Inclusion of Alfalfa Hay in Diets for Non-lactating Dairy Cows During the Prepartum Period

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ABSTRACT

The study objectives were to determine the dry matter intake, urine pH, Ca concentration in blood, Ca output in urine, and incidence of hypocalcemia from pregnant, non-lactating dairy cows during the prepartum period consuming diets containing either grass hay (GH) or alfalfa hay (AH) with the inclusion of either calcium chloride (CL) or polyhalite mineral (PO). Eighty Holstein cows in their 2^{nd} parity or greater were fed an experimental diet according to a 2×2 factorial arrangement of treatments during the prepartum period (21 d before calving). All diets had a dietary cation-anion difference (DCAD) below -190 mEq/kg/DM. Grass hay contained 7.5% CP, 74.9% NDF, 0.36% Ca, 0.02% Na, 1.88% K, 0.38% Cl, and 0.15% S. Alfalfa hay contained 19.6% CP, 45.6% NDF, 1.52% Ca, 0.16% Na, 2.5% K, 0.77% Cl, and 0.32% S. Cows consuming grass hay tended to consume more dry matter than cows consuming alfalfa hay (11.6 vs 10.8 kg/d), but dry matter intake (DMI) was not affected by the acidogenic products. Urine pH decreased below 6.5 for all diets and was greatest for cows consuming the GHPO diet. The concentration of calcium in plasma decreased significantly ($P < 0.01$) around calving but neither the hay type ($P=0.86$) nor the acidogenic product ($P =0.81$) affected it. Urinary calcium output was less for cows consuming the GHPO diet. Cows consuming diets containing alfalfa hay had a greater incidence of normocalcemia (37 and 40% for AHCL and AHPO, respectively) than cows consuming diets containing grass hay (20 and 25% for GHCL and GHPO, respectively). In conclusion, alfalfa hay can be included in prepartum diets without necessarily increasing the incidence of hypocalcemia, and the cation-anion difference of alfalfa hay is a determinant of whether it can be included in the prepartum diet.

ABSTRACT

(General Audience)

Hypocalcemia, low concentrations of calcium in the blood, is more prevalent during the transition from pregnancy to post-pregnancy and can be diagnosed through blood analysis or by observing physical symptoms. The dietary cation-anion difference (DCAD) is the acid-base regulation of the diet and has been used to prevent hypocalcemia during the pre-calving period. The objective of this study was to feed 2 types of hay and 2 types of acidogenic products (**G**rass **H**ay/**C**alcium **C**hloride, **G**rass **H**ay/**PO**lyhalite, **A**lfalfa **H**ay/**C**alcium **C**hloride, and **A**lfalfa **H**ay/**PO**lyhalite) to non-lactating cows in their pre-calving period and observe variables in relation to the incidence of periparturient hypocalcemia. The variables included plasma and urine calcium concentrations pre-calving (21 days) and post-calving (3 days), dry matter intake (DMI) during the pre-calving period, and urine pH. Cows decreased in their dry matter intake toward calving and consumed more grass hay than alfalfa hay (11.6 vs. 10.8 kg/d). All four diets were in a negative DCAD and successfully decreased urine pH for all cows. Calcium concentrations in plasma decreased around calving, but this was not affected by hay type or acidogenic product. Urinary calcium output was greatest in the GHCL-fed cows. The study resulted in further differentiation of cows with plasma calcium concentrations ≤ 5.5 mg/dL without physical symptoms and an animal with ≤ 5.5 mg/dL and a loss of muscle function. Only one cow had a loss of muscle function; however, 13 out of 79 cows had calcium concentrations that according to past research, should've resulted in this. Additionally, 44 out of 79 cows had calcium concentrations between 5.5 and 8.0 mg/dL at least once after calving. In conclusion, alfalfa can be included in pre-calving diets as long as the DCAD is negative.

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ABBREVIATIONS

Grass Hay and Calcium Chloride diet (GHCL)

Grass Hay and Polyhalite diet (GHPO)

Alfalfa Hay and Calcium Chloride diet (AHCL)

Alfalfa Hay and Polyhalite diet (AHPO)

Negative Energy Balance (NEB)

Total Mixed Ration (TMR)

Neutral Detergent Fiber (NDF)

Virginia Tech Dairy Complex (VTDC)

Subclinical Hypocalcemia (SCH)

Dry Matter (DM)

Days in Milk (DIM)

Expected Calving Date (ECD)

Dietary Cation-Anion Difference (DCAD)

Parathyroid Hormone (PTH)

Non-Fiber Carbohydrate (NFC)

Neutral Detergent Soluble Fiber (NDSF)

Cumberland Valley Analytical Service (CVAS)

Chapter 1

Thesis Introduction

When dairy cattle transition between late pregnancy and early lactation, the chances of metabolic diseases occurring rise. These diseases include ketosis, retained placenta, displaced abomasum, metritis, and periparturient hypocalcemia. Low calcium concentrations, otherwise known as hypocalcemia, will occur when calcium is reallocated to the mammary gland to support the production of colostrum and milk. Physical symptoms of calcium concentrations ≤5.5 mg/dL can present themselves in the form of cold ears, sunken eyes, wobbly gait, and lack of appetite. The loss of muscle function is also connected to low calcium concentrations ≤5.5 mg/dL and often results in a "down cow". Prior to the start of physical symptoms, blood analysis can be performed during the first 4 days in milk to diagnose a condition known as subclinical hypocalcemia. Subclinical hypocalcemia postpartum can be prevented through prepartum diet manipulation by limiting dietary calcium, including calcium binders, and lowering the dietary cation-anion difference (DCAD). Limiting the calcium that's available to the cow prepartum helps prepare the metabolism to increase calcium availability through mobilization, increased absorption, and reduced excretion. As the main influence on hypocalcemia, the DCAD can be influenced by the inclusion or exclusion of Sodium (Na^+), Potassium (K^+), Chloride (Cl^-), and Sulfur (S^-) in the diet.

Alfalfa is a forage typically high in energy, protein, and digestibility, as well as a high concentration of potassium. Achieving a negative DCAD is typically done by including inorganic salts (Cl^- or S^-) in the form of acidogenic products; however, with the inclusion of alfalfa, a negative DCAD can be harder to accomplish. Due to potassium influencing DCAD in the positive direction, alfalfa is not typically fed to prepartum cows. The purpose of this study was to evaluate the inclusion of alfalfa in prepartum diets on dry matter intake and urine pH since these are indicators of health status and acidification. The value of the study is to show farmers, nutritionists, and the industry in general that when it is readily available for the area or individual operation, alfalfa hay can be included in prepartum dairy rations as long as a mineral analysis is completed and the DCAD is calculated prior to ration formulation.

Chapter 2

Literature Review

Hypocalcemia

Dairy cattle have a transitional period from a pregnant and nonlactating stage (dry cow) to a non-pregnant and lactating stage (milking cow) that starts 3 weeks before calving and last for 3 weeks after calving. This transition period is characterized by an increased risk of metabolic diseases (DeGaris & Lean, 2008). Under typical management practices, dairy heifers become pregnant at about 12 to 15 months of age and start the lactation cycle immediately after calving at approximately 2 years of age (Ramberg et al., 1984). The lactation cycle includes a continuous rotation of a nonpregnant and lactating stage immediately after calving, a pregnant and lactation stage after conception, and a pregnant and nonlactating stage a few weeks or months before the next calving.

During the transition period, substantial metabolic changes occur in the cow, and the energy balance decreases considerably. Grummer (1995) observed that energy balance decreases towards calving, especially during the last 7 days before calving, and that decrease is exacerbated with diets with lower energy (1.51 vs 1.27 Mcal NE_l/d). The negative energy balance (NEB) can be explained by either a limited supply of energy due to a low dry matter intake (DMI), an increased requirement of energy due to stress, or a combination of both. Dry matter intake remains steady at around 2.0% of body weight throughout the 3 weeks before calving and will reach a decline of about 1.4% of body weight 1 day before calving (Grummer et al., 2004).

Metabolic diseases such as metritis, displaced abomasum, retained placenta, and ketosis can occur because of an NEB. Metritis will occur after parturition due to excessive stress on the body, particularly the barriers of the cervix, vagina, and vulva (Dervishi et al., 2016). Bacteria enter the genital tract through these compromised entries and result in inflammation of uterine layers (Dervishi et al., 2016). A retained placenta is connected to metritis when the fetal membranes fail to expel within 24 hours of calving, leading to a further compromised genital tract. When DMI decreases in the prepartum, a displaced abomasum is more likely to occur due to the reduction of ruminal fill that provides an opportunity for ruminal migration (Shaver, 1997). When energy requirements for maintenance exceed the energy supplied in the diet, the cow will start mobilizing energy from body reserves. The catabolism of body fat results in the release of ketone bodies and this increase is called ketosis or hyperketonemia (Moore & DeVries, 2020). Other metabolic diseases in the transition period are clinical or subclinical hypocalcemia.

Periparturient Hypocalcemia

As gestation is ending, colostrum (colostrogenesis) and milk (lactogenesis) production are initiated. During this time, nutrient requirements shift from the development of the fetus to the production of milk. The animal's metabolism is partitioning and regulating nutrients through a homeorhetic process that helps the animal adapt to the new physiological state of lactogenesis (Bauman & Bruce Currie, 1980). Lactation is the most dramatic alteration of metabolism for the mammary gland to receive nutrients needed for milk synthesis (Bauman & Currie, 1980).

Periparturient hypocalcemia happens when calcium concentration in the blood drops to abnormal levels (< 8.0 mg/dL) and occurs when the cow is unable to meet the metabolic demands for large quantities of calcium within 24 hours of parturition (Joyce et al., 1997b). Periparturient paresis or milk fever is a metabolic disease that occurs in the transition period and is associated with very low concentrations of calcium in the blood (≤ 5.5 mg/dL). The signs of milk fever include recumbency (a "down" cow), cold ears, sunken eyes, wobbly gait, and lack of appetite (Goff, 2008). Recumbency is the decreased functionality of the muscles and is a result of the rapid withdrawal of calcium from the blood that supplies the mammary gland (Hibbs, 1950). Treatment for milk fever is typically an intravenous infusion of calcium gluconate, that is given when the animal is down (Oetzel, 1988). The intravenous treatment is a rapid deposition of calcium into the blood to increase and sustain the elevation of blood calcium concentrations (Braun et al., 2012). With this baseline for a down cow and knowing that the paresis was resolved once concentrations reached normocalcemia concentrations (≥ 8.0 mg/dL), subclinical hypocalcemia (SCH) was determined as a middle ground condition. The middle ground was needed to evaluate strategies to be used for the prevention of milk fever (Neves et al., 2018). To diagnose SCH (≤ 8.0 mg/dL) blood analysis is needed to determine the total calcium concentration. In blood, total calcium is 50% in the ionized form, 40% protein-bound, and 10% complexed with anions (Ott et al., 2021). A ruminal calcium bolus is administered when an animal is suspected of SCH after calving (Hernández-Castellano et al., 2020).

