analyze for VFA production, but the addition of Ca to ruminal cultures low in Ca would be expected to increase microbial fermentation resulting in increased production of VFAs and a subsequent decrease in pH. In the present study, Ca supplementation resulted in an increase ($P < 0.01$) in total VFA production and a decrease in ruminal pH ($P < 0.01$) (Tables 2 and 3).

Methane concentration was not affected by dietary Ca (Table 2). However, methane concentration tended ($P = 0.07$) to be higher in cultures given $CaCO_3$ than in those receiving Ca propionate. Neutral detergent fiber digestibility was not affected by Ca supplementation compared with the control ($P = 0.47$). These results were unexpected in that Hubbert *et al*. (1958) noted that the addition of 50 to 300 μg of Ca (from $CaCl_2$) per mL of fermentation medium appeared to result in a consistent increase of approximately ten percentage units in

cellulose digestion. There was also no difference between $CaCO_3$ and the average effect of both prilled and powdered Ca propionate treatments ($P = 0.47$). However, NDF digestion was higher ($P = 0.03$) for cultures supplemented with prilled Ca propionate compared with cultures receiving powdered Ca propionate. The higher NDF digestion in cultures supplemented with prilled Ca propionate may relate to the higher ruminal soluble Ca concentrations noted in this treatment.

When compared to the control, Ca supplemented as either $CaCO_3$ or Ca propionate ($P < 0.01$) increased total VFA production in ruminal cultures (Table 3). However, the addition of Ca propionate prompted greater ($P < 0.01$) total VFA production as compared with the supplementation of $CaCO_3$. When Ca propionate was provided in the prilled form, total VFA production tended to be greater ($P < 0.07$) compared to that of the powdered form. The greater total VFA production in cultures supplemented with Ca compared to the control was due largely to an increase in ruminal butyrate ($P < 0.01$) and propionate ($P < 0.01$) as well as a tendency for higher ($P = 0.10$) acetate concentration as well. The greater total VFA production in cultures receiving Ca propionate was due primarily to an increase in ruminal propionate ($P < 0.01$). The quantity of Ca propionate added to ruminal cultures provided 4.4 mM of propionate per day. Therefore, the increased propionate production in ruminal cultures supplemented with Ca propionate can be largely accounted for by the propionate added to cultures. Prilled Ca propionate also resulted in greater production of ruminal butyrate ($P < 0.01$), and the isoacids, isovalerate and isobutyrate, ($P < 0.01$) when compared to the powdered form.

Molar percentages of acetate were significantly decreased with the addition of Ca ($P < 0.01$) (Table 4). The proportion of acetate was also lower ($P < 0.01$) in cultures

supplemented with Ca propionate compared with $CaCO_3$ (Table 4). Molar percentages of propionate were significantly increased with the addition of Ca ($P < 0.01$). Supplemental Ca propionate resulted in greater proportions of propionate ($P < 0.01$) compared to those associated with added $CaCO_3$. Thus, the increased ruminal propionate in cultures receiving Ca propionate lowered ($P < 0.01$) the molar ratio of acetate and increased ($P < 0.01$) the molar ratio of propionate (Table 4). In agreement with the present study, Spears *et al.* (2002) noted the addition of Ca propionate also prompted an increase in the molar proportion of ruminal propionate and a decrease in the molar proportion of ruminal acetate in steers. Once again, the dietary contribution from Ca propionate is able to explain the decrease in acetate and increase in propionate molar proportions noted in cultures supplemented with Ca propionate. Prilled Ca propionate was also associated with a lower ($P = 0.10$) proportion of ruminal acetate compared with the powder. The molar percentage of butyrate was shown to increase ($P < 0.01$) with the addition of $CaCO_3$ compared to Ca propionate. However, a form effect of Ca propionate on the proportion of butyrate was evident with the addition of prilled Ca propionate resulting in a greater ($P = 0.03$) molar percentage than the powder. Molar proportions of the isoacids, isobutyrate and isovalerate, were significantly higher ($P < 0.01$) in the control cultures compared with cultures that received Ca supplementation (Table 4). Specifically, those cultures supplemented with $CaCO_3$ showed greater ($P < 0.01$) isoacid molar percentages than those supplemented with Ca propionate (Table 4). However, the physical form of Ca propionate had an effect on the proportions of isoacids with prilled Ca propionate resulting in much greater ($P < 0.01$) concentrations than the powder.

