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IMPACT OF DIETARY CATION-ANION DIFFERENCE ON RUMEN FERMENTATION, DIGESTIBILITY, AND BLOOD PARAMETERS IN ZANDI LAMBS UNDER HEAT STRESS

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ABSTRACT

The dietary cation-anion difference (DCAD) has received increased attention recently; however, information on organic matter digestibility, blood parameters, and rumen fermentation of male Zandi sheep fed with various DCAD diets is scarce. In male lambs under heat stress, the effects of DCAD on these variables were examined in this study. A total of 40 male Zandi lambs were randomly assigned to 5 treatments, each with 8 duplicates, and had an average body weight of 39kg. Diets with various DCAD concentrations of 150, 300, 450, 600, and 750mEq/kg dry matter were provided to lambs. With a 21-day adaptation, this trial lasted 100 days. In terms of dry matter intake, dry matter digestibility, and crude protein digestibility, the results indicated that the control group had the highest values ($P<0.05$). Additionally, the control group had the higher ruminal pH ($P<0.05$). Additionally, the findings demonstrated that the various DCAD concentrations had no effect on the rumen fermentation parameters, including propionic acid, butyric acid, buffering capacity, butyric acid, acetic acid, the ratio of acetic acid to propionic acid, and the sum of acetic acid and propionic acid ($P>0.05$). According to the blood glucose parameter data, the control group's influence on blood glucose level was the most significant ($P<0.05$). Other blood indicators such as cholesterol, phosphate, magnesium, and potassium did not differ significantly ($P>0.05$). Rumen fermentation is unaffected by feeding a different DCAD. However, feeding more DCAD to lambs who are under heat stress could stabilize their production and apparent digestibility.

***Keywords*:** Zandi Sheep, DCAD, Digestion, Rumen Fermentation, Blood Physiology, Heat Stress.

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1. INTRODUCTION

The non-metabolizable dietary ion combinations' potential positive or negative alteration is known as the cation-anion differential (DCAD). The benefit of feeding varied DCAD was originally mentioned by Block (1984), who noticed that treatment with -172.3mEq/kg DM DCAD could prevent hypocalcaemia when compared to a control DCAD of +448.6mEq/kg DM. Studies have shown that transition mammary animals' health and economic well-being could be enhanced by diets with decreased DCAD levels. The interaction of DCAD with vitamin D, 5-hydroxyl-tryptophan, cholecalciferol/calcidiol, and 5-hydroxyl-tryptophan has recently received attention (Rodney et al. 2018; Martinez et al. 2018a, Martinez et al. 2018b, Collazos et al. 2018), calcium (Ca) (Diehl et al. 2018; Collazos et al. 2018; Lopera et al. 2018; Rajeerad et al. 2020). The findings of these trials demonstrated that improving peripheral blood Ca homeostasis by decreased DCAD in combination with the aforementioned parameters was successful. Heat stress increases respiratory CO_2 loss (respiratory alkalosis), Na and K loss, and sweat and urine output (associated with bicarbonate ions). In theory, alterations in blood acid-base balance and associated electrolyte losses can be corrected by modifying the DCAD. For breastfeeding dairy calves to produce the greatest milk, diets with DCAD of 200–370mEq/kg DM have been recommended (Tucker et al. 1988; West et al. 1991; Caixeta et al. 2020), while for hens and pigs to grow the fastest, diets with 250mEq/kg DM have been recommended (Mongin, 1981). Such dietary adjustments may have a direct impact on ruminal pH in ruminants, which could have an impact on digestion, dry matter intake, and ruminal microbial productivity (Nguyen et al. 2020; Yang et al. 2021). Dietary digestibility and dry matter intake may be directly impacted by changes in dietary salt concentrations that alter DCAD. There has not been much focus on how DCAD modifications affect sheep performance. There has not been much focus on how

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DCAD modifications affect sheep performance. As a result, the impact of DCAD on the male lambs' blood metabolites, organic matter digestibility, dry matter intake, digestibility, and fermentation has not been explicitly evaluated. Therefore, the purpose of this study was to investigate how male lambs' blood metabolites and rumen fermentation were affected by increasing DCAD from 150 to 750mEq/kg of DM. Additionally, we did this study to determine if lambs' DMI, production, and rumen fermentation might be enhanced by positive DCAD in a high-temperature setting.

