

EFFECT OF PRE-PARTUM DIETARY CATION-ANION DIFFERENCE ON THE PERFORMANCE OF TRANSITION SAHIWAL CATTLE

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ABSTRACT

Dietary cation anion difference (DCAD) is an important aspect of dairy nutrition, especially in the transition period. Sahiwal cattle is the highest milk producing breed among Zebu cattle. We planned first study on transition Sahiwal cattle to determine the effects of feeding varying levels of negative DCAD. For this purpose, twenty pregnant cows (at the 250th day of gestation) were selected and randomly divided into 5 groups comprising four animals each. Five *iso-caloric* (2100 Kcal) and *iso-nitrogenous* (12%) diets were formulated and each diet was allotted to each group. The animals who received positive DCAD diet (+134.32 mEq/Kg DM) served as control. Diets were supplemented with NutriCAB® to attain 0, -15, -30 and -45 mEq/kg DM DCAD levels. Experimental diets were fed at *ad-libitum* upto parturition and data regarding feed intake were recorded daily. Post-partum incidence of milk fever, dystocia, retention of placenta (RP), mastitis as well as milk production, milk fat percentage and serum Ca levels were recorded. Urine and blood pH were determined weekly during the last month of pregnancy. Results showed that prepartum feed intake and blood pH were not affected ($P>0.05$), while urine pH was significantly reduced ($P<0.05$) by lowering DCAD levels. Post parturient blood calcium level linearly increased ($P<0.05$) with decreasing DCAD. Pre-partum negative DCAD feeding had no significant effect ($P>0.05$) on post-parturient milk production and fat percentage. However, subclinical milk fever, RP and clinical mastitis decreased with decreasing DCAD feeding. It was concluded that negative DCAD feeding raised serum calcium level and reduced the incidence of post-parturient problems in Sahiwal cattle.

Key words: Dairy cattle, transition, metabolic diseases, reproductive disorders, incidence.

Abbreviations used: DCAD = (dietary cation anion difference), Ca = (calcium), RP = (retention of placenta).

INTRODUCTION

Efficient feeding of dairy cows during transition period is a key for successful dairy enterprise. Transition period of the dairy cow is important for milk production and dairy cow health (Keady *et al.*, 2001). This period covers three weeks before and three weeks after parturition. Energy requirement, endocrinological and metabolic changes occur during this period associated with dietary cation anion difference (DCAD) (Piccione *et al.*, 2012). Maintenance, growth, gestation and lactation determine the demand of dietary minerals (Nielsen and Volden *et al.*, 2011). Diets having negative DCAD create acidic conditions in body results in improved parathyroid hormone functions. Minerals play an important role in body physiology and acid-base balance (Afzaal *et al.*, 2004). The pH of biological fluids is directly linked with biochemical reactions essential for life support functions. Positively charged minerals impact alkalogenically; whereas, the negatively charged minerals have acidogenic effect on body fluids (Block, 1994). High pH of blood reduces the receptivity of tissues (bones and kidney) for parathyroid hormones (PTH). This condition results in lower calcium mobilization from bones and poor conversion of Vitamin D into 1, 25-

dihydroxyvitamin D by kidneys, causing negative effect on Ca absorption from gastrointestinal tract (Goff, 2008).

Inability of dairy animal to maintain normal Ca results in milk fever (De Garis and Lean, 2008). Sub-optimum Ca homeostasis increases the risk of periparturient problems such as dystocia, retained placenta (RP), metritis, mastitis (Mulligan *et al.*, 2006). Various prophylactic measures are in vogue to prevent milk fever. These techniques includes oral drenching or feeding of lower Ca diet (<20g/day), administration of vitamin D and feeding of lower DCAD diet (Thilsing *et al.*, 2002). Recent research advocates the benefits of DCAD feeding to attain the desired blood ionic Ca level and to reduce milk fever and parturient problems (Shire and Beede, 2013). Usually, calcium chloride (CaCl₂) is given as anionic salt. It raises the level of chloride ions (Cl⁻) without rising sodium or potassium ions (Na⁺, K⁺) causing acidifying effect (National Research Council, 2001). Irritation, bitterness and poor palatability are the problems associated with CaCl₂ supplementation ((Lawless *et al.*, 2003; Scott and Vijk, 2000; Scott and Vijk, 2002; Tucker *et al.*, 1991). To overcome this problem anionic salts are always mixed with feed to avoid reduction in feed intake (Goff, 2008). We used a commercially available negative DCAD diet "NutriCAB®". This anionic product also contains CaCl₂,

encapsulated in pan coating to ensure better palatability and intestinal release without affecting feed intake.

Sahiwal cattle is the most important cattle milch breed of Pakistan including tropical regions of world; having highest milk production among Zebu cattle with docile temperament, heat tolerant, tick, drought, parasite and bloat resistance and ease of calving characteristics. Keeping in view the above mentioned scenario, we aimed first study to investigate the effects of feeding different levels of negative DCAD diet "NutriCAB®" on productive performance, Ca status and postpartum problems in Sahiwal cattle.

MATERIALS AND METHODS

Experimental animals and feeding: The experiment was conducted at Livestock Experiment Station, Khizerabad, Sargodha, Pakistan for 90 days (30 days before and 60 days after parturition) during August to October, 2015. Twenty pregnant Sahiwal cows (3rd parity) at their last months of pregnancy were selected and randomly divided into five groups comprising four animals each under randomized complete block design (RCBD). The diets were formulated having 2100 Kcal energy and 12% protein according to NRC recommendations (Table 1; National Research Council, 2001). Each diet was randomly allotted except control who received positive DCAD. Diets were supplemented with NutriCAB® (Table 2) to attain 0, -15, -30, -45 mEq/Kg DM DCAD levels (Table 3). The experimental animals were kept on concrete floor in an isolated shed for 15 days of adaptation period. After adaptation cows were tied and experimental diets were provided at *ad-libitum* up to 4 weeks before parturition. After parturition the animals were fed according to their production performance. Concentrate was fed at the rate of 2Kg/animal once in a day and chopped (2cm) fresh Rhode grass was fed at *ad-libitum* basis in individual mangers till parturition. Fresh drinking water was available around the clock throughout experiment. However, just after calving experimental animals were fed normal routine diet with positive DCAD (121.3 mEq/Kg).