Calcium supplementation did not affect C16:0, C18:0, C18:2, CLA *cis*9, *trans*11 or CLA *trans*10, *cis*12 isomers (Table 5). However, the proportion of C18:1 *trans* isomers (P =

0.03) as well as the total proportion of C18:1 isomers (P = 0.04) significantly increased as a result of supplementing Ca. Thus, the increase in total C18:1 in cultures receiving Ca was due to an increase (P = 0.03) in C18:1 *trans* isomers. This indicates that the addition of Ca seemed to enhance the rate of ruminal biohydrogenation by converting a greater proportion of dietary C18:2 to C18:1 and C18:0. Calcium carbonate increased (P < 0.01) C18:0, and proportions of (P < 0.01) C18:1 *cis* and (P = 0.04) C18:2 were reduced compared to Ca propionate addition. This indicates that the addition of $CaCO_3$ enhanced the rate of ruminal biohydrogenation by converting a greater proportion of dietary C18:2 to C18:0. Consequently, this can be explained since $CaCO_3$ resulted in a higher culture pH relative to Ca propionate. Thus, higher ruminal pH is more conducive to lipolysis and biohydrogenation.

The question still remains that the Ca treatments affected biohydrogenation; however, the effect was not the same. Not all microbes are capable of biohydrogenating unsaturated fatty acids to completeness. It is possible that the Ca treatments caused a shift in the microbial species associated with the completeness of biohydrogenation. Microorganisms capable of fatty acid hydrogenation in the rumen are often divided into Groups A and B based on their end-products and patterns of isomerization during biohydrogenation (Harfoot and Hazlewood, 1988). Bacterial species in Group A hydrogenate linoleic acid to trans C18:1 but appear incapable of hydrogenating monoenes. Group B bacteria can hydrogenate a wide range of monenes, including trans-11 C18:1, to stearic acid.

Conclusions

Supplemental dietary Ca resulted in increased soluble Ca concentrations in ruminal cultures. However, soluble Ca concentrations were greater in cultures supplemented with Ca

propionate compared with those supplemented with $CaCO_3$. Ruminal soluble Ca concentrations were also greater for the prilled form than the powdered form of Ca propionate. Despite improvements in ruminal soluble Ca, NDF digestibility was not affected by Ca supplementation compared with control. There was also no difference between $CaCO_3$ and the average effect of both prilled and powdered Ca propionate treatments; however, NDF digestion was greater for the prilled form than the powdered form of Ca propionate. The higher NDF digestion in cultures supplemented with prilled Ca propionate may be related to the higher ruminal soluble Ca concentrations noted in this treatment. Thus, the amount of ruminal soluble Ca will depend on the solubility of Ca present in feedstuffs as well as supplemental sources of Ca. In order to meet the Ca requirement of ruminal microorganisms, Ca probably needs to be present in a soluble form in the ruminal environment. Therefore, Ca sources such as $CaCO_3$ that are poorly soluble at lower ruminal pH may be less effective in meeting the microbial Ca requirement.

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Table 1. Ingredient composition of diets fed to continuous culture vessels.

Ingredient	Control	$CaCO_3$	Ca Propionate	
			Prilled	Powder
	DM %			
Corn silage	35	35	35	35
Soybean Meal	18	18	18	18
Corn, ground	15	15	15	15
Cottonseed Hulls	22	20.42	19.1	19
Whole Cottonseed	10	10	10	10
Mineral Supplement	0.12	0.12	0.12	0.12
Ca Carbonate	---	1.58	---	---
Ca Propionate, Prilled	---	---	2.9	---
Ca Propionate, Powder	---	---	---	3.0

Table 2. Ruminal pH, methane output, and NDF digestibility in continuous culture vessels receiving calcium carbonate or calcium propionate.[1]

	Treatments					*P* values		
						Contrasts		
			Ca Propionate					
Item	Con	$CaCO_3$	Prilled	Powder	SE	Con vs. Ca	$CaCO_3$ vs Ca Prop	Prilled vs Powder
Soluble Ca, mg/L	9.8	16.0	20.8	17.4	1.10	<0.01	0.02	0.04
pH	5.87	5.75	5.67	5.64	0.03	<0.01	<0.01	0.37
Methane, nmol/ml	1276.61	1373.09	1264.27	1245.30	52.59	0.77	0.07	0.80
NDF, digest. %	73.85	74.62	82.41	72.29	2.99	0.47	0.47	0.03

[1]n=3 for soluble Ca and n=5 for culture pH, methane and NDF digestibility.