2. MATERIALS AND METHODS

2.1. Animals and Management

Animals were bred at the research farm of the Islamic Azad University between June 2020 and October 2021, and their breeding was authorized by the university's committee on experimental animal ethics under the designation IUA-2020-P312. Forty Zandi male lambs (a native breed of sheep found in Iran's central regions) were divided into 5 treatments, each consisting of 8 replicates with one lamb in each. This was done using a completely randomized block design. The entire experiment involved feeding lambs inside of their cages. Different DCAD levels were fed to the animals: +150 (control; group 1), +300 (group 2), +450 (group 3), +600 (group 4), and +700 (group 5). The TMR (total mixture ration) food was pelleted with a concentrate-to-roughage ratio of 30:70. To raise DCAD, sodium bicarbonate ($NaHCO_3$) and sodium carbonate (Na+CO+) were added. The experiment lasted for 100 days, with a trial period of 79 days and an adaptation period of 21 days. Lambs were then fed the treatment meals at 9:00 and 18:00 during the study period. Throughout the experiment, all lambs had unlimited access to water. The nutritional elements and chemical components of diets are shown in Table 1.

Table 1: Ingredients and chemical components of diet for lambs (diets were formulated according to the recommendations of NRC 2001 and gradually provided to lambs. This experiment included five diets with the same energy and protein levels).

Items	Group 1 (+150 mEq/kg DM)	Group 2 (+300 mEq/kg DM)	Group 3 (+450 mEq/kg DM)	Group 4 (+600 mEq/kg DM)	Group 5 (+750 mEq/kg DM)
Ingredients (gr/d)					
Alfalfa hay	400	400	400	400	400
Wheat straw	100	100	100	100	100
Corn meal	300	300	300	300	300
barley	300	300	300	300	300
Corn silage	350	350	350	350	350
wheat bran	120	120	120	120	120
Soybean meal	100	100	100	100	100
Salt	10	10	10	10	10
Na_2CO_3	-	7	15	23	31
NaHCO3	-	7	15	23	31
Chemical components					
Dry matter	93.91	93.01	93.23	92.98	92.89
Crude protein	13.41	13.48	13.51	13.54	13.4
Neutral detergent fiber	39.94	39.75	39.52	39.44	39.32
Acid detergent fiber	30.75	30.64	30.52	30.44	30.41
Crude ash	12.28	12.12	12.17	11.98	11.85
DCAD[1] (mmol/kg DM)	+150	+300	+450	+600	+700

[1]DCAD, dietary cation-anion difference.

2.2. Temperature-Humidity Index (THI) Monitoring

To provide enough ventilation and keep them out of the sun and rain, two wet and dry bulb thermometers were installed on the feeding barns at a height of 1.5 meters above the ground the National Research Council then employed the following formula in 2001 to determine the THI: The daily averages of THI, Td, and Tw (Fig. 1) were calculated as follows: THI = (Td + Tw) x 0.72 + 40.6. The temperatures displayed by the dry bulb and wet bulb thermometers, respectively, are Td and Tw. An electronic thermometer was used to track each lamb's rectum temperature (RT), which is situated 4-6cm from the annas, during the experiment.

2.3. Data Collection and Determination of Digestibility

Dietary samples were taken every day for 21–100 days in order to determine the proximate chemical composition of DM, crude protein (CP), crude ash, total fat (Ash), neutral detergent fiber (NDF), and acid detergent fiber (ADF)

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(AOAC 1990). An atomic absorption spectrophotometer was used to test the concentrations of Na, K, and Cl (iCE3000 SERIES, Thermo Fisher Scientific, USA). A silver nitrate titration was used to estimate the Cl concentration. The magnesium nitrate method was used to measure the S level, as was already described. According to Block (1984), the DCAD was calculated using the following equation:

DCAD is equal to S (%)/0.0016, Cl (%)/0.00355, K (%)/0.0039, and Na (%)/0.0023.