Sampling: All experimental animals were provided green fodder early in morning and orts were collected in the next morning to calculate feed intake. Feed intake of each animal was calculated as: (Feed intake (Kg) = Total amount of feed offered – feed refused) (Norrish and Hutton, 1977). Daily feed intake was noted from last month of parturition till calving. Post parturient milk production was recorded up to 60 days. Animal were milked twice a day (4:00 am and 3:00 pm) by manual method. Milk production of animals was recorded on daily basis. Milk fat percentage was recorded weekly during first month of lactation. Approximately 100 ml

fresh milk sample (50 ml of each time) was taken in a beaker for milk fat determination. Incidence of milk fever was observed during 12 hours before and 7 days after calving using clinical signs and monitoring serum Ca level. Cows showed clinical signs or <7.5 mg/dl serum Ca level were considered a case of milk fever (Massey *et al.*, 1993; Oetzel *et al.*, 1998). Blood samples were collected weekly at 28, 21, 14 and 7 day before calving. Urine and blood pH were determined weekly during the last month of pregnancy. Five milliliter of blood was collected in gel containing test tube by jugular vein puncture with 16 gauge intravenous needle and serum was separated for pH determination. Blood samples were collected 6 to 12 hours after feeding the experimental diets (Tucker *et al.*, 1991). Midstream urine sample was collected in 100 ml container weekly (day 28, 21, 14, 7) by spontaneous or manually stimulated urination (Van Dijk and Lourens, 2001). It was performed 3-4 hours after feeding experimental diet. For determination of serum Ca, blood samples were taken within 24 hours after calving and allowed to clot at room temperature. Serum was separated by centrifugation and kept at 4°C in capped polypropylene tubes (Miles *et al.*, 2001). The experimental cows were monitored for post-partum diseases and disorders including milk fever, dystocia, RP and clinical mastitis.

Laboratory analysis: Gerber method was used to evaluate fat percentage at Nestle Collection Centre, Khizerabad, District Sargodha, Pakistan. Approximately 100 ml milk sample (50 ml per time) was taken in a beaker. Ten millilitre of H₂SO₄, 10.94 ml milk, 1ml alcohol was added and shaken after insertion of stopper in Butyrometer. Then, it was placed in Gerber centrifuge machine for 5 minutes at 1500 rpm. The fat contents moved on top and milk fat percentage reading was recorded (James, 1995).

For pH determination, a drop of serum/urine sample was taken with help of dropper and placed on pH strip. After one minute color of strip was matched with standard pH value (Spanghero, 2004). Serum Ca was evaluated with semi-automated chemistry analyser (Microlab 300; Massanyi *et al.*, 2007) at University Medical Complex and Research Centre, University of Sargodha, Sargodha, Pakistan.

Extrapolation of economic losses and benefit cost ratio: Losses due to incidence of milk fever, clinical mastitis, dystocia and retention of placenta (RP) were assessed on the basis of following parameters and equations as well as consultation with local veterinarians (Dematawewa and Berger, 1997; Thirunavukkarasu *et al.*, 2010; Ashraq *et al.*, 2015).

Parameters: Loss of milk production
Treatment expenses
Veterinarian charges

Value of discard milk
 Loss in animal sale value
 Cost of NutriCAB® diet

Equations: Total losses due to milk fever = Value of milk loss + Veterinarian charges + Treatment cost + Loss in animal sale value

Total losses due to retention of placenta = Value of milk loss + Veterinarian charges Treatment cost + Loss in animal sale value

Total losses due to dystocia = Value of milk loss + Veterinarian charges + Treatment cost + Loss in animal sale value

Total losses due to clinical mastitis = Value of milk loss + Veterinarian charges treatment cost + Value of discarded milk + Loss in animal sale value

Benefits from control of a particular disease
 The benefit cost ratio= -----
 Prevention cost of the disease

Statistical analysis: The results were statistically analyzed under RCBD by using general linear model and means of different treatments were separated by Tukey's test (Steel *et al.*, 1997). In addition to compare means polynomial regression analysis was performed to the linear or quadratic effects of treatments. The cost benefit ratio was analyzed by simple addition and subtraction (Thirunavukkarasu *et al.*, 2010).

RESULTS

Average feed intake was not affected by decreasing DCAD levels ($P>0.05$) during last month of pregnancy in Sahiwal cattle (Table 4). During transition period blood Ca had increased linearly ($P<0.05$) with decreasing DCAD levels in Sahiwal cattle. Highest blood Ca was recorded at -45 DCAD and lowest at positive DCAD (Table 4). Postpartum, total and average milk production had no significant change ($P>0.05$) by decreasing DCAD diet during early transition phase (Table 4). Milk fat percentage of Sahiwal cattle did not show any change by decreasing the levels of DCAD in diet ($P>0.05$; Table 4).

Results of present study showed that weekly and average blood pH was not influenced ($P>0.05$) by negative DCAD feeding in Sahiwal cows (Table 4). Urine pH was not affected ($P>0.05$) by decreasing DCAD level during 1st and 2nd week of trial. While in 3rd and 4th week a linear decrease ($P<0.05$) was observed by decreasing DCAD level. However, lowest pH was recorded at -45 DCAD and highest at positive DCAD during 3rd and 4th week of trial (Table 4).

The negative DCAD diet considerably reduced the incidence of postparturient problems in experimental animals viz. subclinical milk fever, RP and clinical mastitis. Whereas; no case of clinical milk fever was observed (Table 5).

Economic losses due to incidence of subclinical milk fever (A) in Sahiwal cattle at Khizerabad, District Sargodha during July to October, 2015: Subclinical milk fever in control group = 25%

Subclinical milk fever in DCAD treated groups = 6.25%

Reduction in incidence = 25 - 6.25 = 18.75%

Number of animals prevented= 03

Per animal economic losses of subclinical milk fever

(A)= 114.3 \$

Benefit of subclinical milk fever (A) prevention = 114.3 \$

Total benefit of subclinical milk fever (A) =

3×114.3=342.9 \$

Per animal cost of feed= 15.75 \$

Economic losses due to RP (B) in Sahiwal cattle at Khizerabad, District Sargodha during July to October, 2015:

RP in positive DCAD group = 25%

RP in negative DCAD group = 0%

Reduction in incidence of RP= 25 - 0 = 25

Number of animals prevented= 04

Per animal economic losses of retention of placenta

(B)=07.9 \$

Benefit of retention of placenta (B) prevention=07.9 \$

Total benefit of retention of placenta (B)= 4×7.9=31.6 \$

Per animal cost of feed= 15.75 \$

Economic losses due to incidence dystocia in Sahiwal cattle at Khizerabad, District Sargodha during July to October, 2015: Cases of dystocia in positive DCAD group = 0%

Cases of dystocia in negative DCAD group = 0%

The DCAD diet had no effect on dystocia.