Table 3. Production of volatile fatty acids (VFA) in continuous culture vessels receiving calcium carbonate or calcium propionate (n=5).

	Treatments					*P* values		
						Contrasts		
			Ca Propionate					
Item	Con	$CaCO_3$	Prilled	Powder	SE	Con vs. Ca	$CaCO_3$ vs Ca Prop	Prilled vs Powder
	_____ mmol/d _____							
Total VFA	56.20	59.53	64.57	61.76	1.10	<0.01	<0.01	0.08
Individual VFA								
Acetate	29.25	30.58	30.56	30.17	0.61	0.10	0.78	0.65
Propionate	12.24	12.32	17.19	17.23	0.48	<0.01	<0.01	0.96
Butyrate	10.31	12.10	11.98	10.37	0.32	<0.01	0.02	<0.01
Isobutyrate	0.59	0.60	0.65	0.50	0.02	0.78	0.25	<0.01
Valerate	1.05	1.18	1.23	1.24	0.08	0.05	0.56	0.96
Isovalerate	2.77	2.75	2.96	2.25	0.09	0.27	0.19	<0.01

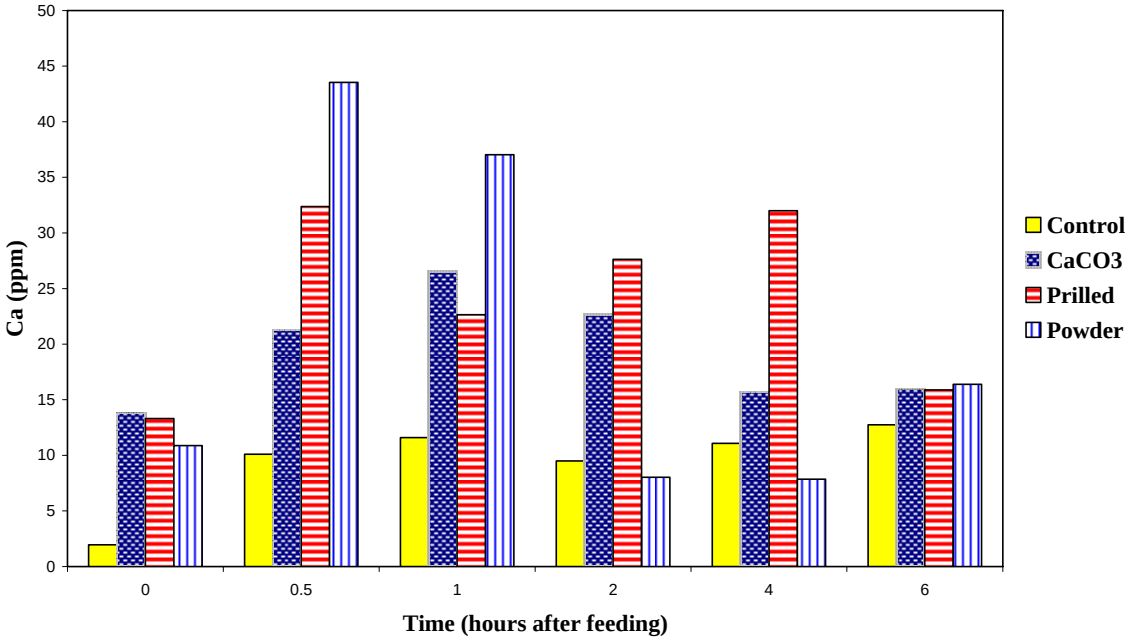
Table 4. Molar percentages of volatile fatty acids in continuous culture vessels receiving calcium carbonate or calcium propionate (n=5).