Every 14 days for a total of 100 days, every lamb was weighed separately. Each lamb's DMI was tracked daily and computed using the allowance for refusals.

2.4. Measurement of Plasma Metabolites

For the examination of blood serum's biochemical parameters, blood samples were taken every month. The samples were drawn from the jugular vein and put into EDTA vacuum tubes with intravenous vein and EDTA. The pH of the samples was measured, and then the blood plasma was separated by centrifugation and frozen for further analysis. All samples were determined in terms of minerals such as sodium, potassium and magnesium, phosphorus, glucose, and blood cholesterol through the proposed AOAC methods.

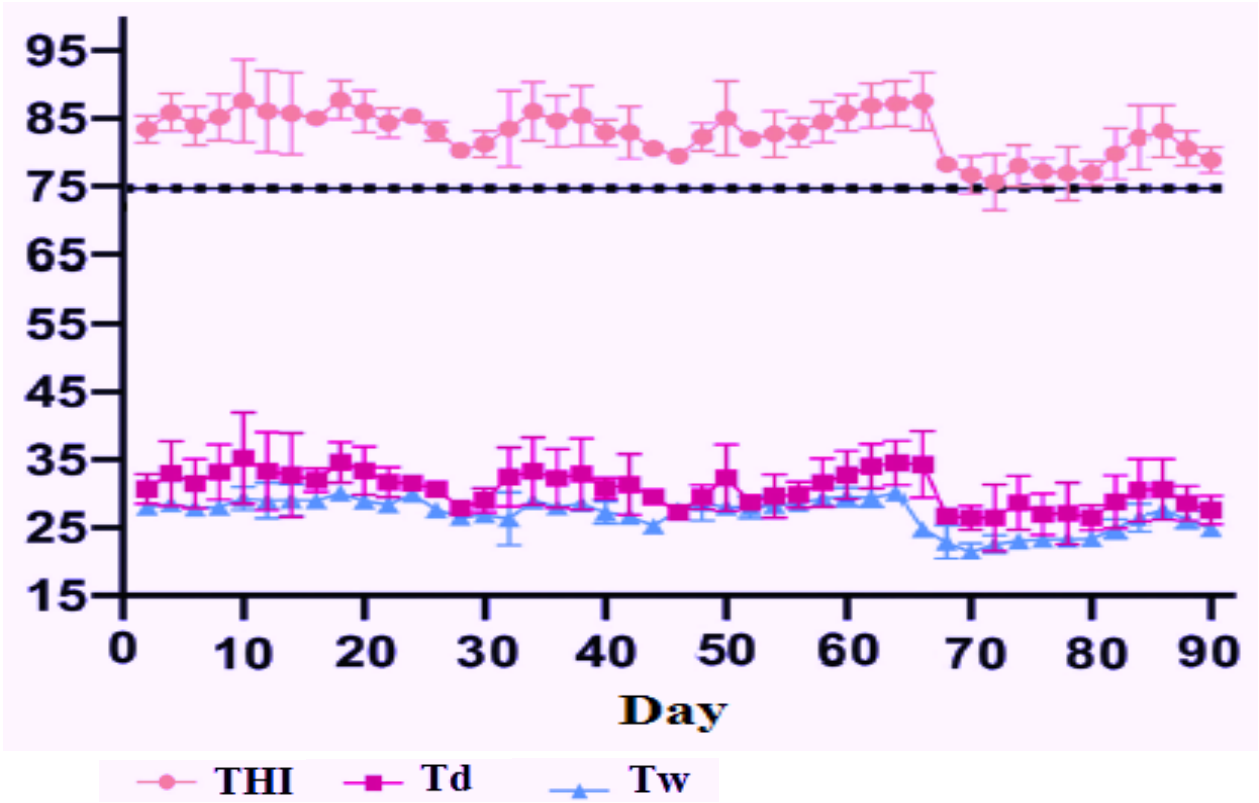

Fig. 1: The daily average temperature-humidity index (THI), dry bulb temperatures (Td) and wet bulb temperatures (Tw) during the experimental period. The horizontal line at 75 indicates the threshold for heat stress.