Economic losses due to incidence of clinical mastitis (C) in Sahiwal cattle at Khizerabad, District Sargodha during July to October, 2015:

Incidence of clinical mastitis in control group = 25%

Incidence of clinical mastitis in DCAD treated group = 0%

Reduction in incidence of clinical mastitis = 25 - 0 = 25

Number of animals prevented= 04

Per animal economic losses of retention of placenta (B) = 51.6 \$

Benefit of retention of placenta (B) prevention = 51.6 \$

Total benefit of retention of placenta (B) = 4×51.6=206.4 \$

Per animal cost of feed= 15.75 \$

Total loss prevented/benefit (D) due to sub-clinical milk fever (A), retention of placental (B) and clinical mastitis (C) in Sahiwal cattle at Khizerabad, District Sargodha during July to October, 2015=(D)=

342.9+31.6+206.4 = 580.9 \$

Over all benefit of using NutriCAB® supplementation:

Total cost of feed supplement (E) = 252 \$ (@ 15.75 per animal)

Profit due to over milk increase using NutriCAB[®] supplementation (F) = 104.8 \$
 Total profit G (D+F) = 580.9 + 104.8 = 685.7
 Net profit (G-E) = 685.7 - 252 = 433.7 \$ (for 16 experimental animals except control)

Benefit cost ratio:

Disease	Benefit (\$)	Cost (\$)	Ratio
Milk fever	114.3	15.75	7.3
Retention of placenta	7.9	15.75	0.5
Clinical mastitis	51.6	15.75	3.3

DISCUSSION

Average feed intake was not affected by decreasing DCAD levels ($P > 0.05$) during last month of pregnancy in Sahiwal cattle (Table 4). Similar findings are reported by Herson *et al.* (2010) and Weichert *et al.* (2013). Modified palatability of commercial products or higher herd dry matter intake or lesser inclusion level of anionic salt would be the outcome for unaffected feed intake (Hutjens, 1991; Oetzel *et al.*, 1991; DeGroot *et al.*, 2010). On the contrary, some researchers depicted that feed intake decreased by lowering DCAD diet (Chan *et al.*, 2006; Luebke *et al.*, 2011). Reduction in feed intake might be due to systemic acidosis or physiological and endocrinological changes during transition phase (Holtenius *et al.*, 2003; Plaizier *et al.*, 2008).

During transition phase, blood Ca had increased linearly ($P < 0.05$) with decreasing DCAD levels in Sahiwal cattle. Highest blood Ca was recorded at -45 DCAD and lowest at positive DCAD (Table 4). These findings are supported by various researchers who reported that feeding of lower DCAD diet before calving have substantial effect on blood Ca status (Grunberg *et al.*, 2011; Oba *et al.* 2011; Reece 2009). Results of current study are contradictory to the outcomes, who stated that the blood Ca status remained unaffected by varying DCAD levels during transition phase (Goff and Horst, 2004; Rezac *et al.*, 2014). The Ca and K contents in feed including ecological and environmental stress could be involved for impassive Ca level (Nieves *et al.*, 2009).

Postpartum average milk production had no significant change ($P > 0.05$) by decreasing DCAD diet during early transition phase (Table 4). This might be due to generation of slight acidic environment in rumen by lower DCAD diet (Atkinson, 2014) or genetic makeup (Koivula *et al.*, 2005). Outcomes of current study are similar with Martinez *et al.* (2012); Weich *et al.* (2013) and Silva (2015) who demonstrated that prepartum negative DCAD feeding had no effect on postpartum average milk production. Contradictory results were reported by Ganjkanlou *et al.* (2010) and Jawor *et al.* (2012) who reported that post parturient milk production was increased by prepartum negative DCAD diet. Increased milk production might be associated with

nutrient composition of postpartum diet and level of production (Rekhis *et al.*, 2001; Suttle and McLauchlan, 2010).

Milk fat percentage of Sahiwal cattle did not show any change by decreasing the levels of DCAD in diet ($P > 0.05$; Table 4). Result of this finding are in agreement with finding of Chamberlin *et al.* (2013) who mentioned that fat percentage was not affected by different DCAD feeding during early transition period. Conversely, positive DCAD of diet had direct effect on fat percentage (Hu and Kung-Jr, 2009). The improvement in fat percentage after calving might be related to increase in DMI by positive DCAD diet (Hu and Murphy, 2004); this increased the rumen pH and stimulate fermentation process to synthesize volatile fatty acids (Kolver and deVeth, 2002); consequently de novo synthesis of fatty acid takes place in mammary cells (Roche *et al.*, 2005).

Results of present study showed that weekly and average blood pH was not influenced ($P > 0.05$) by negative DCAD feeding in Sahiwal cows (Table 4, 6). Similar findings were reported by Grunberg *et al.* (2011) and Ganjkanlou *et al.* (2010) while contradictory results were reported by Goff (2008); they described that negative DCAD diet had direct impact on blood pH. The high concentration of anionic salts in diet supported the strong ion difference theory; this help to determine the mechanism of acid-base disorders (Gelfert *et al.*, 2006; Neligan and Deutschman, 2014). Change in Na^+ , K^+ and Cl^- ions (strong ions) of extracellular fluid causes alteration in acid based balance (Stewart, 1983).

Urine pH was not significantly affected ($P > 0.05$) by decreasing DCAD level during 1st and 2nd week of trial. While in 3rd and 4th week a linear decrease ($P < 0.05$) was observed by decreasing DCAD level (Table 7). However, lowest pH was recorded at -45 DCAD and highest at positive DCAD during 3rd and 4th week of trial (Table 4). These results are in accord with the finding of Grunberg *et al.* (2011) and Wu (2011) who stated that diet with lower DCAD level had significant effect on urine pH. The possible reason of reduction in urine pH was the acidifying effect of negative DCAD diet (Penner *et al.*, 2008; Rerat *et al.*, 2009). Monovalent ions possess high bioavailability that strictly impact blood acid base status (Goff *et al.*, 2004). However, Lean and DeGaris [2010] stated that varying DCAD levels had no impacts on urine pH. Improper consumption of anionic salt could be the possible reason for this result (Block, 2011).