	Treatments					*P* values		
			Ca Propionate			Contrasts		
Item	Con	$CaCO_3$	Prilled	Powder	SE	Con vs. Ca	$CaCO_3$ vs Ca Prop	Prilled vs Powder
	_____ mol % _____							
Acetate	52.01	51.40	47.34	48.77	0.35	<0.01	<0.01	0.10
Propionate	21.79	20.68	26.65	27.68	0.48	<0.01	<0.01	0.13
Butyrate	18.36	20.32	18.53	17.05	0.48	0.62	<0.01	0.03
Isobutyrate	1.05	1.01	1.00	0.82	0.03	<0.01	<0.01	<0.01
Valerate	1.86	1.98	1.91	2.05	0.12	0.41	0.95	0.43
Isovalerate	4.93	4.61	4.58	3.67	0.13	<0.01	<0.01	<0.01
A:P	2.40	2.50	1.79	1.78	0.04	<0.01	<0.01	0.95

Table 5. Fatty acid profile in continuous culture vessels receiving calcium carbonate or calcium propionate (n=5).

	Treatments					*P* values		
						Contrasts		
			Ca Propionate					
Item	Con	$CaCO_3$	Prilled	Powder	SE	Con vs. Ca	$CaCO_3$ vs Ca Prop	Prilled vs Powder
	_____ % of total _____							
C16:0	21.57	21.18	21.44	21.37	0.45	0.66	0.71	0.92
C18:0	15.15	18.93	14.91	11.99	1.29	0.93	<0.01	0.13
C18:1, Total	34.69	38.27	40.35	39.34	1.75	0.04	0.51	0.69
C18:1 *trans*	20.27	26.67	25.62	23.67	1.78	0.03	0.40	0.45
C18:1 *cis*	14.43	11.60	14.73	15.69	0.67	0.59	<0.01	0.34
C18:2	19.90	12.09	16.08	20.01	1.99	0.12	0.04	0.18
CLA *cis*9, *tran*11	0.22	0.18	0.31	0.32	0.08	0.55	0.19	0.99
CLA *trans*10, *cis*12	0.11	0.09	0.29	0.17	0.07	0.36	0.12	0.21

Fig. 1 Soluble Ca in rumen cultures over time.



RUNNING HEAD: Calcium Effects on Ruminal Soluble Calcium and Ruminal Fermentation

Effect of Calcium Source and Level on Ruminal Soluble Calcium and Ruminal Fermentation in Growing Angus Steers[1]

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Introduction

Ruminal microbial digestion of fiber has been shown to be improved with the addition of Ca (Burroughs *et al.*, 1950d). In 1958, Hubbert *et al.* speculated that ruminal microorganisms have a requirement for Ca due to a consistent increase in cellulose digestion as a result of adding 50 to 300 μg of dietary Ca (from $CaCl_2$) per milliliter of fermentation medium. However, Ca needs to be present in a soluble form in the ruminal environment in order to meet the Ca requirement of ruminal microorganisms. The solubility of Ca present in feedstuffs as well as supplemental sources of Ca will determine the amount of ruminal soluble Ca. It has been hypothesized that Ca may function as a bridge between bacteria and plant cell walls, both of which tend to be negatively charged (Lopez-Guisa and Satter, 1992). Calcium may also improve fiber digestibility by alleviating the antimicrobial effects of fatty acids on ruminal fermentation via formation of Ca soaps (Galbraith *et al.*, 1971). However, little is known regarding the effect of different sources of Ca on ruminal fermentation and ruminal soluble Ca levels. Spears *et al.* (2002) found that supplemental Ca supplied from Ca propionate caused ruminal soluble Ca concentrations to increase greatly with increased dietary Ca in steers fed a corn-cottonseed hull-based diet. However, ruminal soluble Ca concentrations were unaffected due to increasing dietary Ca from $CaCO_3$ (Spears *et al.*, 2002). Kegley *et al.* (1991) noted that ruminal soluble Ca concentrations only increased in steers by 11% when dietary Ca was increased from 0.35 to 0.71% as a result of adding limestone to a corn silage diet. Therefore, Ca sources such as $CaCO_3$ that are poorly soluble at lower ruminal pH may be less effective in meeting the microbial Ca requirement. Thus, the present study was conducted to determine the effect of Ca level and source (Ca propionate vs $CaCO_3$) on ruminal fermentation in Angus steers.