Blood calcium concentrations can vary within the first 4 days in milk (DIM), and blood analysis should be completed throughout this entire period. When evaluating total blood calcium concentrations in 263 multiparous Holsteins diagnosed with SCH, McArt and Neves (2020) discovered that a nadir was reached at 1 DIM (~6.4 mg/dL), and concentrations did not reach prepartum concentrations (~9.2 mg/dL) until 4 DIM (~8.8 mg/dL). When assessing the blood calcium concentration within 4 DIM, we can correlate the timing of the blood sample to the proper diagnosis of SCH and differentiate hypocalcemia from milk fever (Neves et al., 2018).

Hypocalcemia is also associated with suppressed immune response and function that will lead to a higher incidence of bacterial infections like metritis and mastitis (Kimura et al., 2006; Martinez et al., 2012). Hypocalcemia can increase mastitis incidence due to the reduction in muscle contractions in the teat sphincter that delays teat closure post-miking (Goff, 2008). Drackley (1999) discovered that cows with health concerns during the transition period produced 7.2 kg less milk daily than their healthy counterparts between 1 and 20 DIM. Milk production in cows previously diagnosed with milk fever was monitored, and for some time post-calving (4 to 6 weeks) the loss ranged from 1.1 to 2.9 kg/d (Rajala-Schultz et al., 1999). Supporting data from Martinez et al. (2012) found that 66% of the incidence of metritis was associated with SCH due to reductions in neutrophils concentration and functionality in the blood within 0 to 3 DIM.

Throughout the United States, 5% of dairy cows will develop milk fever (Goff, 2008), and Reinhardt et al. (2011) found 47% of second-lactation cows to have some degree of SCH. Clinical hypocalcemia has often been linked with age, with 1.4, 5.7, and 16.1% occurrence for the second, third, and ≥fourth parity cows respectively (Venjakob et al., 2017). Subclinical hypocalcemia can present in 29, 49.4, and 60.4% of second, third, and ≥fourth lactation respectively (Venjakob et al., 2017). Breed associations with clinical and subclinical hypocalcemia have been studied as well. When studying 406 Jersey and 872 Holstein parturitions, Chiwome et al. (2017) found that Jerseys developed milk fever at 14.78% compared to 4.82% for Holstein cows. The parity and production connection to hypocalcemia confirm the point that periparturient hypocalcemia is more prevalent as cows mature in age and increase milk production.

Calcium Homeostasis

Throughout the lactation cycle, calcium accumulates in skeletal tissue, is excreted in feces and urine, is transported to the fetus, and secreted in milk (Ramberg et al., 1984). At the onset of lactation, the mammary gland has an increase in calcium uptake, inevitably decreasing the calcium available in the blood (Hernández-Castellano et al., 2020). A typical 680 kg Holstein dairy cow would have 9.2 mg/dL of calcium present in the blood, and at parturition, that concentration decreases to 6.9 mg/dL (Hernández-Castellano et al., 2020). Calcium can be replaced and reintroduced to the extracellular fluid through dietary calcium, resorption of the 98% that is stored in bone, and renal reabsorption (NASEM, 2021).When the loss of calcium exceeds intake, plasma concentrations decrease, and when loss and intake are balanced, concentrations are maintained at 8.5-10 mg/dL (normocalcemia) (Goff, 2008). Parathyroid hormone (PTH) is secreted from the parathyroid glands when plasma calcium concentrations start to decrease. The release of PTH acts on the kidney to increase reabsorption, enhance intestinal absorption, and increase resorption from the bone (NASEM, 2021)

Magnesium is an example of a nutrient that is commonly analyzed alongside calcium. Normal values of magnesium in the plasma of dairy cows range between 1.8 and 2.4 mg/dL (Goff, 2008). Evidence from van de Braak et al. (1987) suggests that when blood magnesium concentrations reach <1.5 mg/dL in the prepartum period, the risk of milk fever increases. Low concentration of magnesium in the blood has been linked to the reduction of PTH production and a decreased sensitivity of tissues to PTH (Hernández-Castellano et al., 2020). Phosphorus is another mineral that has been linked to calcium metabolism; however, it is more importantly linked to negative environmental impacts. Lean et al. (2006) found that increasing the dietary concentration of phosphorus from 0.30 to 0.40% , increases the risk of milk fever by 18%. Cohrs et al. (2018) also found that when feeding a prepartum diet with 0.28% phosphorus, the resulting plasma calcium concentrations were lower.

Minerals supplied through the diet will come into contact with the epithelial cells on the lining of the gastrointestinal tract (Goff, 2018). Tight junctions form between the epithelial cells and are normally impenetrable by minerals (Goff, 2018). If the free ionized state of a mineral exceeds the concentration in the extracellular fluid, the electrical potential difference across the tight junctions will decrease resistance and increase movement (Goff, 2018). This force is known as paracellular absorption and is also influenced by the size of the mineral atom and its associated electrical charge (Goff, 2018). A diet with a high concentration of a target mineral will encourage the paracellular absorption. Intestinal transcellular transport consists of calcium entering through the transient receptor potential vanilloid (TRPV) and moving into the bloodstream through the Na^+/Ca^{2+} exchanger by binding to a calcium-binding protein known as calbindin-D_{9k} (Hernández-Castellano et al., 2020).

When dietary calcium is insufficient for the requirements of the animal, calcium will be drawn from the bone. The three processes, formation, sustenance, and resorption are continuously happening in bones to protect the skeletal system and balance mineral concentrations (Lerner, 2012). Osteocytes are responsible for bone mass regulation (sustenance), osteoblasts form new bones (formation), and osteoclasts are responsible for resorption (Lerner, 2012). When the concentration of calcium in the blood decreases, the calcium-sensing receptors on the parathyroid chief cells are activated. Activation and proliferation of the osteoclasts occur at high levels of PTH and increase the cell population which will increase the release of calcium from the bone (Hernández-Castellano et al., 2020).

The kidney regulates mineral utilization by secreting them into the extracellular fluid pool and excreting them through urine (Horst, 1986). Renal 1-α hydroxylase, an enzyme that catalyzes the hydroxylation of calcidiol to calcitriol, is the renal regulator of calcium when stimulated by PTH (Hernández-Castellano et al., 2020). Calcitriol, otherwise known as the active form of vitamin D (Hernández-Castellano et al., 2020) is responsible for calcium absorption across the small intestinal mucosa through the active transcellular pathway (Schröder & Breves, 2006). Parathyroid hormone sensitivity in the body could be affected by pH, hinting that providing an acidogenic diet during the prepartum period can increase sensitivity and calcitriol production.

Strategies to Prevent Postpartum Hypocalcemia

Limiting dietary calcium at the end of gestation maintains calcium homeostasis and prepares the cow for the mineral losses that are associated with milk and colostrum (Kronqvist et al., 2011a). Calcium absorption increases when cows consume calcium-deficient diets (Horst, 1986). Kronqvist et al. (2012) and Thilsing-Hansen et al. (2002) found that to prevent milk fever, intake of calcium has to be < 20 g/d. A calcium-deficient prepartum diet can be difficult to achieve with a high-forage diet, and Kerwin et al. (2019), and Richardson et al. (2021) substantiates this when they formulated a control prepartum diet that included corn silage and grass hay and accomplished a calcium concentration of 1.68%. The mechanistic pathway for reduced dietary calcium starts with the decreased calcium that is absorbed in the intestine, which then decreases plasma calcium concentrations; consequently, increasing the secretion of PTH (Horst, 1986). The secretion of PTH increases 1α-hydroxylase concentrations in the kidney and vitamin D in the blood and leads to further bone calcium resorption (Horst, 1986).

Calcium binding is an approach that will replicate a calcium-deficient prepartum diet since a true calcium-deficient diet is not readily achievable for the prepartum period (Kerwin et al., 2019). By including a binding compound in the diet, dietary calcium is bound within the rumen, and calcium absorption rates into blood decrease (Kerwin et al., 2019). Zeolite A can bind to calcium, magnesium, and phosphorus in varying rumen pHs' (Thilsing et al., 2006); therefore, making it appropriate for inclusion in the prepartum period. When zeolite A, which contains sodium aluminum silicate, was fed 4 weeks prepartum, it improved calcium concentrations on 1 and 2 DIM (Thilsing-Hansen & Jørgensen, 2001). Kerwin et al. (2019) found that cows fed a diet containing zeolite A were 3 times less likely to contract SCH (serum calcium concentrations <8.5 mg/dL) within 1 DIM.

To raise blood pH, dietary cations like K^+, Na^+, Ca^{++}, and Mg^{++} are absorbed in the blood, and dietary anions like Cl^-, SO_4^{2-}, and PO_4^{3-} will lower blood pH when absorbed (NASEM, 2021). The cation-anion balance, mainly sodium, potassium, chloride, and sulfur, can be accomplished by mixing these minerals with other ingredients in the diet based on the equation, DCAD= (mEq Na^+ + mEq K^+) – (mEq Cl^- +mEq S^2) (Goff, 2006). Normally, cows are in a state of metabolic alkalosis due to forages typically being high in potassium (NASEM, 2021). Metabolic alkalosis impairs bone resorption (Block, 1994) and the production of 1,25-dihydroxyvitamin D by PTH (Goff et al., 1991; NASEM, 2021). A prepartum diet with the addition of anions (negative DCAD) has been found as the best preventative method for hypocalcemia (Joyce et al., 1997b). These acidogenic diets reduce the charge ion difference in the blood which induces metabolic acidosis (Hernández-Castellano et al., 2020). Acidification increases the responsiveness to PTH (DeGaris & Lean, 2008), which increases the calcitriol concentrations (Hernández-Castellano et al., 2020), resulting in the increased bone mobilization of calcium (Block, 1984).