The negative DCAD diet considerably reduced the risk of postparturient problems in experimental animals viz. subclinical milk fever, RP and clinical mastitis. Whereas; no case of clinical milk fever was observed (Table 5); as supported by various researchers (Crnkic *et al.*, 2010; Ghattas, 2014; Klos *et al.*, 2015; Sakha *et al.*, 2014). However, results of present study are inconsistent with the finding of Melendez *et al.* (2004),

Hu *et al.*(2007), Gulayet *al.*(2005) who reported that negative DCAD diet did not reduce the incidence of post parturient problems. Various factors including age, breed, milk production, hormonal changes and type of forages (feeding of forages containing high Kand Ca) during early transition (Rehageand Kaske, 2004; Taylor *et al.*, 2008). Demineralization process of bones in dairy cows

decrease with increasing age (Sjaastad *et al.*, 2010). Whereas; Goff (2008), Mulligan and Doherty (2008) and Oetzel (2011) described that hypocalcaemia is directly associated with dystocia, RP and mastitis because of the significant role of Ca in muscle functioning and immune system (Shire and Beede. 2013; Santos *et al.*, 2011).

Table 1. Ingredient composition of experimental ration

Ingredient	Percentage	CP	ME (Mcal/Kg)
Cotton seed Cake	12	2.58	0.2568
Linseed Cake	4	1.2888	0.1152
Sorghum	20	3.15	0.568
Rhode grass	27	2.43	0.5481
Wheat Bran	7	0.959	0.1645
Wheat Straw	27	0.648	0.3888
Mustard Cake	3	0.969	0.912
Total	100	12.0248	2.1326

Items (%)	DCAD ¹			
	0	-15	-30	-45
Positive	1	1	0	0
Retention of placenta	1	0	0	0
Dystocia	0	0	0	0
Clinical mastitis	1	0	0	0

Ingredient composition of post-partum concentrate

Table 2. Analytical constituents of NutriCAB.

Composition		
Sr. No	Constituents	Percentage
1	Calcium	28
2	Phosphorus	0.1
3	Sodium	0.2
4	Potassium	0.1
5	Chloride	48
6	Sulphur	0.01

Table 3. Allotment of treatments to the experimental groups.

Experimental group	NutriCABg/day per cow	Levelsmeq/Kg
Group A	156	-45
Group B	143	-30
Group C	129	-15
Group D	117	0
Group E	0	+134.32

Table 4. Dietary means for feed intake, blood calcium, milk production, blood and urine pH in Sahiwal cattle.

Items	DCAD ¹					SE	Significance	
	Positive	0	-15	-30	-45		Linear	Quadratic
Dry matter intake (Kg)	10.97	10.97	10.94	10.97	10.94	0.036	0.53	0.97
Blood Ca mg/dL	8.475 ^a	8.925 ^{bc}	9.700 ^{ab}	10.100 ^{ab}	10.350 ^a	0.3958	<0.01*	0.96

Total milk (L in 60 days)	287.9	259.7	290.5	318.9	311.9	27.435	0.57	0.98
Milk production (L/day)	4.8	4.3	4.8	5.3	5.1	0.4573	0.62	0.90
Milk fat (%age)	4.0	4.0	4.0	4.1	4.1	0.353	0.46	0.87
Average blood pH	7.93	7.93	7.93	7.87	7.68	0.1271	*0.041	0.12
Average urine pH	7.93	7.93	7.93	7.87	7.68	0.1271	*0.041	0.12

DCAD¹ (Dietary Cation Anion Difference) + ive, 0, -15, -30 and -45 meq/Kg of dry matter indicate inclusion of NutriCAB[®] at the rate of 0, 117, 129, 143 and 156g/day per animal, respectively.

^{abc} Within a row, means sharing different superscripts differ significantly (P<0.05).

SE = standard error

Table 5. Effect of dietary cation anion difference on incidence post parturient problems of transition Sahiwal dairy cattle.

Ingredients	Percentage	CP%	ME (Mcal/Kg)
Cotton seed cake	15	5.85	0.38
Canola meal	9	3.4	0.25
Maize grain	43	3.87	1.21
Wheat bran	16	2.43	0.36
Molasses	12	0.53	0.23
Mineral mixture	1	-	-
Salt	1	-	-
Sodium Bicarbonate	1	-	-
DCP	2	-	-
Total	100	16.08	2.42

DCAD¹ (Dietary Cation Anion Difference) + ive, 0, -15, -30 and -45 meq/Kg of dry matter indicate inclusion of NutriCAB[®] at the rate of 0, 117, 129, 143 and 156g/day per animal, respectively.

Table 6. Effect of dietary cation anion difference on blood pH of transition Sahiwal dairy cattle.

Blood pH	DCAD¹					SE	Significance	
	Positive	0	-15	-30	-45		Linear	Quadratic
Week one	8.0	8.0	8.0	8.0	8.0	0	0.24	0.80
Week two	8.0	8.0	8.0	8.0	8.0	0	0.82	0.89
Week three	8.0	8.0	8.0	7.75	7.5	0.2236	0.52	0.72
Week four	7.75	7.75	7.75	7.75	7.25	0.3651	0.48	0.67
Average pH	7.93	7.93	7.93	7.87	7.68	0.1271	0.04	0.12

DCAD¹ (Dietary Cation Anion Difference) + ive, 0, -15, -30 and -45 meq/Kg of dry matter indicate inclusion of NutriCAB[®] at the rate of 0, 117, 129, 143 and 156g/day per animal, respectively.

NS = non-significant (P >0.05). * = Significant (P <0.05).

Table 7. Effect of dietary cation anion difference on urine pH of transition Sahiwal dairy cattle.

Urine pH	DCAD¹					SE	Significance	
	Positive	0	-15	-30	-45		Linear	Quadratic
Week one	8.0	8.0	8.0	8.0	8.0	0.1826	0.73	0.83
Week two	8.0	8.0	8.0	8.0	7.7	0.1581	0.056	0.97
Week three	8.0 ^a	7.7 ^{ab}	7.7 ^{ab}	7.25 ^{ab}	7.0 ^b	0.2661	0.02	0.67
Week four	7.75 ^a	7.3 ^{ab}	7.0 ^{ab}	6.8 ^{ab}	6.3 ^b	0.4378	0.02	0.45
Average pH	8.06 ^a	7.75 ^{ab}	7.68 ^{ab}	7.5 ^{ab}	7.25 ^b	0.1790	0.041	0.12

DCAD¹ (Dietary Cation Anion Difference) + ive, 0, -15, -30 and -45 meq/Kg of dry matter indicate inclusion of NutriCAB[®] at the rate of 0, 117, 129, 143 and 156g/day per animal, respectively.