2.5. Rumen Fluid Collection and Study of Volatile Fatty Acids

Each lamb had rumen fluid samples taken from them using a stomach tube and syringe. The multiple tubes approach was employed in the rumen fluid pool to reduce excessive saliva contamination. The mouth gag's outer rubber tube (i.d =2.5cm) specifications were given. The collection tube (110cm) that was inserted into the rumen was the inner rubber tube (o.d =1.2cm). Fluid samples totaling 25mL were obtained 2.5 hours after morning feeding and a pH meter was used to determine the pH right away. Then, once the ruminal fluid samples had been filtered through two layers of cheesecloth, 1mL of 6 N HCl was added for preservation. The samples were then frozen at -20°C in preparation for further measurements of osmolality, volatile fatty acid (VFA), and NH3-N. Using an osmometer, the ruminal fluid's osmolality

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was assessed. According to Thammacharoen et al. (2001), the VFAs were created and examined. Using the salicylate-hypochlorite technique, NH3-N was identified.

2.6. Allantoin Excretion Analysis and Urine Collection

Using plastic containers with 10% sulfuric acid solution added to decrease nitrogen loss, total urine was collected and measured the same week as a fecal collection (15mL H_2SO_4 10% in 90mL urine). The ultimate pH of the urine was kept below 3. After the experiment, Chen and Gomes (1992) combined all of the urine from each day (30mL), frozen it at -20°C, and utilized it to measure allantoin and nitrogen excretion using colorimetric methods. The difference between nitrogen input (from FI) and nitrogen output was used to compute nitrogen retention.

2.7. Statistical Analysis

The MIXED models in SAS 9.4 (SAS Institute Inc.) were used to evaluate experimental data. A randomized complete block design was used for data analysis. This experiment is carried out using the subsequent statistical model.

$$Yij = \mu + Ti + Eij$$

In this regard, Yij is the value of each variable; μ is the mean of the related trait, Ti effect of treatment, and Eij error rate. And then LSD test method was used to compare the mean of each trait. *Statistical significance* was defined as $P<0.05$.

3. RESULTS

3.1. THI and Rectal Temperature of Lambs

The readings on the dry bulb thermometer for the time period ranged from 29.03 to 39.25°C, with a mean of 33.94°C. The mean wet-bulb temperature was 25.61°C, with measurements ranging from 20.16 to 30.01. The calculated THI has a range of 75.13 to 86.55, with a mean of 81.23. The THI remained continuously higher than 75 for the duration of the entire experiment. The control group's rectal temperature did not differ significantly from those of the other groups. Between DCAD groups, there were no discernible variations in rectal temperature.

3.2. DCAD effect on Plasma Metabolites

The results of blood biochemical parameters showed a significant ($P<0.05$) difference between treatments in glucose, cholesterol, and sodium parameters in lambs (Table 2). The results demonstrated that the control group had the highest ($P<0.05$) effect on blood glucose levels, however, groups 3, 4, and 5 did not differ in blood glucose levels. There was a significant difference between group 5 comparisons of the other group in blood cholesterol, with the highest effect on blood cholesterol. There were no significant differences between blood phosphorus, magnesium and potassium levels ($P>0.05$). Nevertheless, group 5 had the greatest blood levels of phosphorus, magnesium, and potassium. The control group had the lowest blood levels of phosphorus, magnesium, and potassium (Table 2).

Table 2: Effect of varying dietary cation-anion difference on the plasma metabolites and nitrogen balance of male lambs

Items	Group 1 (+150 mEq/kg DM)	Group 2 (+300 mEq/kg DM)	Group 3 (+450 mEq/kg DM)	Group 4 (+600 mEq/kg DM)	Group 5 (+750 mEq/kg DM)	P-Value
Ca (mmol/L)	2.82^{a}	2.71^{ab}	2.64^{ab}	2.61^{ab}	2.42^{b}	<0.05
Glc (mmol/L)	5.15^{a}	4.21^{b}	3.36^{c}	3.55^{c}	3.40^{c}	<0.05
CHOL (mmol/L)	16.46^{b}	16.55^{b}	15.22^{b}	15.13^{b}	18.12^{a}	<0.05
P (mmol/L)	1.45	1.56	1.57	2.05	2.17	0.75
Mg (mmol/L)	1.13	1.00	1.76	1.30	1.76	0.82
K (mmol/L)	3.39	3.30	3.44	3.50	3.52	0.84
Na(mmol/L)	124^{a}	108^{abc}	111^{ab}	94^{bc}	84^{c}	<0.05
Cl (mmol/L)	112.50	112.00	111.50	111.00	110.23	0.32
Nitrogen balance (g/d)	9.65	9.40	11.80	12.39	12.06	0.83