To control hypocalcemia, urine pH should decrease from the starting point of 8.2 and be maintained around 6.2 and 6.8 (Goff, 2006, 2008). This adjustment in urine pH is based on anion supplementation to acidify the blood in the prepartum period. High acidity (pH less than 6) can have an effect on the viability of the fetus and result in stillborn births (Melendez et al., 2021). Similar trends of lower plasma total Ca and higher β-hydroxyl-butyrate (BHB) have been identified for animals with urine pH < 6.0 and > 7.0 (Melendez et al., 2021). Measuring prepartum BHB concentrations in the blood is an indicator of hyperketonemia, which increases the risk of other health problems early in lactation (Benedet et al., 2019). Due to the lower plasma calcium concentrations and the higher BHB concentrations, Melendez et al. (2021) suggests that the target urine pH should be between 6.0 and 7.0 to avoid over-acidification.

Challenges associated with feeding acidogenic products are typically connected to a decrease in dry matter intake. For an acidogenic product to be a regulator of the cation-anion balance, it has to contain chloride and sulfate (sulfur) in greater proportions than sodium and potassium (Ferreira et al., 2019). Polyhalite ($K_2SO_4 \cdot 2CaSO_4 \cdot MgSO_4 \cdot 2H_2O$), is an abundant and natural rock salt mineral (Ferreira et al., 2019; Peryt et al., 2005; Wollmann et al., 2008) that is considered an acidogenic product based on its potassium and sulfate equivalents. With a DCAD of -249 mEq kg/DM and about 500 g/cow/d of polyhalite for 21 days, Ferreira et al. (2019) were able to decrease urine pH to about 5, inducing metabolic acidosis. Richardson et al. (2021) observed maintenance of dry matter intake, a decrease in urine pH, and an increase in calcium output when feeding a diet with a DCAD of -172 mEq/kg DM with the inclusion of polyhalite. Both studies (Ferreira et al., 2019; Richardson et al., 2021) found that acidogenic products can be used during the transition period without facing the previously mentioned challenges.

Forages

Carbohydrates account for 60 to 70% of the DM provided in rations for dairy cattle and are the primary source of energy (Poorkasegaran & Yansari, 2014). Carbohydrates can be classified by their solubility in neutral detergent (NDF) (NASEM, 2021). Structural carbohydrates, specifically NDF, limit the intake and stimulate chewing and rumination (Allen, 1997), consequently promoting rumen health. Less energy is supplied through structural carbohydrates, and more is supplied through non-fiber carbohydrates (NFCs) (Wei et al., 2021). The components of NFC consist of organic acids, sugars, starch, and neutral detergent soluble fiber (NDSF) (Wei et al., 2021). As a plant matures, the stem-to-leaf ratio increases, the protein content decreases, and fiber concentrations increases (NASEM, 2021). More lignin is present in the fiber of a mature plant making the plant less digestible (NASEM, 2021). Energy, NDF, and starch are correlated because increasing NDF will reduce starch and energy supplied to the animal (NASEM, 2021).

Ration formulation should change throughout the dry period, with the first 5 weeks (far-off period) targeting maintenance needs and the last 3 weeks (close-up period) preparing for lactation (Dann et al., 2006). From 200, 250, and 275 days of gestation, the NASEM (2021) energy and protein requirements are 1.4, 3.5, and 5.4 NE_L, Mcal/d, and 125, 320, and 489 metabolizable protein, g/d respectively. As the fetus continues to require more nutrients, the DMI of the dam can decrease by 10 to 30% (Kamiya et al., 2006). Energy and DMI can be increased by including more starch or a higher quality forage (NASEM, 2021). A close-up diet typically has more energy supplied by increasing starch and reducing the forage and fiber (NASEM, 2021).

Corn grain is mostly starch (65-70% DM), sugar (12-40% DM), and NDSC (25-44% DM) (Poorkasegaran & Yansari, 2014) and has been included at 21.3% DM in close-up diets (Mashek & Beede, 2000). More starch has been connected to an increase in DMI, and Akhlaghi et al. (2022) found that a lower starch diet reduced DMI (29.2% vs 22.3%). Fiber digestibility is another contributor to changes in DMI, and Oba and Allen (1999) found that improving NDF digestibility also improved DMI (20.5 vs. 19.9 kg/d). A NASEM (2021) model estimated DMI for a 1500lb far-off cow and found that as NDF was increased in a prepartum diet from 30% to

50%, intakes would decrease by 12% and that a more significant reduction would occur in the close-up period. Bermuda hay, grass hay, wheat straw, and corn silage are close-up ingredients (Table 2:1) that can be included respectively at 10 % of DM (Zimpel et al., 2018), 37% of DM (Weiss et al., 2015), 26.15 % of DM (Dann et al., 2006), and 32.10 % of DM (Glosson et al., 2020) to meet NASEM requirements previously mentioned.

NASEM (2021) recommends calcium at 0.39%, phosphorus at 0.21%, potassium at 0.69%, magnesium at 0.14%, sodium at 0.17%, chloride at 0.14%, and sulfur at 0.20% in the close-up diet. Deviation from the suggested concentrations can reduce DMI, reduce rumen and intestine motility, have poor productivity in the future, and increase susceptibility to other diseases (Goff, 2006). Dietary magnesium should be 0.35-0.40% (Goff, 2006), and sulfur above 0.22% (Goff, 2006). Puggaard et al. (2011) found that a low phosphorus diet (0.24 vs 0.34%) will lower NDF digestibility.

Alfalfa Quality and Management

Alfalfa (*Medicago sativa*) is a perennial clover-like legume that is consistently a source of protein (more rumen degraded protein) and fiber (more fiber without added starch) in lactating dairy cattle TMR rations (Mullins et al., 2009). Leaves from a budding alfalfa plant and a flowering plant were compared for proteins and metabolites, and Fan et al. (2018) found that flowering alfalfa contains less crude protein and increased NDF. The nutrient composition of immature and mature alfalfa was also analyzed by NASEM (2021), with mature alfalfa having 18.1% crude protein and 46.6% NDF, and immature alfalfa having 21.5% crude protein and 37.7% NDF.

Calcium concentrations increase with the quality and maturity of alfalfa, justifying its inclusion in lactating rations to combat the demand for calcium (Joyce et al., 1997a). A report from Cumberland Valley Analytical Service (CVAS) including 7,373 legume forage samples from May 20, 2021, to May 23,2022 in the United States, stated that this type of forage contains on average 0.22% sodium, 2.53% potassium, 0.75% chloride, 0.31% sulfur, and 1.53% calcium (DM bases).

Alfalfa is a deep-rooted plant with a long growing season that requires lots of irrigation (Yang et al., 2019). Salinity and irrigation are often managed together and are critical for high-forage mass production (Ferreira et al., 2015). Nitrogen, potassium, and calcium are the main nutrients in alfalfa but concentrations can be affected by salinity adjustment of the soil during the management of the seedling (Ferreira et al., 2015). Alfalfa grows best in soils with a pH of 6.5 to 7.5, and growth will be stunted if the soil pH is lower. Liming is the most important alfalfa management practice to increase soil pH and is typically performed before sowing (3 weeks) (Grewal & Williams, 2003). Liming adds calcium and magnesium to the soil, increases stand establishment and increases the activity of nitrogen-fixing bacteria (Grewal & Williams, 2003)**.** Grewal and Williams (2003) showed that liming increased calcium concentrations in alfalfa shoots while also increasing phosphorus for functionality and management of the plant. Potassium fertilization (sometimes referred to as potash) is variable based on the alfalfa cultivar, soil type, and maturity (Jungers et al., 2019). Fertilization of the alfalfa with a chloride-based fertilizer could result in decreased DCAD through an increase in plant chloride (Henning, 2004).

Intake and Digestibility of Grasses and Legumes

When comparing alfalfa hay to grass hay, DMI and NDF are important considerations. Broderick et al. (2002) found that cows consuming alfalfa silage had a DMI of ~25 kg/day vs cows consuming ryegrass silage with a DMI of ~17kg/day. Kilmer et al. (1979) also observed the DMI of lactating dairy cows on two diets with 77% forage and 23% concentrate, and those consuming alfalfa hay had greater DMI (164 g/kg body weight$^{\cdot 75}$) than those consuming orchard grass (136 g/kg body weight$^{\cdot 75}$). As alfalfa matures, NDF accounts for ~25% of legume mass (Buxton & Redfearn, 1997). Alfalfa tends to contain less fiber, leading it to be more digestible than grasses (Buxton & Redfearn, 1997). The rationale behind digestibility differences between alfalfa and grasses is grasses have a midrib for support that increases fiber concentrations in the leaves as they mature (Buxton & Redfearn, 1997).

Alfalfa in the Transition Period?

Dry cow rations are often based on forage, and the dietary potassium concentrations should be limited to reach a negative DCAD (Rérat et al., 2009). Hays low in potassium (1.3 vs 3.3% DM) are known to reduce metabolic alkalosis (DCAD of 195 vs 514 mEq/kg of DM) and increase the cow’s ability to maintain calcium homeostasis (Rérat et al., 2009). Roche et al. (2002) also found that when decreasing dietary potassium concentrations, urinary pH, and therefore DCAD, decreased as well. Rérat and Schlegel (2014) found that a diet with 1.62 %K that was supplemented with anionic salts (0.5 kg/d) lowered urine pH to 7.5. NASEM (2021) has reported mature alfalfa hay to have a concentration of 2.34% K compared to a mature grass-legume mixture that is predominately grass that has 1.55% K. The potassium concentration in alfalfa can be a disadvantage for its inclusion in prepartum diets. To reach negative DCAD levels, a forage-based diet high in potassium requires an impractical amount of anionic salts (Roche et al., 2002). More anionic salts are needed for diets with higher potassium, but this raises palatability concerns. Diets that are not palatable are not ideal for cows in the prepartum period because they are already decreasing in DMI as they near parturition.