^{abc} Within a row, means sharing different superscripts differ significantly (P<0.05). NS = non-significant (P >0.05). * = Significant (P <0.05).

Conclusion: Blood ionic Ca had improved by providing negative DCAD supplementation. This helped to reduce the incidence of postparturient problems (subclinical milk

fever, RP, and mastitis) in moderate milk producing cattle breed i.e. Sahiwal cattle. The supplementation of anionic

salt would be economical for dairy animals with high incidence of clinical milk fever.

REFERENCES

- Afzaal, D., M. Nisa, M. A. Khan and M. Sarwar, 2004. A review on acid base status in dairy cow: Implications of dietary cation anion balance. *Pakistan Vet. J.*, 24: 199-202.
- Ashfaq, M., A. Razzaq, Shamsheer-ul-Haq and G. Muhammad, 2015. Economic analysis of dairy animal diseases in Punjab: A case study of Faisalabad district. *J. Anim. Plant Sci.*, 25: 1482-1495.
- Atkinson, O., 2014. Prevalence of Subacute Ruminant Acidosis (SARA) on UK dairy farms. *Cattle Practice*. 22: 1-9.
- Bender, S., C.C.Gelfert and R.Staufenbiel, 2003. Use of urine samples for monitoring acid-base-equilibrium in herd health management of dairy cattle herds. *Tieraerztliche Praxis*. 31: 132-142.
- Block, E., 1994. Manipulation of dietary cation-anion difference (DCAD) on nutritionally related production diseases, productivity, and metabolic responses of dairy cows. *J. Dairy Sci.*, 77: 1437-1450.
- Block, E., 2011. Revisiting Negative Dietary Cation-Anion Difference Balancing for prepartum cows and its Impact on Hypocalcaemia and Performance. Princeton: ARM AND HAMMER Animal nutrition.
- Brown, G.J., 2013. A review of the economic effect of bovine dystocia. Department of reproduction animal studies, university of Pretoria; Geoff.brown@up.ac.za. Page no.2.
- Chamberlin, W. G., Middleton, J. R., Spain, J. N., Johnson, G. C., Eilersieck, M. R., and Pithua, P., 2013. Subclinical hypocalcemia, plasma biochemical parameters, lipid metabolism, postpartum disease, and fertility in postparturient dairy cows. *J. Dairy Sci.* 96: 7001-7013.
- Chan, P. S., West, J. W. and Bernard J. K., 2006. Effect of prepartum dietary calcium on intake and serum and urinary mineral concentrations of cows. *J. Dairy Sci.* 89:704-713.
- Cifuentes, M., and Rojas, C. V., 2008. Antilipolytic effect of calcium-sensing receptor in adipocytes. *Mol. Cell. Biol.* 319: 17-21.
- Crnkic, C., Muratovic, S., Piplica, S., Kavazovic, A. and Kutlaca, S., 2010. Blood plasma mineral profile and health status in postpartum cow fed anionic diet before parturition. *Turk. J. Vet. Anim. Sci.* 34: 255-260.
- DeGaris, P. J., and Lean, I. J., 2008. Milk fever in dairy cows: A review of pathophysiology and control principles. *Vet. J.* 176: 58-69.
- DeGaris, P. J., Lean, I. J., Rabiee, A. R. and Stevenson, M. A., 2010. Effects of increasing days of exposure to prepartum diets on the concentration of certain blood metabolites in dairy cows. *Aust. Vet. J.* 88:137-145.
- DeGroot, M. A., Block, E., and French, P. D., 2010. Effect of prepartum anionic supplementation on periparturient feed intake, health, and milk production. *J. Dairy Sci.* 93: 5268-79.
- Dematawewa, C. M. and Berger, P. J., 1997. Effect of dystocia on yield, fertility and an economic evaluation of dystocia scores for Holsteins. *J. Dairy Sci.* 80: 754-61.
- Dubuc, J., Duffield, T. F., Leslie, K. E., Walton, J. S. and LeBlanc, S. J., 2010. Risk factors for postpartum uterine diseases in dairy cows. *J. Dairy Sci.* 93: 5764-5771.
- Elrod, C. C., and Butler, W. R., 1993. Reduction of fertility and alteration of uterine pH in heifers fed excess ruminally degradable protein. *J. Anim. Sci.* 71: 694-701.
- Espino, L., Santamarina, F. M. L., Santamarina, G., Goico, A. and Fidalgo, L. F., 2005. Effect of dietary cation anion difference on blood cortisol and ACTH level in reproducing ewes. *J. Vet. Med.* 52: 8.
- Frick, K. K., Krieger, N. S., Nehrke, K. and Bushinsky, D. A., 2009. Metabolic acidosis increases intracellular calcium in bone cells through activation of the proton receptor OGR1. *J. Bone Miner. Res.* 24: 305-313.
- Ganj Khanlou, M., Nikkhan, A. and Zali, A. 2010. Effect of dietary cation-anion balance on milk production and blood mineral of Holstein cows during the last two months of pregnancy. *African J. Biotech.* 9: 5983-5988.
- Gelfert, C.C., Passfeld, M., Lohtien, A., Montag, N., Baumgartner, W. and Staufenbie, R., 2006. Experimental studies on the impact of an increased dose of anionic salts on the metabolism of dairy cows. *Quarterly Vet.* 28: 130-139.
- Ghattas, T. A., 2014. Influences of dietary cation anion difference (DCAD) in late pregnancy on the incidence of postpartum disorder in Holstein dairy cows. *J. Egypt. Vet. Med. Assoc.* 74: 163-181.
- Goff, J. P., 2000. Pathophysiology of calcium and phosphorus disorders. *Vet. Clin. N. Am. Food Anim. Pract.* 16: 319-337.
- Goff, J. P., 2008. The monitoring, prevention and treatment of milk fever and subclinical hypocalcemia in dairy cows. *Vet. J.* 176: 50-57.