DCAD, dietary cation-anion difference; CHOL, cholesterol; Na, sodium, K, potassium; Mg, magnesium; P, phosphorus. K, potassium; Mg, magnesium; P, phosphorus.

3.3. Rumen Fermentation Parameters and DCAD Effect

The ruminal pH varied significantly between treatments ($P<0.01$). The control group's ruminal pH was found to be the lowest. As well, the highest ruminal pH was observed in group 5 ($P<0.05$) (Table 3). The variation of DCAD did not affect total volatile fatty acids, acetate, propionate, butyrate, valeric, isovaleric, acetate/ propionate, urea

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nitrogen, and ammonia. Nevertheless, numerically, the highest total volatile fatty acids, acetate, propionate, butyrate, valeric, isovaleric, acetate/ propionate, urea nitrogen, and ammonia, were observed in the control group. Moreover, the lowest total volatile fatty acids, acetate, propionate, butyrate, valeric, isovaleric, acetate/propionate, urea nitrogen, and ammonia, were observed in group 5 (Table 3).

3.4. Effect of DCAD on Digestibility and Intake of Dry Matter

There were significant differences between treatments in terms of dry matter intake, digestibility, and crude protein ($P<0.05$; Table 4). Table 4 displays the mean comparison of treatments on dry matter intake. The control group had the lowest intake of dry matter and the lowest crude protein digestibility ($P<0.05$). Additionally, group 4 had the highest apparent digestibility of DMI, CP, and organic matter ($P<0.05$). Group 1 had the lowest crude protein digestibility ($P<0.05$; Table 4).

Table 3: Effects of DCAD and concentrate level (conc) on the rumen fermentation parameters of male lambs

Items	Treatments					
	Group 1 (+150 mEq/kg DM)	Group 2 (+300 mEq/kg DM)	Group 3 (+450 mEq/kg DM)	Group 4 (+600 mEq/kg DM)	Group 5 (+750 mEq/kg DM)	P-Value
pH	6.54^{b}	6.68^{ab}	6.64^{ab}	6.71^{ab}	6.82^{a}	<0.05
Total VFA (mmol/L)	75.15	72.21	71.36	72.65	73.40	0.33
Acetate (%)	61.46	56.55	58.22	55.13	54.12	0.44
Propionate (%)	19.44	19.10	18.53	18.01	17.83	0.24
Butyrate (%)	13.60	13.31	13.10	13.04	13.04	0.75
Valeric (%)	0.34	0.33	0.33	0.31	0.30	0.84
Isovaleric (%)	1.14^{a}	0.96^{b}	0.98^{b}	0.84^{b}	1.01^{ab}	<0.05
A/P[1]	3.74	3.20	3.10	3.08	3.01	0.86
Ammonia (mg/L)	112.50	112.00	111.50	111.00	110.23	0.32

[1]A/P: Acetic acid/propionic acid.

Table 4: Effects of dietary cation and anion difference on dry matter intake, organic matter digestibility and crude protein digestibility in lambs

Items	Treatments					
	Group 1 (+150 mEq/kg DM)	Group 2 (+300 mEq/kg DM)	Group 3 (+450 mEq/kg DM)	Group 4 (+600 mEq/kg DM)	Group 5 (+750 mEq/kg DM)	P-Value
DMI (g/kg BW)	31.60^{b}	36.75^{a}	38.11^{a}	38.21^{a}	37.11^{a}	<0.05
Nutrient intake (g/kg BW/d)						
Organic matter	30.46^{b}	34.67^{a}	35.35^{a}	35.13^{a}	34.12^{a}	<0.05
Crude protein	4.44^{b}	5.46^{a}	6.53^{a}	6.42^{a}	6.83^{a}	<0.05
Neutral detergent fiber	16.60	16.90	19.10	19.07	19.04	0.09
Acid detergent fiber	8.34	8.56	9.33	9.59	9.30	0.84
Apparent digestibility (%)						
Dry matter	73.74^{b}	74.40^{a}	77.10^{a}	78.75^{a}	80.01^{a}	<0.05
Organic matter	61.50^{b}	68.05^{a}	73.50^{a}	74.86^{a}	76.23^{a}	<0.05
Crude protein	65.87^{b}	73.03^{a}	77.05^{a}	78.08^{a}	78.22^{a}	<0.05