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Table 2:1 Literature review for forage usage in prepartum research for the last 31 years

#	Reference	Yr	State	Forage	Tmts	CP %	NDF%	Starch %	Ash%	DCAD	Ca %	Mg %
1	Zhang et Al	2022		CS; WS	1	15.1	49.3	14.1	9.39	6	0.4	0.43
					2	16.6	47.7	12.7	8.59	−24.0	0.44	0.44
					3	16.4	46.9	12.8	11.8	−24.1	1.97	0.43
2	Zimpel et al.	2021		CS;BH	1	14.8	45.2	-	6.4	224	0.68	0.47
					2	15.3	44.1	-	6.7	-46	0.63	0.45
					3	15.1	44.5	-	6.4	-148	0.74	0.4
3	Richardson et al	2021		CS; GH	1	142	387	184	72	196	16.8	2.6
					2	141	383	185	76	7	12.6	3.4
					3	135	384	194	80	-172	9	4
					4	137	381	184	77	-167	17	2.5
4	Serrenho et al	2021		CS; Straw	1.1	14.4	44.7	-	-	100	1	0.5
					1.2	14.4	44.8	-	-	-100	0.7	0.4
					2.1	14.1	42.9	-	-	145	1.3	0.4
					2.2	14.3	47.3	-	-	-100	1.4	0.4
					3.1	15	43.6	-	-	47.5	0.7	0.5
					3.2	15	41.8	-	-	-112	1.3	0.5
					4.1	14.1	41.8	-	-	67	1.8	0.4
					4.2	14.3	42.5	-	-	-121	1.7	0.4
5	Rajaeerad et al	2020		CS; WS	1	141	422	-	-	86	2.4	4.4
					2	141	405	-	-	95	12.3	4.2
					3	141	397	-	-	-112	12.1	4.9
					4	141	420	-	-	-115	12.8	4.4
6	Maier et al.	2020	California	CS; AH;WS	1	15.4	38.4	13.9	9.6	-12.7	1.2	0.38
7	Glosson et al.	2020		CS; WS	1	15.1	49.3	14.1	9.39	6	0.4	0.43
					2	16.6	47.7	12.7	8.59	−24.0	0.44	0.44
					3	16.4	46.9	12.8	11.8	−24.1	1.97	0.43
8	Caixeta et al.	2019		CS; GH	1	14.6	46.3	-	-	-200	0.5	0.4
					2	14.6	45.6	-	-	-228	0.67	0.44
9	Goff et al.	2019	Iowa	CS; WS; Legume Hay	1	11.7	44.8 (41.6)	-	-	196	0.66	0.47
					2	11.8	44.8 (41.6)	-	-	-9	0.65	0.46
10	Zimpel et al.	2018		CS; BH	1	14.2	39.1	21.7	5.3	196	0.57	0.39
					2	13.5	38.8	21.7	7	194	0.58	0.38
					3	13.5	39.8	22.2	7.8	192	0.5	0.4

					4	13.5	39.9	22.1	5.7	-114	0.55	0.4
					5	13.5	39.4	22	7.8	-113	0.51	0.4
11	Melendez et al.	2018		CS; Fescue/ crabgrass hay; Pasture	1	13.59	44.2	12.66	8.56	-25.7	0.79	0.82
					2	13.38	45.07	12.06	9.95	-26.1	0.77	0.65
12	Slater et al.	2018		CS; Straw	1	14.9	42.7	19	7.7	14	0.56	0.3
					2	15.3	41.4	19.1	7.8	-5.5	0.59	0.32
13	Farnia et al.	2017	Iran	Legume Hay; CS	1	13.4	33.9	18.9	-	-11.3	1.1	0.43
					2	14	34.9	19.4	-	5.03	0.51	0.32
14	Martinez et al.	2017		CS; BH	1	13.5	37.8	20.2	-	145	0.61	0.39
					2	12.9	39	20.1	-	130	0.62	0.37
					3	13.5	38.3	20.8	-	−129	0.54	0.38
					4	13.4	38.2	20.9	-	−124	0.55	0.39
15	Lean et al.	2017		Brown mid-rib corn silage; WS	1	13	44.3	17	-	18.3	1.54	0.47
					2	13.2	44	16	-	5.9	1.57	0.48
					3	13.2	43.2	16.3	-	−7.4	1.57	0.5
16	Rodney et al.	2017		CS; BH	1	13.5	37.8	-	-	145	0.61	0.39
					2	12.9	39	-	-	130	0.62	0.37
					3	13.5	38.3	-	-	-129	0.54	0.38
					4	13.4	38.2	-	-	-124	0.55	0.39
17	Neves et al.	2017		CS; Haylage; Dry Hay; WS	1	14.3	35.2	22.1	-	-6.9	1.78	0.45
					2	15.2	31.1	15.7	-	7.3	1.48	0.39
					3	14.2	33.33	17.5	-	14.1	0.86	0.36
18	Weiss et al.	2015	Ohio	GH; GS; CS; AS	1	14.6	41.8	-	-	165	0.88	0.31
					2	14.5	41.8	-	-	-139	1	0.36
					3	14.5	41.6	-	-	-138	0.9	0.37
19	Wu et al.	2014	Georgia	CS; AH	1	14.6	42.3	2.8	7.1	3.98	0.97	0.52
20	Goff et al.	2013		CS; GH	1	13.9	42.4	-	-	-181	0.76	0.42

					2	13.8	42.1	-	-	188	0.76	0.41
21	Weich et al.	2013		CS; WS	1	16.65	42.54	12.25	9.01	-15.79	0.8	0.39
					2	17.33	41.35	11.03	8.8	12.34	0.82	0.39
22	Rezac et al.	2013		CS; WS	1	15	49.4	-	-	17.7	0.84	0.36
					2	14.9	49.7	-	-	2.5	0.8	0.37
					3	15.2	49.1	-	-	0.4	0.62	0.34
23	Razzaghi et al.	2012	Iran	AH; CS	1	13	48.7	-	-	-108	0.76	0.31
					2	13.2	48.3	-	-	106	0.48	0.23
24	DeGroot et al.	2010	Oregon	CS; AH; OH	1	14.7	35.7	-	-	22.1	0.99	0.22
					2	14.7	35	-	-	-12.1	1	0.2
					3	14.9	34.6	-	-	-11.3	1	0.21
					4	14.7	35.6	-	-	-9.8	1	0.22
24	Ramos-Nieves et al.	2009		CS; WS	1	15.9 (0.9)	46.1 (2.0)	17.5 (3.1)	-	11	1.43 (0.26)	0.47 (0.08)
					2	15.9 (0.7)	44.6 (3.0)	18.0 (3.1)	-	-15	1.35 (0.24)	0.51 (0.04)
25	Penner et al.	2008		TH	1	12.5	49.6	-	7	1.6	0.74	0.35
					2	12.9	47.4	-	7.1	14.5	0.72	0.35
26	W.X. Wu et sl	2008		WRG; CS	1	12.9	41.2	-	-	127	0.85	0.36
					2	13.2	40.9	-	-	30	0.85	0.36
					3	13.5	40.6	-	-	-63	0.86	0.35
					4	13.8	40.4	-	-	-154	0.86	0.35
27	Chan et al.	2005	Georgia	CS; AH	1	13.9	35.1	-	-	-6.02	0.99	0.24
					2	13.2	35.4	-	-	-5.57	1.5	0.26
28	Moore et al.	2000	Michigan	CS; AH	1	16.5	31.6	-	-	15	0.44	0.2
					2	16.5	30	-	-	0	0.97	0.24
					3	16.3	28	-	-	-15	1.5	0.28
29	Tucker et al.	1991		AH; SS	1	19.25	41.05	-	-	9.35	1.58	0.36
					2	17.8	37.6	-	-	-3.41	1.62	0.35

CS=Corn Silage; WS=Wheat Straw; BH=Bermuda Hay; GH=Grass Hay; AH=Alfalfa Hay; GS=Grass Silage; AS=Alfalfa Silage; OH=Oat Hay; TH=Timothy Hay; SS=Sorghum Silage; WRG=Wild Ryegrass

CHAPTER 3

Evaluating the inclusion of alfalfa hay in diets fed to pregnant and non-lactating Holstein cows during the prepartum period

INTRODUCTION

To prepare for lactation, dairy cows experience physiological challenges during the transition period from gestation to lactation. Appropriate management during this time is critical for maximized productivity in the future and disease prevention. The probability of metabolic diseases is higher during this period, including periparturient hypocalcemia (DeGaris & Lean, 2008). One of the main characteristics of the transition period is the demand for nutrients that can be compromised with the lower dry matter intake (DMI) that typically occurs during this period.

Many factors affect DMI including nutrient balance, forage quality, feeding method, palatability, moisture, stress, physical environment, and management. During the transition period, DMI tends to decline 21 days prepartum and remain at a steady decline until the end of gestation (Grummer, 1995; Hayirli et al., 2003). The decline of DMI is inevitable, but by increasing the energy supplied in the diet, energy balance can be maintained (McNamara et al., 2003). Including a high forage transition cow diet during the end of the transition period (3 weeks) can control energy intake (Vickers et al., 2013).

Alfalfa hay is a highly valued forage with crude protein concentrations of 18.8% and 34.5% for NDF (Leonardi & Armentano, 2003). The benefits of alfalfa justify it for lactating dairy rations, but it is commonly avoided for transition diets. The high levels of potassium in alfalfa prevent the dietary cation-anion difference (**DCAD**) from reaching a negative balance which is beneficial for the transition period. The DCAD is the difference between the sum of the cations (sodium (Na) and potassium (K)) and the sum of the anions (chloride (Cl) and sulfur (S)). To accomplish a proper negative DCAD with the inclusion of alfalfa, acidogenic products have to be included at high doses. This approach typically raises concerns due to its association with reduced dry matter intake.

In the last weeks of the transition period, the absorbed calcium requirement can exceed 10 g/d (NASEM, 2021). As the animal freshens, the loss of calcium increases with the start of milk and colostrum production (Kronqvist et al., 2011a). Blood calcium concentrations should be maintained at 8.5-10 mg/dL (normocalcemia) within 24 hours of parturition to meet the demand. Blood calcium concentrations can vary postpartum (typically within the first 3 days in milk (DIM)), and side effects of low calcium concentrations start at ≤ 8.0 mg/dL. Subclinical hypocalcemia (≤ 8.0 mg/dL) can only be diagnosed through blood analysis, and was found as the middle ground condition to further understand milk fever (Neves et al., 2018). Periparturient paresis and/or milk fever occurs when calcium concentrations are very low (≤ 5.5 mg/dL). The symptoms of milk fever include cold ears, sunken eyes, wobbly gait, lack of appetite, while periparturient paresis is the loss of muscle function (Goff, 2008).