Materials and Methods

Experimental Design

Sixteen Angus steers initially averaging 274 kg (trial 1) or 252 kg (trial 2) were divided by weight and randomly allotted to three treatments. The North Carolina State University Institutional Animal Care and Use Committee approved care and handling of the animals and sampling procedures. In order to accommodate moving animals into metabolism crates, the study was conducted over two trial periods. Eight steers were randomly assigned to one of the following treatments in each trial: 1) control (no supplemental Ca), 2) $CaCO_3$ and 3) Ca propionate (prilled). Trial 1 was conducted from September 15 to October 14, 2003; trial 2 was conducted from October 6 to November 4, 2003.

Ingredient composition of diets is shown in Table 1. Each steer was supplemented with 1.36 kg of a corn-corn gluten feed supplement per day. The supplement provided 14.8 grams of Ca per day for steers in treatments two and three. A low Ca chopped orchardgrass hay (Table 2) containing 0.2% Ca was also fed to all steers based on what each animal would consume in a 24-h period.

Each trial was preceded by a 14-d adjustment period. During this time, steers were housed in covered pens and individually fed once per day using electronic Calan gate feeders (American Calan, Northwood, NH). Following the 14-d adjustment period, steers were placed in metabolism crates in order to obtain a total collection of urine and feces. Steers were acclimated to the metabolism crates for 5-d followed by a collection period of 5-d in order to obtain a total collection of urine and feces. The steers' feed intake in the metabolism crates were equalized across treatments. Urine preserved with 150 ml of 6 N HCl was

measured daily and a 1% aliquot was retained and composited over the 5-day collection period. Total fecal ouput was measured daily, mixed, and a 10% aliquot was retained and composited across days. Samples of feed and feces obtained during the collection periods were dried at 55˚C and subsequently ground in a Wiley mill to pass through a 2 mm screen.

Jugular blood samples were obtained on days 0, 14, and 28 of the experimental period for determination of plasma Ca concentration. Blood samples were centrifuged at 2500 rpm for 20 min at 19˚C and plasma was frozen at -20˚C until analyses were conducted. Ruminal fluid samples (150 to 200 ml) were obtained via stomach tube at the conclusion of the collection period to evaluate ruminal volatile fatty acids and ruminal soluble Ca concentrations. Blood and ruminal fluid were collected 2-h postfeeding. Ruminal fluid was strained through four layers of cheesecloth, then centrifuged at 17,000 rpm for 20 min at 4˚C. The supernatant was collected and used for Ca and VFA determination. A sample of strained ruminal fluid was acidified with 25% *meta*-phosphoric acid, centrifuged and the supernatant fluid was stored (-20˚C) for VFA analysis.

Analytical Procedures

Feed, fecal and urine samples were prepared for Ca determination by wet ashing with nitric acid and hydrogen peroxide using a microwave digestion (Mars™, CEM, Matthews, NC) procedure described by Gengelbach et al. (1994). Plasma and ruminal fluid supernatant were diluted with 5% nitric acid and centrifuged at 2500 rpm for 20 min prior to Ca determination. Calcium was measured by flame atomic absorption spectrophotometry (Model AA-6701 F, Shimadzu, Kyoto, Japan). Ruminal VFA were measured by gas liquid chromatography (Model 3380, Varian, Walnut Creek, CA) using Nikol™ fused silica column, 30 m x 0.25 mm x 0.25 µm. Feed and fecal samples were analyzed for NDF using

an Ankom 200 fiber extractor (Ankom Technologies, Fairport, NY) according to the method of Van Soest et al. (1991).

Statistical Analysis

Data from the two trials were combined and analyzed statistically using the GLM procedure of SAS (1985). The model included treatment, trial, and trial x treatment. Differences among treatment means were determined using single degree of freedom contrast. Comparisons made included: 1) control vs Ca supplemented treatment and 2) $CaCO_3$ vs. Ca propionate.