DCAD, dietary cation and anion difference; DMI, dry matter intake; BW, body weight; DM, dry matter.

4. DISCUSSION

The ratio of primary cations (Na+ and K+) to main anions (Cl- and S2-) per kg of dry matter (DM) is known as the dietary cation-anion difference (DCAD; mEq/kg DMI) (Riond 2001). In recent years, the DCAD level has become increasingly important for formulating diets for animals (Shahzad et al. 2008; Edwards et al. 2010; Heer et al. 2017; Cardoso et al. 2020; Hassanien et al. 2022). Santos et al. (2019) and Lean et al. (2019) have recently used meta-analyses to investigate the various effects of DCAD on cattle performance, production, and health. They concluded that prenatal DCAD causes less disease and adversely affects total blood calcium levels during childbirth. The results of this investigation revealed a considerable distinction between the relevant treatments, including crude protein, organic matter digestibility, and dry matter intake (DMI). According to the data, the lambs in the control group consumed the driest matter. In line with this, group 5 had the lowest dry matter intake. Similar to other groups, the control group had the highest organic matter and crude protein digestibility whereas group 5 had the lowest. The results of this study demonstrated that supplementation affected male lambs' nutrient digestibility. The increase in

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digestibility may refer to the lack of the rumen microbial population requirements in the basic diet (Mallaki et al. 2015). Some factors such as supplement concentration, supplement source (organic or mineral), diet balance (ratio of forage to concentrate), and base diet supplement concentration were the factors that differentiated nutrient digestibility in different studies (Bougouin et al. 2019; Glosson et al. 2020; Samarin et al. 2022).