A negative DCAD increases calcium and the responsiveness to the parathyroid hormone, facilitating the mobilization of calcium from the bone (DeGaris & Lean, 2008). To achieve this negative DCAD, an acidogenic product can be added to the diet which will reduce the charge ion difference of the animal, and induce metabolic acidosis (Hernández-Castellano et al., 2020). Measuring calcium concentrations in the urine provides the best method of testing the effectiveness of feeding a ration with an acidogenic product, and urine pH can also provide a practical and inexpensive method for monitoring the acidification and effectiveness of the acidogenic diet.

Polyhalite ($K_2SO_4 \cdot 2CaSO_4 \cdot MgSO_4 \cdot 2H_2O$), is an abundant and natural rock salt mineral (Ferreira & Teets, 2020; Peryt et al., 2005; Wollmann et al., 2008), that is considered an acidogenic product based on its potassium and sulfate equivalents. In a previous study, about 500 g/cow/d of polyhalite was incorporated in the diets (DCAD of -249 mEq kg/DM) of non-lactating Holstein cows to induce metabolic acidosis (Ferreira & Teets, 2020). Richardson et al. (2021) observed maintenance of dry matter intake, a decrease in urine pH, and an increase in calcium output with the inclusion of polyhalite with a DCAD of -172 mEq/kg DM in the diet as a continuation of the Ferreira & Teets study. These preliminary studies show that acidogenic products can properly induce metabolic acidosis during the transition period without decreasing DMI. In this study, we hypothesized that alfalfa hay can be included in prepartum diets for pregnant and non-lactating cows without increasing hypocalcemia as long as a negative DCAD can be obtained with acidogenic products. Therefore, the objectives of this study were to determine the dry matter intake, urine pH, Ca concentration in blood, Ca output in urine, and incidence of hypocalcemia from pregnant and non-lactating dairy cows during the prepartum period consuming diets containing either grass hay **(GH)** or alfalfa hay **(AH)** with the inclusion of either calcium chloride **(CL)** or polyhalite mineral **(PO)**.

MATERIALS AND METHODS

Animals, Housing, and Diets

The study was conducted at Virginia Tech Dairy Complex in Blacksburg, VA, from February 2021 to March 2022. All procedures were approved by the Institutional Animal Care and Use Committee of Virginia Tech (Protocol No. 20-158). Eighty pregnant and non-lactating

Holstein cows (34±7 days relative to expected calving date (**ECD**) and 749±73 kg BW) approaching their second calving or greater (Table 3:1) were housed in 2 pens and trained to eat through an electronic gate (American Calan Inc., Northwood, NH). Each pen contained 8 individual feeding tubs with their specific door. Ten cohorts of 8 cows each were selected as cows approached their calving date. Cow were selected based on their proximity to calving and their parity (2^{nd} calving or greater). When a cow in a cohort reached 35 d before her expected calving date (d -35), the whole cohort was transferred from a pasture paddock to a pen within in a compost-bedded pack barn. Once in the pen, cows were fed once daily (8:00 am) a far-off diet for 14 d as an acclimation period. Dry matter intake was not recorded during the acclimation period.

When a cow in a cohort reached 21 d before her expected calving date (d -21), cows were fed 1 of the 4 experimental diets (Table 3:2) according to a 2 × 2 factorial arrangements of treatments, in which hay type (alfalfa hay **AH**, vs. grass hay **GH**) and the acidogenic products (calcium chloride **CL**, vs. polyhalite **PO**) where the experimental factors. Grass hay (Table 3:3) was grown on-site and harvested in round-bales that were chopped using a hay chopper (Roto Grind 760, Burrows Enterprises, LLS; Greeley, CO). Large square-bales of AH (Table 3:3) were obtained from the Great Plains region and chopped using a vertical mixer (NDEco FS600; Sioux Falls, SD). Calcium chloride was the acidogenic product commonly included in prepartum diets (i.e., positive control) at the Virginia Tech Dairy Complex, and PO was an acidogenic product under evaluation for prepartum diets (Ferreira et al., 2019; Richardson et al., 2021).

The experimental diets were formulated using CPM Dairy (version 3.0.8.1; CAHP Software Information; Philadelphia, PA). Cow input for ration formulation included a BW equal to 720 kg, 37 months of age, 260 days pregnant, and a body condition score of 3.5. Formulation constraints included DMI (≥100% requirement), metabolizable energy ((≥100% requirement), metabolizable protein (≥100% requirement), dietary forage (≥60% DM), hay inclusion (20% forage), dietary NDF (35%<NDF<40%), dietary NFC (≤40%), and dietary cation-anion difference (DCAD; approximately -160 mEq/kg DM). Rations were formulated after obtaining the Na, K, Cl, and S concentrations of the corn silage and the alfalfa and grass hays. Mineral concentrations for concentrates were obtained from the feed library of CPM Dairy. The acidogenic products were then included into pelleted concentrate mixtures prepared by a commercial feed mill (Big Spring Mill, Inc; Elliston, VA).

The experimental diets were mixed and delivered daily (~8:00 am) using a mobile mixer (Data Ranger American Calan) equipped with a scale. Feed refusals (~5%) were collected daily with the same equipment, and DMI was estimated daily as the difference between feed delivered and feed refused. A preliminary mixing test was performed to ensure adequate mixing with small amounts of total mixed ration (**TMR**) (i.e., amount enough for feeding 2 cows). For this, 1 L of a 50 mM solution of $LaCl_3$ was sprayed onto 15 kg of pelleted concentrate and mixed. Then, a small batch (~52 kg) of representative TMR was prepared and mixed for 3 minutes, dispensed on a tarp, and sampled (n = 10) for analysis of La concentration (Richardson et al., 2021). With a coefficient of variation equal to 8.2%, we considered this mixing process appropriate for mixing small batches.

Sample Collection and Analysis

Body Weight and Body Condition Scoring

With each cohort, all 8 animals were weighed using the farms’ scale starting on days -24, -23, and -22 and continued weekly. Body Condition Scoring (BCS) was determined weekly by the graduate student at the time of sampling using the 5-point scoring system (Edmonson et al., 1989).

Forage and Feed Samples

Forages were collected weekly and stored in the freezer to make a composite sample. Representative samples were collected for TMR and refusals for each experimental diet. These samples were collected twice weekly from 2 feed tubs for every diet (4 diets) and stored in a -20°C freezer to form a composite sample for each period. For each cohort, a sample of each hay (alfalfa and grass), corn silage, and each pelleted concentrate was collected and sent to Cumberland Valley Analytical Services (CVAS) for mineral analyses.

To maintain proper DMI, the DM concentration each TMR, each hay, and corn silage were analyzed on a weekly basis. All composite samples were analyzed for starch, neutral detergent fiber (NDF), organic matter, and crude protein. All composited samples were dried to constant weight at 55 ◦C in a forced-air oven and ground to pass through a 1-mm screen of a Wiley mill (Thomas Scientific, Swedesboro, NJ, USA). Ash concentration was determined after burning samples in a furnace (Thermolyne 30400, Barnstead International, Dubuque, IA, USA) for 3 h at 600 ◦C (Method 942.05, (AOAC, 2019)). Crude protein concentration was calculated as percent N × 6.25 after combustion analysis (Method 990.03, (AOAC, 2019)) using a Vario El Cube CN analyzer (Elementar Americas, Inc., Mount Laurel, NJ, USA). Neutral detergent fiber was determined using the Ankom200 Fiber Analyzer (Ankom Technology, Macedon, NY, USA). Sodium sulfite and α-amylase (Ankom Technology) were included for NDF analysis (Ferreira & Mertens, 2007). Starch concentration was determined using the acetate buffer method of Hall (2009)with α-amylase from *Bacillus licheniformis* (FAA, Ankom Technology) and amyloglucosidase from *Aspegillus niger* (E-AMGDF, Megazyme International, Wicklow, Ireland).

Blood Samples

Blood samples were collected from the coccygeal vein or artery using vacutainers containing heparin as anti-coagulant. Blood samples were collected before feeding on day -21 and continued weekly until day 0 of the last cow. Blood samples were collected every other day in the last 2 relative to each cow's ECD. All samples were retrospectively assigned days relative to the true calving date (-24, -17, -10, -3). Blood samples were collected at calving (d0) and at days 1, 2, and 3 post-calving. To ensure d0 samples were taken within +/-12 hours of calving, cows were monitored through 3 surveillance cameras (Ring Spotlight Cam Plus; Santa Monica, CA). Blood samples were then centrifuged at 1,500 g for 10 minutes to harvest plasma. Samples were stored at -20°C until calcium analysis.

Plasma samples were diluted (1:25) in a 7 mM solution of $LaCl_3$ as described previously (Richardson et al., 2021). Diluted samples were analyzed for total calcium using inductively coupled plasma atomic emission spectrometry (Spectro Arcos II ICP-AES, SPECTRO Analytical Instruments GmbH, Germany) using 318 µm of wavelength.

Urine Samples

Urine samples were collected starting on day -21 and continued weekly until day 9 of the last cow. As a cow entered into the last 2 weeks of her ECD, urine samples were collected every other day. A midstream urine sample was collected by manual stimulation of the vulva. A pH measurement was obtained from this sample immediately after collection using a portable pH meter (Hanna Checker; Hanna Instruments; Woonsocket, RI), which was calibrated before each sampling. After collection, urine samples were filtered through grade 1 qualitative filter papers

(Whatman, GE Healthcare Bio-Sciences, Pittsburg, PA). Filtered urine samples were stored at -20°C until calcium analysis. All samples were retrospectively assigned days relative to the true calving date (-24, -17, -10, -3, and 0). Day 0 samples were not collected for cohorts 1-4 as an oversight, but for cohorts 5-10 the d0 sample was collected +/-12 hours of calving. The pH measurements were retrospectively assigned relative to the first cow in each cohort's expected calving date (-21, -14, -7, 0).