- Goff, J. P., and Horst, R. L., 2012. Comparison of low versus high calcium “anionic” diets for prevention of hypocalcemia and milk fever. *J. Dairy Sci.*, 95(Suppl.2): 444.
- Goff, J. P., Ruiz R. and Horst, R. L., 2004. Relative acidifying activity of anionic salts commonly used to prevent milk fever. *J. Dairy Sci.* 87: 1245-1255.
- Grunberg, W., Donkin, S. S. and Constable, P. D. 2011. Periparturient effects of feeding a low dietary cation-anion difference diet on acid-base, calcium, and phosphorus homeostasis and on intravenous glucose tolerance test in high-producing dairy cows. *J. Dairy Sci.* 94: 727-745.
- Gulay, M. S., Hayen, M. J., Head, H. H., Wilcox, C. J. and Bachman, K. C., 2005. Milk production from Holstein half udders after concurrent thirty- and seventy-day dry periods. *J. Dairy Sci.* 88: 3953-3962.
- Herson, M. J., Hansen, G. R. and Arthington J. D., 2010. Effect of dietary cation-anion difference on measures of acid-base physiology and performance in beef cattle. *J. Anim. Sci.* 88: 374-382.
- Holtenius, K., Agenas, S., Delavaud, C. and Chillard Y., 2003. Effects of feeding intensity during the dry period 2: Metabolic and hormonal responses. *J. Dairy Sci.* 86: 883-891.
- Hu, W. and Kung-Jr. L., 2009. Effect of dietary ratio of Na: K on feed intake, milk production, and mineral metabolism in mid-lactation dairy cows. *J. Dairy Sci.* 92: 2711-2718.
- Hu, W. and Murphy, M. R., 2004. Dietary cation-anion difference effects on performance and acid-base status of lactating dairy cows: A meta-analysis. *J. Dairy Sci.* 87: 2222-2229.
- Hu, W., Kung-Jr, L. and Murphy, M. R., 2007a. Relationship between dry matter intake and acid-base status of lactating dairy cows as manipulated by dietary cation anion difference. *Anim. Feed Sci. Tech.* 136: 216.
- Hutjens, M. F. 1991. Feed additives. *Vet Clinics North Am. Food Anim. Practice.* 7: 525.
- Keady, E. D., Roessler, R., Kahi, A. K., Piepho, H. P. and Zarate, V., 2011. Production objectives and breeding goals of Sahiwal cattle keepers in Kenya and implications for a breeding programme. *Trop. Anim. Health Prod.* DOI 10.1007/s11250-011-9928-8.
- James, C. S. 1995. Determination of fat content of dairy product by the Gerber method. In: *Analytical chemistry of Food*. Blackie Academic and professional, an important of chapman and Hall, Glasgow, U. K. pp 93-95.
- Jawor, P. E., Huzzey, J. M., LeBlanc, S. J. and VonKeyserlingk. M. A. G., 2012. Associations of subclinical hypocalcemia at calving with milk yield, and feeding, drinking, and standing behaviours around parturition in Holstein cows. *J. Dairy Sci.* 95: 1240-1248.
- Kara, C., 2013. Physiological and metabolic changes during the transition period and the use of calcium propionate for prevention or treatment of hypocalcemia and ketosis in periparturient cows. *J. Biological and Environmental Sci.* 7: 9-17.
- Keady, T. W. J., Mayne, C. S., Fitzpatrick, D. A. and McCoy, M. A., 2001. Effect of concentrate feed level in late gestation on subsequent milk yield, milk composition, and fertility of dairy cows. *J. Dairy Sci.* 84: 1468-1479.
- Klibs, N. G., 2011. Identifying and Treating Uterine Disease in Dairy Cows. *Proceedings 47th Florida Dairy Production Conference*, Gainesville, March 30, 2011.
- Klos, B., Kaliciak, M., Walkowiak, K. and Adamski. M., 2015. The influence of negative cation-anion balance in cows on the frequency of milk fever. *Acta Sci. Pol. Zootechnica.* 14: 91-98.
- Koivula, M., Mantysaari, E. A., Negussie, E. and Serenius T., 2005. Genetic and phenotypic relationships among milk yield and somatic cell count before and after clinical mastitis. *J. Dairy Sci.* 88: 827-833.
- Kolver, E. S. and deVeth, M. J., 2002. Prediction of ruminal pH from pasture-based diets. *J. Dairy Sci.* 85: 1255-1266.
- Kronqvist, C., 2011. Minerals to dairy cows with focus on calcium and magnesium balance. *Doctoral Thesis*. Page no. 11.
- Kurosaki, N., Yamato, O., Mori, F., Imoto, S. and Maede. Y., 2007. Preventive effect of mildly altering dietary cation-anion difference on milk fever in dairy cows. *J. Vet. Med. Sci.* 69: 185-192.
- Lawless, H. T., Rapacki, F., Horne, J., Hayes, A. and Wang. G., 2003. The taste of calcium chloride in mixtures with NaCl, sucrose and citric acid. *Food Quality and Preference.* 15: 83-89.
- Lean, I. J. and DeGaris, P. J., 2010. *Transition Cow Management: A review for nutritional professionals, veterinarians and farm advisers*. Dairy Australia.
- Lemann, J., Bushinsky, D. A. and Hamm, L. L., 2003. Bone buffering of acid and base. *Am. J. Physiol. Renal.* 285: F811-F832.
- Liesegang, A., Singer, K. and Boos, A., 2008. Vitamin D receptor amounts across different segments of the gastrointestinal tract in Brown Swiss and Holstein Friesian cows of different age. *J. Anim. Physiol. Anim. Nutr.* 92: 316-323.
- Luebbe, M. K., Erickson, G. E., Klopfenstein, T. J., Greenquist M. A. and Benton J. R., 2011. Effect