Results and Discussion

Total DM intake averaged 4.94 kg and 4.16 kg per day for trials one and two, respectively (Table 3). Therefore, steers in trial one consumed 1.8% of their body weight while steers in trial two consumed 1.7% of their body weight. Ca supplementation increased ($P = 0.03$) ruminal soluble Ca concentrations; however, concentrations were much higher ($P < 0.01$) in steers supplemented with Ca propionate compared to $CaCO_3$ (Table 4). Previously, when supplemental Ca was supplied from Ca propionate, ruminal soluble Ca also increased with increased dietary Ca (Spears *et al.*, 2002). However, increasing dietary Ca from $CaCO_3$ had little effect on ruminal soluble Ca concentrations (Spears *et al.*, 2002). Also in agreement with the present study, ruminal soluble Ca concentrations in steers only increased by 11% when dietary Ca was increased from 0.35 to 0.71% by the addition of limestone to a corn silage diet (Kegley *et al.*, 1991).

Dry matter intake and digestibility during the digestion period were both similar across treatments (Table 4). Steers consumed on average 5.50 kg DM per day including 4.14 kg hay (DM basis) and 1.36 kg supplement (DM basis) per day. All three treatments yielded

DM intakes of approximately 2.1% of the average steer body weight. NDF intake and digestibility were also not affected by Ca level or source. In contrast to these results, Spears *et al.* (2002) reported that steers supplemented with Ca propionate had higher NDF digestion then those supplemented $CaCO_3$. The lack of a significant increase in digestion in the present study as a result of Ca supplementation may be explained in that adequate amounts of soluble Ca may have existed in the supplement and orchardgrass hay to meet the microbes' Ca requirement. In the previous study, addition of $CaCO_3$ to a corn-cottonseed hull-based diet, low in Ca, to increase dietary Ca to 0.25 or 0.75% resulted in ruminal soluble Ca concentrations of only 5.8 and 7.2 mg/L, respectively (Spears *et al.*, 2002). However, in the present study, steers fed the control supplement and orchardgrass hay had ruminal soluble Ca concentrations that were approximately six times higher. Most of the Ca in the control treatment was derived from the orchardgrass hay. This suggests that the Ca present in the hay was fairly soluble in the ruminal environment. Steers fed greenchop forage containing 0.35 to 0.60% Ca (DM basis) had ruminal soluble Ca concentrations ranging from 71 to 115 mg/L (Spears *et al.*, 1989). Studies in cattle fed corn silage based diets also have indicated fairly high ruminal soluble Ca concentrations (Kegley *et al.*, 1991). This suggests that Ca present in forages contributes greatly to ruminal soluble Ca concentrations.

Previous work has shown that the addition of Ca results in increased fiber digestion. In fact, experiments with ruminants have shown that fiber digestion can be favorably altered by Ca supplementation. Hubbert *et al.* (1958) noted that the addition of 50 to 300 μg of dietary Ca (from $CaCl_2$) per milliliter of fermentation medium appeared to result in a consistent increase of approximately ten percentage units in cellulose digestion. In addition to directly contributing to the Ca requirement of the bacteria, Ca may function as a bridge

between negatively charged bacteria and plant cell walls encouraging increased fiber digestion (Lopez-Guisa and Satter, 1992). However, in a present study, it can be postulated that if adequate ruminal soluble Ca concentrations existed in steers fed the control diet to maximize ruminal digestion, increasing ruminal soluble Ca would not be expected to improve ruminal digestion. Therefore, microbial digestion does not increase as a result of adding supplemental Ca.

Plasma Ca concentrations were not affected ($P > 0.15$) by Ca supplementation (Table 5).

Total VFA were not affected by Ca level or source (Table 6; $P > 0.21$)). However, the addition of Ca propionate decreased ($P < 0.01$) molar percentages of acetate due to the increase in propionate being fed. In fact, the proportion of acetate ($P < 0.01$) was lower with supplemented Ca propionate compared with $CaCO_3$ (Table 5). In agreement with the present study, Spears *et al.* (2002) determined that the lower molar proportion of acetate was associated with steers supplemented with Ca propionate compared to those given $CaCO_3$. The addition of Ca significantly increased ($P < 0.01$) the molar percentages of propionate. Supplemental Ca propionate resulted in much greater proportions of propionate ($P < 0.01$) compared to $CaCO_3$ addition. Steers supplemented with Ca propionate had a lower ($P < 0.01$) acetate:propionate ratio than those supplemented with $CaCO_3$. In agreement with the present study, Spears *et al.* (2002) observed that steers fed Ca propionate had a lower ruminal acetate:propionate ratio than those receiving supplemental Ca as $CaCO_3$. The dietary contribution from supplemented Ca propionate may be able to explain the decrease in acetate and increase in propionate molar proportions observed in steers supplemented with Ca propionate.