Filtered urine samples were diluted (1:50) in a solution of 2% nitric acid (v/v). Diluted samples were analyzed for total calcium using inductively coupled plasma mass spectrometry (Spectro Arcos II ICP-AES, SPECTRO Analytical Instruments GmbH, Germany) using 318 μm wavelength. The concentration of calcium in urine was normalized using urinary creatinine concentration, which were determined using a colorimetric assay (Creatinine kit 500701, Cayman Chemical, Ann Arbor, MI, USA) after a 1:25 dilution of urine. Calcium output was estimated as described in Eq. [1],

$$\text{Ca output} = 0.029 \times \text{BW} \times \text{Ca urine} \quad [1]$$

where Ca output is the daily output of calcium in g/d, 0.029 is the constant daily clearance of creatinine in g/kg BW (Valadares et al., 1999), BW is the body weight of the cow in kg, and Ca urine is the concentration of calcium in urine normalized by the concentration of creatinine in urine as g Ca/g creatinine.

Statistical Analysis

Prior to designing the experiment, a statistical power analysis was performed using the POWER procedure of SAS (SAS version 9.4, SAS Institute Inc., Cary, NC, USA). Considering a statistical power equal to 0.80 and a probability of committing Type I error (α) equal to 0.10, 38 cows per treatment within a main effect were deemed sufficient to detect a 2.5-kg/d difference in

DMI between two treatments. One cow consuming the AHCL died of pulmonary emphysema while in the experiment, leaving 79 experimental units for the final statistical analysis.

Variables were analyzed using MIXED procedure of SAS (SAS version 9.4) using repeated measures (cow was the subject and day was the repeated measure). The statistical model for evaluating DMI in the pre-partum period included BW as a covariate [fixed; degrees of freedom (df) = 1], the effect of hay (fixed; df = 1), the effect of acidogenic product (fixed; df = 1), the interaction of hay × acidogenic product (fixed; df = 1), the random error associated to cows, the effect of day (fixed; df = 21), the day × hay type interaction (fixed; df = 21), the day × acidogenic product interaction (fixed; df = 21), the day × hay type × acidogenic product interaction (fixed; df = 21), and the random error associated to repeated measures. The statistical models for evaluating urine pH, Ca concentration in plasma and urinary Ca output in the pre-partum period included the effect of hay type (fixed; df = 1), the effect of acidogenic product (fixed; df = 1), the interaction of hay type × acidogenic product (fixed; df = 1), the random error associated to cows, the effect of day (fixed; df = 3, df = 7, and df = 4 for urine pH, Ca concentration in plasma, and urinary Ca output, respectively), the day × hay type interaction (fixed; df = 3, df = 7, and df = 4), the day × acidogenic product interaction (df = 3, df = 7, and df = 4), the day × hay type × acidogenic product interaction (fixed; df = 3, df = 7, and df = 4), and the random error associated to repeated measures.

RESULTS

On a dry matter basis, grass hay contained 7.5% CP and 74.9% NDF, whereas the alfalfa hay contained 19.6% CP and 45.6% NDF (Table 3:3). In regard to minerals, grass hay contained 0.36% Ca, 0.02% Na, 1.88% K, 0.38% Cl, and 0.15% S, whereas alfalfa hay contained 1.52%

Ca, 0.16% Na, 2.5% K, 0.77% Cl, and 0.32% S (Table 3:3). The grass hay had a cation-anion difference equal to 289 mEq/kg DM, whereas the alfalfa hay had a cation-anion difference equal to 290 mEq/kg DM (Table 3:3).

Cows consuming grass hay tended to consume more dry matter than cows consuming alfalfa hay (11.6 vs 10.8 kg/d; $P = 0.07$; Figure 3:1). Dry matter intake did not differ between acidogenic products (11.2 kg/d; $P = 0.21$), and no interaction existed between hay type and acidogenic products ($P = 0.41$; Figure 3:1). Dry matter intake decreased towards calving ($P < 0.01$), and no interactions existed between day and hay or acidogenic product ($P > 0.51$).

Feeding the diets with a negative DCAD decreased urine pH ($P < 0.01$), and this occurred for all four diets (Figure 3:2). As reflected by the interaction between hay type and acidogenic product ($P = 0.08$), urine pH decreased to a lesser extent for the cows consuming the GHPO diet than for the rest of the cows, which lead to significant main effects for hay type ($P < 0.01$) and anionic product ($P < 0.01$).

The concentration of calcium in plasma decreased substantially around calving ($P < 0.01$; Figure 3:4) but neither the hay type ($P = 0.86$) nor the acidogenic product ($P = 0.81$) affected it. No interaction existed between these factors ($P = 0.37$) or between time and any of these factors ($P > 0.65$). For urinary calcium output (Figure 3:3), an interaction existed between hay type and acidogenic product ($P = 0.01$), and the urinary calcium output was the greatest for cows consuming the GHCL diet.

Only one cow had acute signs of clinical hypocalcemia (i.e., peripartum paresis). However, 13 out of the 79 cows had calcium concentrations in plasma below 5.5 mg/dL at least once between 0 and 3 d after calving (Figure 3:5). In addition, 44 out of 79 cows had calcium concentrations in plasma between 5.5 and 8.0 mg/dL at least once between 0 and 3 d after calving (Figure 3:5).

DISCUSSION

This study was designed to evaluate alfalfa, and how its inclusion affected hypocalcemia rates and DCAD when it is included in prepartum diets. According to NASEM (2021), a mature legume has 18.1% CP and 46.6% NDF, so based on the 19.6% CP and 45.6%NDF of the alfalfa hay in the present study (Table 3:3), it can be classified as mature. Mature alfalfa is commonly avoided in the transition period due to its higher potassium (K) concentrations. Accomplishing a negative dietary cation-anion difference (DCAD), with high concentrations of potassium can be problematic since the balance [$(Na_{(\%)}/0.023 + K_{(\%)}/0.039) - (Cl_{(\%)}/0.0355 + S_{(\%)}/0.016)] \times 10 = DCAD_{(mEq/kg/DM)}$] includes potassium as a main cation. As expected, the alfalfa hay had a greater concentration of K than grass hay (2.50 vs. 1.88% K; Table 3:3), which would lead to greater inclusions of acidogenic products to obtain a similar negative DCAD. The concentration of K in the alfalfa hay used in this study was 7% greater than the mean concentration reported by NASEM (2021) for mature legume hay (2.50 vs. 2.34% K), whereas the concentration of K in the grass hay used in this study was 15% greater than the mean concentration reported by NASEM (2021) for mature grass hay (1.88% vs. 1.63% K).

Despite the greater concentration of K, alfalfa hay had a DCAD similar to that of grass hay (290 and 289 mEq/kg DM, respectively; Table 3:3), which contradicts our expectations of a greater DCAD for alfalfa hay. According to NASEM (2021) the DCAD is 387 mEq/kg DM for mature legume hay and 187 mEq/kg DM for mature grass hay, which agrees with our expectation. However, in this study the DCAD of the alfalfa hay was lower than expected and the DCAD of the grass hay was greater than expected. The unexpected DCAD differences of the forages can be attributed to two factors, soil mineral concentrations (Goff et al., 2007) and fertilization (Goff et al., 2007; Penner et al., 2008). Goff et al. (2007) sought to manipulate DCAD by raising the chloride content of alfalfa to produce a low DCAD. With the addition of 56, 112, and 168 kg of $CaCl_2$ to the soil, chloride content in alfalfa increased to 0.77, 0.87, and 0.89% Cl, and DCAD decreased to 229, 211, 194 mEq/kg of DM (Goff et al., 2007). The results of Goff et al. (2007) suggest that chloride application to the soil can increase chloride content and lower DCAD in alfalfa. Similarly, Penner et al. (2008) evaluated the effect of fertilization of timothy field with $CaCl_2$ and observed that the concentration of chloride increased (0.17 to 0.75% Cl) and the DCAD of the timothy decreased (145 to 16 mEq/kg DM). Even though we ignored them, the soil mineral concentrations, the fertilization practices, or both could explain the similar DCAD of the hays in this study.

Calcium chloride stimulates parathyroid hormone (PTH) and increases calcium resorption from bone, calcium reabsorption in the kidneys, and calcium absorption in the intestine (Verhoef et al., 2021); therefore, making it a sufficient acidogenic product. By including an acidogenic product in transition diets, with legume hay as a factor, we successfully reduced the DCAD and urine pH. Acidification is identified with a pH of 6.2 to 6.8 (Goff, 2006) and the results of our study show that with the DCADs we accomplished, (-209, -207, -190, and -194 mEq/kg/DM; Table 3:2) all animals were in an acidotic state with an average of 6.4 for urine pH (Figure 3:2). A counterargument for inducing metabolic acidosis is the depressed DMI that is commonly reported alongside it. The results from our study show that the grass-fed cows tended to consume less DMI than the alfalfa-fed cows (11.6 vs 10.8 kg/d; P = 0.07; Figure 3:1) and this reduction argues Charbonneau et al. (2006), where they found that the lower the DCAD reaches, the more significant the reduction in DMI. The NDF concentration in the four diets are similar and the grass diets have a lesser DCAD than the alfalfa diets, so we account for the reduction in DMI of the two alfalfa diets to the larger particle size of the hay. This theory correlates with Leonardi and Armentano (2003) where animals tended to consume a smaller hay particle over a larger one. Further research on the relationship between incubation time, particle size, and distribution of particle size pools could be beneficial, but since the reduction was not significant (P=0.07), this may not be necessary.

A freshened cow has a demand increase of 55 g Ca/d to then be utilized by the cow for milk and colostrum production (Goff et al., 2014; Seely et al., 2021). The skeletal supply that is typically available accounts for 6 to 10 g of the calcium in the body (Wilkens et al., 2020), but this is not enough to satisfy the increase in demand. Parathyroid hormone has an inversely proportional relationship with calcium concentrations that reestablish calcium homeostasis in the blood (Kronqvist et al., 2011a; Shappell et al., 1987). The release of PTH and calcitriol are responsible for the calcium balance that occurs through bone tissue mobilization, kidney reabsorption, and intestinal uptake (Kronqvist et al., 2011b; Megahed et al., 2018; Ramberg et al., 1976). The animals in this study had the expected reductions of blood calcium concentrations on day 0, 1, and 2 and treatments did not influence blood calcium concentrations ($P < 0.01$). Megahed et al. (2018) studied periparturient multiparous cattle and their plasma calcium concentration 9 hours before partition, and similar to the present study, saw a total calcium decrease starting on 3 days prepartum and then a gradual increase for 3 days postpartum.