- of dietary cation-anion difference on urinary pH, feedlot performance, nitrogen mass balance, and manure pH in open feedlot pens. *J. Anim. Sci.* 89: 489-500.
- Martinez, N., Risco, C. A., Lima, F. S., Bisinotto, R. S., Greco L. F., Ribeiro, E. S. Maunsell, K. N. Galvao, and J. E. P. Santos. 2012. Evaluation of periparturient calcium status, energetic profile and neutrophil function in dairy cows at low or high risk of developing uterine disease. *J. Dairy Sci.* 95:7158-7152.
- Massanyi, P., Kovacic, J., Toman, R., Lukac, N., Stawarz, R., Capcarova, M. and Kolesarova, A., 2007. Concentration of selected serum parameters after cadmium administration. *Physiological Res.* 56: 22.
- Massey, C.D., Wang, C., Donovan, G. A. and Beede, D. K., 1993. Hypocalcemia at parturition as risk factor for left displacement abomasum in dairy cows. *JAVMA.* 203: 252-253.
- Melendez, P., McHale, J., Bartolome, J., Archbald, L. F. and Donovan M G. A., 2004. Uterine involution and fertility of Holstein cows subsequent to early postpartum PGF 2α treatment for acute puerperal metritis. *J. Dairy Sci.* 87: 3238-3246.
- Miles, P. H., Wilkinson N. S. and McDowell, L. R. 2001. Analysis of minerals for animal nutrition research. Department of Animal Sciences, University of Florida, Gainesville, FL.
- Mulligan, F. J., O'Grady, L., Rice, D. and Doherty, M., 2006. Production diseases of the transition cow: Milk fever and subclinical hypocalcaemia. *Irish Vet. J.* 59: 697-702.
- Mulligan, F., and Doherty, M., 2008. Production diseases of the transition cow. *Vet. J.* 176: 3-9.
- Neligan, P.J. and Deutschman, C.S. Perioperative Acid-Base Balance. In: Miller RD, Eriksson LI, Fleisher L, Wiener-Kronish JP, Cohen NH. *Miller's Anesthesia*, 8th ed. Philadelphia, PA: Elsevier Saunders; 2014: Ch. 60, pp. 1811-1829
- Nielsen, N. I., and Volden, H., 2011. Animal requirements and recommendations. In Volden, H. (ed): *Nor For-The Nordic feed evaluating system* EAAP publication No. 130, Wageningen Academic Publishers, p 105-111.
- Nieves, R. J. M., Thering, B. J., Waldron, M. R., Jardon, P. W. and Overton, T. R., 2009. Effects of anion supplementation to low-potassium prepartum diets on macro mineral status and performance of periparturient dairy cows. *J. Dairy Sci.* 92: 5677-5691.
- Norrish, K. and Hutton J. T., 1977. Plant analyses by x-ray spectrometry. 1. Low atomic number elements, sodium to calcium. *X-Ray Spectrometry* 6: 6-11.
- NRC (National Research Council). 2001. *Nutrient Requirements of Dairy Cattle.* pp 105-107; 184-185. 7th revised. Natl. Acad. Press, Washington, DC.
- Oba, M., Oakley, A. E. and Tremblay G. F., 2011. Dietary Ca concentration to minimize the risk of hypocalcemia in dairy cows is affected by dietary cation-anion difference. *Anim. Feed Sci. Tech.* 164: 147-143.
- Oetzel, G. R., 2011. Non-infectious diseases: Milk fever. In *Encyclopedia of Dairy Sciences.* Vol.2. F. J. W. Fuquay, P. F. McSweeney, P. L. H., ed. Academic Press, San Diego.
- Oetzel, G., 2013. Cows can suffer from milk fever even though you don't see it. Tri-State Dairy Nutrition Conference, University of Wisconsin, USA.
- Oetzel, G. R., Olson, J. D., Curtis, C. R. and Fettman M. J., 1988. Ammonium chloride and ammonium sulphate for prevention of parturient paresis in dairy cows. *J. Dairy Sci.* 71:3302-3309.
- Oetzel, G. R., Fettman, M. J., Hamar, D. W. and Olson, J. D., 1991. Screening of anionic salts for palatability, effects on acid-base status, and urinary calcium excretion in dairy cows. *J. Dairy Sci.* 74: 965-971.
- Penner, G. B., Tremblay, G. F., Dow, T. and Oba, M., 2008. Timothy hay with a low dietary cation anion difference improve calcium homeostasis in periparturient Holstein cow. *J. Dairy Sci.* 91: 1959-1968.
- Peter, J., DeGaris, P. J. and Lean I. J., 2007. Milk fever in dairy cows: A review of pathophysiology and control principles. *Bovine Research Australasia*, Camden. 2570, Australia.
- Phillippo, M., Reid, G. W. and Nevison, I. M., 1994. Parturient hypocalcaemia in dairy cows; effect of dietary acidity on plasma mineral and calcitropic hormones. *Res.Vet. Sci.* 56: 303-309.
- Piccione, G., Messina, V., Marafioti, S., Casella, S., Giannetto, C. and Fazio, F., 2012. Changes of some haematochemical parameters in dairy cows during late gestation, postpartum, lactation and dry periods. *Vet. Med. Zootech.* 58: 59-64.
- Plaizier, J. C., Krause, D. O., Gozho, G. N. and McBride, B. W., 2008. Subacute ruminal acidosis in dairy cows: The physiological causes, incidence and consequences. *Vet. J.* 176: 21-31.
- Radostits, O. M., Gay, C. C., Hinchcliff, K. W. and Constable, P. D., 2007. *Veterinary Medicine. A textbook of the diseases of cattle, horses, sheep, pigs, and goats.* Parturient Paresis (Milk Fever). Saunders Elsevier, New York. Ed. (10th): 1627-1643.
- Reece, W. O., 2009. *Functional Anatomy and Physiology of Domestic Animals*, Fourth Edition.