Our results revealed a significant difference between treatments in ruminal pH. The results showed no significant difference between treatments in total volatile fatty acids, acetate, propionate, butyrate, valeric, isovaleric, acetate/propionate, urea nitrogen, and ammonia. Furthermore, the control group had the greatest ruminal pH. Additionally, group 5's ruminal pH was found to be the lowest. Based on the findings of this investigation, the kind of cation source used in the diet of lambs has a direct effect on the anion-cation balance of the diet; it can affect ruminal fermentation parameters. The concentration of ammonia nitrogen produced in the rumen is one of the indicators to study the conditions of rumen fermentation. A good diet provides the nitrogen needed for maximum microbial protein, prevents excess ammonia-related waste, and provides sufficient non-degradable protein. Ammonia is one of the nitrogenous compounds used by microbes in the rumen to synthesize protein. Rumen ammonia is produced from nitrogenous substances in the diet, urea in saliva, and penetrating urea through the rumen wall (Afsahi et al. 2020; Lee et al. 2020). Since the concentration of ammonia decreases with increasing DCAD level, it can be stated that increasing DCAD probably increases the ruminal acidity. The decomposition of crude protein strongly influences the production of ammonia in the diet by microorganisms and the decomposition of the microbial population due to nitrogen recycling under adverse conditions; it can be concluded that this increase in acidity balances the culture medium in favor of further synthesis. Dudareva et al. (2004) displayed that reducing rumen ammonia production is beneficial in increasing microbial protein production or reducing rumen protein breakdown. Doepel and Hayirli (2011) reported that adding sodium bicarbonate to the diet did not affect ruminal ammonia levels, which was consistent with the results of this study. Reducing or eliminating protozoa from the rumen prevents the energy cycle between bacteria and protozoa, which reduces the breakdown of bacterial proteins. As a result, the flow of microbial nitrogen from the rumen increases, and consequently, the concentration of ammonia decreases (Kavanagh et al. 2019; Thanh et al. 2020). Harrison et al. (2012) reported that potassium carbonate could be used as an effective buffer to stabilize ruminal pH, increase dry matter intake, increase volatile fatty acid production, and increase the acetate-to-propionate ratio, consistent with the results of this research. Iwaniuk and Erdman (2015) reported that although DCAD affects the ratio of acetate to propionate, it has no significant effect on the molar percentage of propionate. Shahzad et al. (2008) displayed that increasing the level of anion-cation difference in the diet maintains the fermentation pattern to produce balanced acetate and butyrate, which in turn increases the synthesis of fatty acids of domestic origin, which provides up to 25% of milk fat. Therefore, it is observed that increasing DCAD has a positive effect on reducing environmental pollution related to methane production, which is an important point in modern practical nutrition. Previous studies have shown that increasing DCAD via potassium carbonate increases dry matter intake (Apper-Bossard et al. 2010). The highest apparent dry matter digestibility and the area below the digestibility curve (fluctuations of dry matter digestibility at different times) are related to the treatment containing potassium carbonate and magnesium carbonate with DCAD 905+mEq level; it can be concluded that the addition of potassium carbonate to dairy cows' diet improves the rumen fermentation and provides potassium and calcium to the microbial population, increasing dry matter digestibility (West et al. 1987; Zhang et al. 2022). Iwaniuk and Erdman (2015) also reported increased dry matter digestibility with increasing DCAD. After looking at how adding potassium and lasalocid affected the digestibility of insoluble fiber in neutral detergent, Funk et al. (1986) came to the conclusion that doing both at once increased the digestibility of insoluble fiber in neutral detergent. We demonstrated that increasing DCAD enhanced ruminal acidity, which improved insoluble fiber digestion in a neutral detergent, followed by an improvement in dry matter digestibility, which was corroborated by the prior work (Martinez et al. 2018). Blood measures can also be used to gauge an animal's metabolic health. Regarding glucose, cholesterol, and salt levels, there was a substantial difference between treatments. Comparing the mean of treatments on the blood glucose showed the control group had the highest effect, and groups 3, 4, and 5 did not differ significantly. Because blood glucose concentration in ruminants is more affected by the amount of ruminal fluid propionate (McDonald et al. 2010). Additionally, there was no discernible change in blood cholesterol levels across groups 1, 2, 3, and 4, with group 5 having the most impact. There were no appreciable differences between the groups in the blood plasma's phosphorus, magnesium, and potassium concentrations. However, in terms of numbers, group 5 had the highest concentration of phosphorus, magnesium, and potassium. The blood salt level was most significantly impacted by the control group. Therefore, various factors such as diet, source, and amount of supplementation that affect ruminal propionate production (Spears et al. 2004) can change the blood parameters. Because excess sodium and potassium are eliminated by the kidneys, small variations in plasma sodium and potassium may be attributed to dietary changes in these minerals. West et al. (1991) reported similar findings, stating that raising the level of DCAD (-116 to +312mE/kg DM) had no appreciable impact on plasma sodium and potassium levels.

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5. Conclusion

We summarized the rumen fermentation and digestibility mechanisms that are related to the DCAD diet's impact on lambs under heat stress. The findings revealed a substantial difference between treatments in terms of the lambs' 100-day consumption of protein digestibility, organic matter, and dry matter. The group with the greatest ruminal pH was the control group, while the group with the lowest ruminal pH was group 5. The results indicated no significant difference between lamb groups in total volatile fatty acids, acetate, propionate, butyrate, valeric, isovaleric, acetate/propionate, urea nitrogen, and ammonia. Based on the cation-anion differential, some plasma variables were also dramatically changed. These findings might indicate whether it is feasible to feed different DCADs to male lambs.

Author Contributions

All research protocols and animal experiments in this study designed, conducted experiments by M Khani, A Fattah, S Joezy-Shekalgorabi, SR Ebrahimi-Mahmoudabad and contributed to data acquisition and interpretation of the experimental results. GJ Cho contributed to the writing of the manuscript. All authors have read and agreed to the published version of the manuscript.

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