In the present study, the postpartum observations and the plasma calcium results mostly did not concur. The clinical hypocalcemia blood calcium concentration cutoff is ≤5.5 mg/dL (Goff, 2008) and we observed 16% of the experimental animals in this category (Figure 3:5). Forty-six percent of the clinically hypocalcemic cows were asymptomatic (no signs of wobbly gait, cold ears, sunken eyes, or recumbency). Fifty- four percent of the experimental animals developed subclinical hypocalcemia (Figure 3:5) (5.6-7.9 mg/dL (Goff, 2008)), with 79% of those being asymptomatic. The study led to further differentiation between milk fever (≤5.5 mg/dL) and periparturient paresis (loss of muscle function), due to many of animals falling under this concentration while still maintaining muscle function.

CONCLUSIONS

The conclusion of this study is that the inclusion of alfalfa hay in prepartum diets for pregnant and non-lactating cows may not increase hypocalcemia rates when compared to prepartum diets including grass hay. This conclusion is based on whether the DCAD of alfalfa hay is also comparable to grass hay. In this regard, more research evaluating the fertility of the soil and the use of acidifying fertilizers is needed to better understand how to incorporate alfalfa hay in prepartum diets for pregnant and non-lactating dairy cows in the prepartum period.

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FIGURES AND TABLES

Table 3:1 Count of cows by parity and treatment.†

Parity	GHCL	GHPO	AHCL	AHPO
2	13	10	7	8
3	3	7	4	2
4	2	0	6	5
5	1	3	2	3
6	0	0	0	0
7	1	0	0	2
Average	2.8	2.8	3.2	3.6

† GHCL = grass hay and calcium chloride as acidogenic product, GHPO = grass hay and polyhalite as acidogenic product, AHCL = alfalfa hay and calcium chloride as acidogenic product, AHPO = alfalfa hay and polyhalite as acidogenic product.

Table 3:2 Ingredient inclusion and nutritional composition of prepartum diets containing either grass hay (GH) or alfalfa hay (AH) and calcium chloride (CL) or polyhalite (PO).

	GHCL	GHPO	AHCL	AHPO
Ingredient Inclusion, % DM				
Corn silage	38.1	38.0	40.5	39.8
Grass hay	22.7	22.6	-	-
Alfalfa hay	-	-	20.7	20.4
Corn grain	10.9	10.8	7.6	7.5
Soybean meal (48% CP)	10.9	10.4	4.2	4.0
Dry distillers' grains w/solubles	7.3	6.8	7.2	7.1
Soybean hulls	7.3	7.2	17.1	16.8
Calcium chloride (dihydrate)	2.5	-	2.5	-
Polyhalite	-	3.8	-	4.0
Mycotoxin binder†	0.182	0.181	0.180	0.177
Selenium product‡	0.027	0.027	0.027	0.027
Mineral and vitamin mix¶	0.045	0.045	0.045	0.044
Vitamin ADE mix††	0.100	0.099	0.099	0.097
Vitamin E (IU/g)	0.036	0.036	0.036	0.035
Ionophore‡‡	0.014	0.014	0.013	0.013
Nutritional Composition,¶¶ % DM				
Organic matter	92.1 ± 0.9	92.3 ± 0.3	92.6 ± 0.8	92.4 ± 0.5
Crude protein	13.1 ± 0.7	12.9 ± 2.4	14.2 ± 1.2	14.4 ± 1.4
Neutral detergent fiber	41.2 ± 3.3	41.9 ± 2.4	39.2 ± 1.7	37.5 ± 2.2
Starch	22.0 ± 7.2	21.6 ± 6.7	20.4 ± 2.1	21.7 ± 6.8
Calcium	1.10 ± 0.12	0.85 ± 0.05	1.38 ± 0.02	1.20 ± 0.14
Sodium	0.07 ± 0.01	0.09 ± 0.01	0.09 ± 0.05	0.11 ± 0.02
Potassium	1.29 ± 0.17	1.63 ± 0.10	1.33 ± 0.18	1.54 ± 0.22
Chloride	1.31 ± 0.53	0.26 ± 0.03	1.56 ± 0.04	0.91 ± 0.44
Sulfur	0.32 ± 0.26	0.95 ± 0.06	0.21 ± 0.02	0.61 ± 0.22
DCAD, mEq/kg DM	-209 ± 30	-207 ± 45	-190 ± 34	-194 ± 57

† Mycosorb (Alltech, Inc; Nicholasville, KY).

‡ Sel-Plex 600 (Alltech, Inc).

¶ Contained 22.3% calcium; 7.5% magnesium; 2.8% potassium; 3.9% sulfur; 1.5% manganese; 1.5% zinc; 9,500 ppm iron; 2,500 ppm copper; 200 ppm iodine; 200 ppm cobalt; 66 ppm selenium; 227,273 IU/kg Vitamin A; 136,364 IU/kg Vitamin D3; 636 IU/kg Vitamin E.

†† Contained 3500 IU/kg Vitamin A; 950 IU/kg Vitamin D3; 2000 IU/g Vitamin E.

‡‡ Rumensin 90 (Elanco Animal Health; Greenfield, IN).

¶¶ Mean ± SD (n = 8).

Table 3:3 Nutritional composition† of forages.

	Corn silage	Grass hay	Alfalfa hay
Dry matter, % as-fed	36.3 ± 3.0	86.0 ± 9.8	86.8 ± 7.4
Organic matter, % DM	97.1 ± 0.4	93.9 ± 0.7	90.2 ± 1.1
Crude protein, % DM	7.8 ± 0.7	7.5 ± 1.3	19.6 ± 2.0
Neutral detergent fiber, % DM	38.6 ± 3.7	74.9 ± 2.6	45.6 ± 4.4
Starch, % DM	33.9 ± 3.4	1.9 ± 0.9	2.4 ± 0.9
Calcium, % DM	0.20 ± 0.04	0.36 ± 0.04	1.52 ± 0.13
Sodium, % DM	0.01 ± 0.01	0.02 ± 0.02	0.16 ± 0.10
Potassium, % DM	0.75 ± 0.14	1.88 ± 0.21	2.50 ± 0.45
Chloride, % DM	0.08 ± 0.04	0.38 ± 0.09	0.77 ± 0.16
Sulfur, % DM	0.10 ± 0.04	0.15 ± 0.02	0.32 ± 0.02
Cation-Anion Difference, mEq/kg DM	99.20± 3.21	288.88±4.44	289.75± 5.89

† Mean ± SD (n = 8).

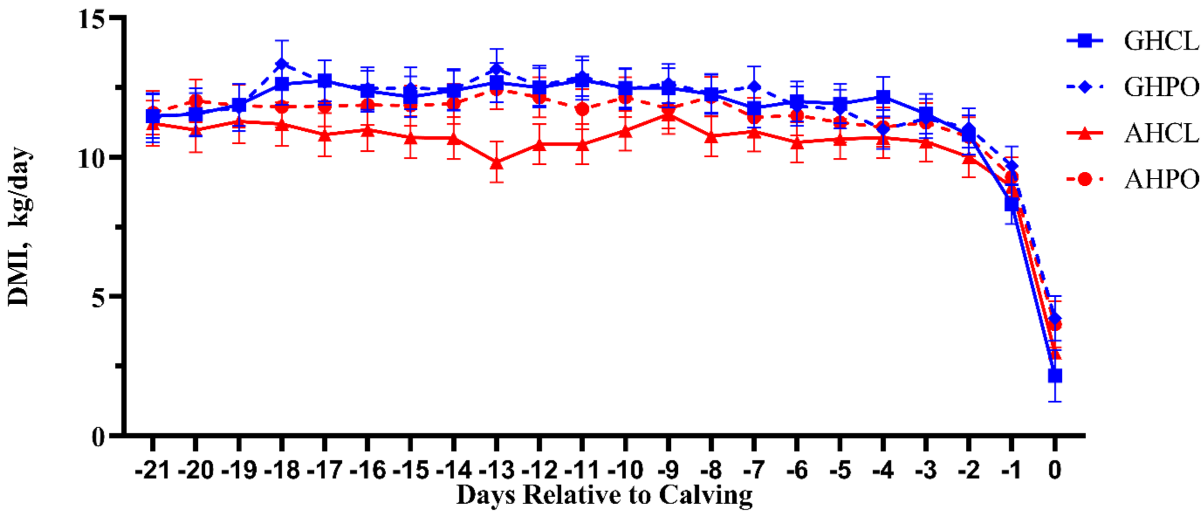

Figure 3:1 *Dry Matter Intake of pregnant and non-lactating dairy cows during the prepartum period. Cows consuming the Grass Hay/Calcium Chloride (GHCL; Square; DCAD=-208.9 mEq/kg of DM), Grass Hay/Polyhalite (GHPO; Diamond; DCAD= -206.7 mEq/kg of DM); Alfalfa Hay/Calcium Chloride (AHCL; ; Triangle; DCAD= -190.1 mEq/kg of DM); Alfalfa Hay/Polyhalite (AHPO; Circle; DCAD= -194 mEq/kg of DM). Tendency for an effect of diet (P<0.09); time effect (P<0.01). Error bars represent standard errors of the means (SEM).*