- Rehage, J., and Kaske, M., 2004. Interaction between milk yield and productive diseases in dairy cows. *Proc. Soc. Nut. Physiol.* 13: 177-182.
- Rekhis, J., Kouki, C. K., Dhaouadi, B., and Khlif, K., 2001. Mineral supplementation in Tunisian small holder dairy farms. *New Zealand Vet. J.* 49: 78-80.
- Rerat, M., Philipp A., Hess, H. D. and Liesegang A., 2009. Effect of different potassium level in hay on acid base status and mineral balance in periparturient dairy cow. *J. Dairy Sci.* 92: 6123-6133.
- Rezac, D. J., Block E., Weber, D., Brouk, M. J. and Bradford, B. J. 2014. Effects of prepartum dietary cation-anion difference and acidified coproducts on dry matter intake, serum calcium and performance of dairy cows. *J. Anim. Sci.* 92: 666-675.
- Roche, J. R., Petch S. and Kay J. K., 2005. Manipulating the dietary cation-anion difference via drenching to early-lactation dairy cows grazing pasture. *J. Dairy Sci.* 88: 264-276.
- Roxtrom, A., Strandberg, E., Berglund, B., Emanuelson, U. and Philipsson, J., 2000. Genetic and environmental correlations among female fertility traits and milk production in different parities of Swedish Red and White Dairy Cattle. *Acta Agraria Scandinavia, Sect A, In: Anim. Sci.* 51: 7-14.
- Sakha, M., Mahmoudi, M. and Nadalian, M. G., 2014. Effects of dietary cation-anion difference on milk fever, subclinical hypocalcemia and negative energy balance in transition dairy cows. *Res. Opin. Anim. Vet. Sci.* 4: 69-73.
- Santos, J. E. P., Bisinotto, R. S., Ribeiro, E. S., Lima, F. S., Greco, L. F., Staples, C. R. and Thatcher, W. W., 2011. Applying nutrition and physiology to improve reproduction in dairy cattle. In: *Reproduction in Domestic Ruminants VII.* (Eds M. C. Lucy, J. L. Pate, M. F. Smith, and T.E. Spencer) pp. 387-404. (Nottingham University Press: Nottingham, UK).
- Scott, D. J., and Vijk, V. N., 2000. Comparison in dairy cattle of mucosal toxicity of calcium formate and calcium chloride in oil. *N. Z. Vet. J.* 48:24-6.
- Scott, D. J. and Vijk, V. N., 2002. An investigation of the safety of oral calcium formate in dairy cows using clinical, biochemical and histopathological parameters. *N. Z. Vet. J.* 50: 195-8.
- Seifi, H. A., Mohri, M., Farzaneh, N., Nemati, H., and Nejhad S. V., 2010. Effects of anionic salts supplementation on blood pH and mineral status, energy metabolism, reproduction and production in transition dairy cows. *Res. Vet. Sci.* 89: 72-77.
- Shahzad, M. A., Sarwar, M. and Nisa, M., 2008. Influence of varying dietary cation-anion difference on serum minerals, mineral balance and hypocalcemia in Nili Ravi buffaloes. *Lives Sci.* 113: 52-61.
- Sharif, M., Shahzad, M. A., Nisa, M. and Sarwar, M., 2010. Dietary cation-anion difference: Impact on productive and reproductive performance in animal agriculture. *African J. Biotech.* 9: 7976-7988.
- Sharif, M., Nisa, M., Sarwar, M. and Shahzad, M. A. 2008. Influence of varying levels of dietary cation-anion difference on ruminal characteristics, acid base status and milk yield of early lactating animals (a review). *Pak. J. Agric. Sci.* 45: 288-296.
- Shire, J. A. and Beede, D. K., 2013. DCAD Revisited: Prepartum use to optimize health and lactational performance. *Proc. 28th Southwest Nutr. And Management Conf.* pp 1-18.
- Silva, T. S., 2015. Evaluation of dietary cation anion difference DCAD for lactating buffalo. Thesis (Ph.D.). Faculty of animal Science and Food Engineering, University of Soa Paulo, Pirassununga. Page No. 9.
- Sjaastad, O. V., Hove, K. and Sand, O., 2003. *Physiology of domestic animals.* Scandinavian veterinary press, Oslo, Norway.
- Spanghero, M., 2004. Prediction of blood and urinary pH in non-lactating dairy cows fed anionic diets. *Anim. Feed Sci. Tech.* 116: 83.
- Steel, R. G. D., Torrie, J. H. and Dickey, D. A., 1997. *Principle and procedure of statistics. A biometric approach 3rd ed.* McGraw Hill Book Co. Inc. New York. USA. pp. 666. Turkish J. Vet. Anim. Sci. 23: 451-456.
- Stewart, P. A. 1983. Modern quantitative acid-base chemistry. *Can. J. Physiol. Pharm.* 61: 1444-1461.
- Suttle, N. F. and McLaughlan M., 2010. *Proceedings of the Nutrition Soc.*, 35: 22A.
- Taylor, M. S., Knowlton, K. F., McGilliard, M. L., Seymour, W. M., and Herbein, J. H., 2008. Blood mineral, hormone, and osteocalcin responses of multiparous Jersey cows to an oral dose of 25-hydroxyvitamin D3 or vitamin D3 before parturition. *J. Dairy Sci.* 91: 2408-2416.
- Taylor, V. J., Hattan, A. J., Bleach, E. C., Beever, D. E. and Wathes, D. C., 2001. Reproductive function in average and high yielding cows. *Occ. Publ. No. 26, Brit. Soc. Anim. Sci.* 495-498.
- Thilising, H. T., Jorgensen, R.J. and Ostergaard, S., 2002. Milk fever control principles: a review. *Acta Vet. Scand.*, 43: 1-19.
- Thirunavukkarasu, M., Kathiravan, G., Kalaikannan, A. and Jebarani W., 2010. Quantifying Economic

- Losses due to Milk Fever in Dairy Farms. *Agricultural Economics Res. Review.* 23: 77-81.
- Tucker, W. B., Hogue, J. F., Waterman, D. F., Swenson, T. S, Xin, Z., Hemken, R. W., Jackson, J. A., Adams, G. D. and Spicer, L. J., 1991. Role of sulfur and chloride in the dietary cation anion balance equation for lactating dairy cattle. *J. Anim. Sci.* 69:1205-1213.
- Vagnoni, D. B. and Oetzel, G. R., 1998. Effect of dietary cation anion difference on acid base status of dry cow. *J. Dairy Sci.* 81: 1643-1652.
- Van Dijk, C. J. and Lourens, D. C., 2001. Effects of anionic salts in a pre-partum dairy ration on calcium metabolism. *J. South African Vet. Assoc.* 72: 76-80.
- Weich, W., Block, E. and Litherland, N. B., 2013. Extended negative dietary cation-anion difference feeding does not negatively affect postpartum performance of multiparous dairy cows. *J. Dairy Sci.* 96: 5780-5792.
- Wildman, C. D., West, J. W. and Bernard, J. K. 2007. Effect of dietary cation-anion difference and dietary crude protein on performance of lactating dairy cows during hot weather. *J. Dairy Sci.* 90: 1842-1850.
- Wu, W. X., 2011. The correlation between dietary cation-anion difference and acid-base balance of body fluid in dairy cow. *Chinese J. Anim. Nut.* 23: 2198-2202.