Molar proportions of butyrate, valerate, and the isoacids, isobutyrate and isovalerate, were not affected ($P > 0.10$) by the addition of Ca (Table 5). In ruminal microorganisms grown in continuous flow culture fermentors, Ca addition to a control diet containing 0.18% Ca also did not affect molar proportions of butyrate and valerate; however, molar proportions of the isoacids were reduced (Baird *et al.*, 2004).

Conclusions

Ca supplementation increased ruminal soluble Ca concentrations; however, concentrations were much higher in steers supplemented with Ca propionate compared to $CaCO_3$. Regardless of improvements in ruminal soluble Ca, NDF intake and digestibility were not affected by Ca supplementation compared with control. However, the addition of Ca propionate also did not elicit an increase in DM or fiber digestion. This may be due to adequate amounts of soluble Ca existing in the supplement and orchardgrass hay to meet the microbes' Ca requirement. Thus, if adequate ruminal soluble Ca concentrations existed in steers fed the control diet to maximize ruminal digestion, increasing ruminal soluble Ca would not be expected to improve ruminal digestion. Therefore, microbial digestion does not increase as a result of adding supplemental Ca above and beyond NRC levels.

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Table 1. Ingredient composition of diets fed to steers.

Ingredient	Control	$CaCO_3$	Ca Propionate
	DM %		
Corn gluten feed	66.7	66.7	66.7
Corn, ground	33.0	30.4	27.7
Vitamin premix	0.087	0.087	0.087
Trace Mineral premix	0.22	0.22	0.04
$CaCO_3$	--	2.64	--
Ca Propionate	--	--	5.5

Table 2. Chemical composition of orchardgrass hay.

DM, %	95.63
Crude Protein, % DM	6.39
NDF, % DM	86.05
Ca, % DM	0.20

Table 3. Effect of calcium level and source on ruminal soluble Ca concentration and DM and NDF digestion and intake.

	Treatment				Contrasts (*P* value)	
	Control	$CaCO_3$	CaProp	SE	Control vs. Ca	$CaCO_3$ vs. CaProp
Rumen soluble Ca, ppm	33.38	33.01	63.07	3.83	0.03	<0.01
DM intake, kg/d	5.45	5.49	5.56	0.09	0.54	0.56
DM digestion, %	60.44	58.74	60.19	0.94	0.47	0.26
NDF intake, kg/d	3.61	3.80	3.69	0.26	0.70	0.75
NDF digestion, %	56.36	55.16	56.10	3.77	0.89	0.85

Table 4. Effect of calcium source on plasma calcium concentrations.

	Treatment				Contrasts (*P* value)	
	Control	$CaCO_3$	CaProp	SE	Control vs. Ca	$CaCO_3$ vs. CaProp
Day 0	9.98	9.94	10.14	0.28	0.88	0.59
Day 14	9.52	9.99	9.94	0.36	0.40	0.93
Day 28	9.59	9.87	10.08	0.18	0.16	0.39

Table 5. Molar percentages of volatile fatty acids in steers receiving calcium carbonate or calcium propionate.

	Treatment				Contrasts (*P* value)	
	Control	$CaCO_3$	CaProp	SE	Control vs. Ca	$CaCO_3$ vs. CaProp
Total VFA, mM	84.56	80.72	75.50	3.71	0.22	0.31
A:P	3.38	3.32	2.20	0.04	<0.01	<0.01
Acetic, mol/100 mol	68.20	67.39	60.99	0.35	<0.01	<0.01
Propionic, mol/100 mol	20.36	20.29	27.75	0.33	<0.01	<0.01
Isobutyric, mol/100 mol	0.87	1.01	0.95	0.07	0.22	0.46
Butyric, mol/100 mol	8.16	8.51	7.57	0.44	0.84	0.14
Isovaleric, mol/100 mol	1.38	1.50	1.51	0.07	0.22	0.90
Valeric, mol/100 mol	1.15	1.27	1.23	0.04	0.10	0.55