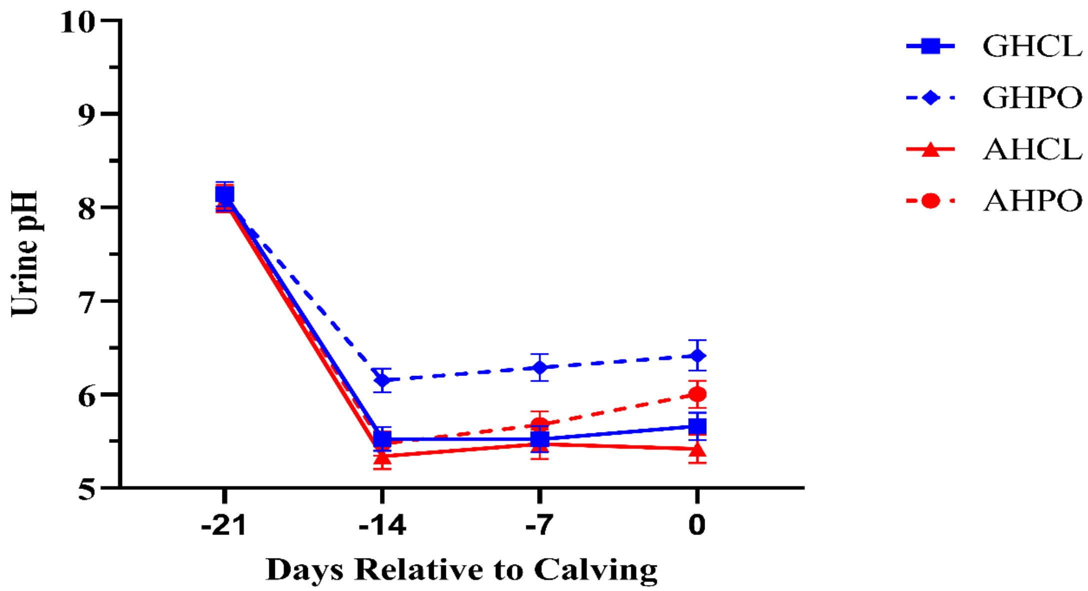

Figure 3:2*Urine pH of pregnant and non-lactating dairy cows during the prepartum period. Cows consuming a common diet until day -21. Cows consuming the Grass Hay/Calcium Chloride (GHCL; Square; DCAD=-208.9 mEq/kg of DM), Grass Hay/Polyhalite (GHPO; Diamond; DCAD= -206.7 mEq/kg of DM); Alfalfa Hay/Calcium Chloride (AHCL; ; Triangle; DCAD= -190.1 mEq/kg of DM); Alfalfa Hay/Polyhalite (AHPO; Circle; DCAD= -194 mEq/kg of DM). Main effect of day (P<0.01); Main effect of diet (P<0.01); Main effect of acidogenic product (P<0.01); Acidogenic product by day interaction (P< 0.0032). Error bars represent standard errors of the means (SEM).*

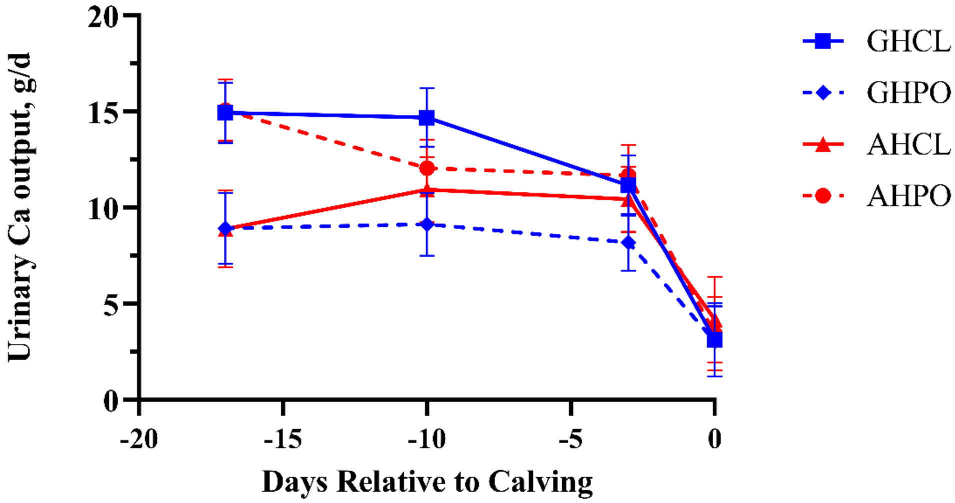

Figure 3:3 *Urine calcium output of pregnant and non-lactating dairy cows during the prepartum period. Cows consuming a common diet until day -21 Cows consuming the Grass Hay/Calcium Chloride (GHCL; Square; DCAD= -208.9 mEq/kg of DM), Grass Hay/Polyhalite (GHPO; Diamond; DCAD= -206.7 mEq/kg of DM); Alfalfa Hay/Calcium Chloride (AHCL; ; Triangle; DCAD= -190.1 mEq/kg of DM); Alfalfa Hay/Polyhalite (AHPO; Circle; DCAD= -194 mEq/kg of DM). Main effect of day (P<0.01); Tendency for an effect of diet (P<0.07); Main effect of the interaction between diet and day (P < 0.04). Error bars represent standard errors of the means (SEM).*

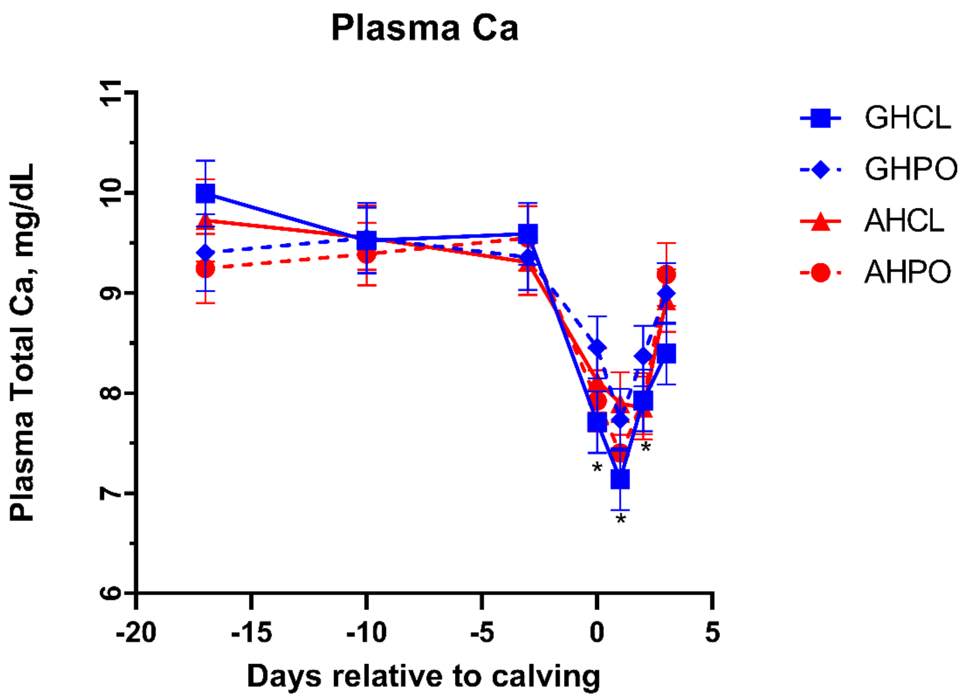

Figure 3:4 *Plasma total calcium of pregnant and non-lactating dairy cows during the prepartum period. Cows consuming a common diet until day -21 Cows consuming the Grass Hay/Calcium Chloride (GHCL; Square; DCAD= -208.9 mEq/kg of DM), Grass Hay/Polyhalite (GHPO; Diamond; DCAD= -206.7 mEq/kg of DM); Alfalfa Hay/Calcium Chloride (AHCL; ; Triangle; DCAD= -190.1 mEq/kg of DM); Alfalfa Hay/Polyhalite (AHPO; Circle; DCAD= -194 mEq/kg of DM). Main effect of day (P < 0.001). Error bars represent standard errors of the means (SEM).*

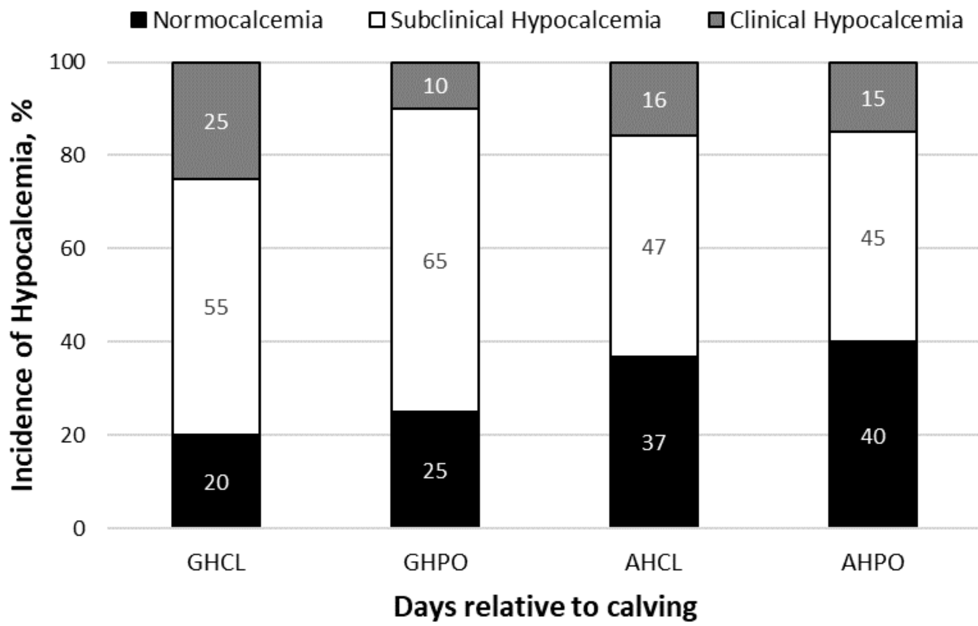

Figure 3:5 *Incidence or normocalcemia ([Ca] > 8.0 mg/dL during the first 4 days after calving), subclinical hypocalcemia (5.5 < [Ca] < 8.0 mg/dL at least once in the first 4 days after calving), and clinical hypocalcemia ([Ca] < 5.5 mg/dL at least once in the first 4 days after calving) in pregnant and non-lactating dairy cows during the prepartum period. Cows consumed diets containing grass hay and calcium chloride as an acidogenic product (GHCL; DCAD = -209 mEq/kg DM), grass hay and polyhalite as an acidogenic product (GHPO; DCAD = -207 mEq/kg DM), alfalfa hay and calcium chloride as an acidogenic product (AHCL; DCAD = -190 mEq/kg DM), or alfalfa hay and polyhalite as an acidogenic product (AHPO; DCAD = -194 mEq/kg DM).*