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Effects of diludine on the production, oxidative status, and biochemical parameters in transition cows

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Abstract: Twenty Friesian Holstein cows in the transition period were exposed to two dietary treatments, one fed with diludine (5 g day^{-1}cow^{-1}) and second fed without diludine, in order to examine effects of diludine on performance, antioxidative status, and animal health. Milk, urine, and blood samples were harvested. Oxidative stress related indices (superoxide dismutase (SOD), Malondialdehyde (MDA) and total antioxidant capacity (TAOC)), immunoglobulin A and M, acetone, acetoacetate, β-hydroxybutyric acid, and insulin were analyzed. Various animal health indicators like alanine aminotransferase (ALT) and alkaline phosphatase showed improvements resulting in increased milk yield and milk fats. Oxidative stress during parturition time got reduced. Two types of ketosis (primary and secondary) did not appear in both groups. Addition of diludine might improve the anti-oxidative status and reduce β-hydroxybutyrate, which might contribute to animal health and milk production. In the parturition time, feed with diludine decreased SOD activity and increased MDA abundance. The level of TAOC was slightly higher in the post parturition time than that in the pre-natal period of two groups.

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1. Introduction

Dairy cows face a critical phase during transition where homeostasis is challenged by physiological and endocrine challenges, which impose a significant metabolic stress (Goff and Horst, 1997). Oxidative stress occurs because of the diminished antioxidants or high production of reactive free radicals such as reactive oxygen species or reactive nitrogen species. The increased production of free radicals is the most attempted target of supplementation intervention in relation to disease prevention. In many situations, the body can adjust to enhanced oxidative stress by upregulation of antioxidant defense systems (Cline et al., 1999). If the oxidative stress can be alleviated, it often has no adverse contribution to disease pathology. If the antioxidant defense induction is absent or inadequate then a possible cellular and tissue damage often occurs.

Oxidative stress is at all instances not damaging. The use of supplementation is required when it is damaging to cells, tissues, proteins, cellular membranes, and mitochondria (Mandelker, 2011). The knowledge on the role of oxidants and antioxidants in physiological and pathological conditions is increasing (Celi et al., 2011).

To overcome this problem we ought to supplement the additives into dairy cow's ration. Dihydropiridine, a new additive, can provide a better physiological effects on dairy cows. Dihydropiridine had been shown to reduce oxidative stress during the transition period (Hisar et al., 2011). One kind of dihydropyridine is diludine (2, 6-dimethyl-3,5-dietheoxycarbonyl-1,4-dihydropyridine) is used in agriculture as a carotene stabilizer in grass meal and as a stimulator of the growth of farm animals (Spurzs, 1971). Moreover, this compound is a highly effective

stabilizer of vitamin A in oil solutions. A study of the biotransformation of diludine is valuable for the full pharmacological and toxicological evaluation of the drug (Odynets et al, 1986). Diludine is agent used to remove mutagenic effects caused by environmental pollutants, improves productivity and safeguards the reproductive system of parents and maintains the quality of off- springs (Hisar et al., 2011).

2. Materials and Methods

The study was conducted at Wuhu Dairy Farm, Wuhan, Hubei, China. The animals used in the study included twenty Chinese Holstein Cows in the transition period with an average weight of 575 ±4.2 kg with average milk production of 22.76 ±1.88. Cows were randomly allocated 2 dietary treatments (Table-1) in a complete randomized design. The applied treatments were formulated as feed without diludine and feed with diludine (5 % of concentrate feed per cow daily) 15 days before parturition to 15 days after parturition.

Table 1: Dietary treatments to experimental dairy cows.

Items	Treatment
Ingredients	**T0/T1**
corn3	45,0%
Bran	3,4%
soya bean meal	15,0%
cottonseed meal	8,0%
PKE	10,0%
corn DDGS	10,0%
$CaHCO_3$	1,0%
Powder	1,0%
Bicarb	0,0%
Salt	1,0%
WY01-1	1,0%
beet pulp	4,0%
Novasilplus	0,1%
XP	1,0%
Diludine	T1 contains 0.05% diludine
Composition	
DM (%)	88
CP (% DM)	18.5
Ether extract (% DM)	3.08
NDF (% DM)	47
ADF (% DM)	28
Ash (% DM)	7.12
NE_L (Mcal/kg DM)	1.69

T0 indicates feed without diludine; T1 indicates feed with diludine 5 g $day^{-1}cow^{-1}$. ADF: acid detergent fiber; CP: crude protein; DM: Dry matter; NDF: Neutral detergent fiber; NE_L: Net energy for lactation.

Sampling: Blood, urine and feed were sampled every week, while milk was sampled thrice a day during the experiment. Serum was obtained after centrifugation at 3000 rpm for 15 min and stored at -20°C until analyzed (Wang et al., 2008). Total superoxide dismutase (TSOD), malondialdehyde (MDA), total antioxidant capacity (TAOC) and insulin were tested using determination kits of Cell BioLabs Inc. Immunoglobulin A (IgA), Immunoglobulin M (IgM) and insulin was determined using ELISA kits from Kamiya biomedical company according to manufacturer's directions. Blood profile was carried out in the laboratory under the supervision of experienced clinicians. Milk was stored at 4°C for the study of fat, protein and lactose by infrared analysis (Laporte and Paquin, 19 Tsenkova et al. 1999) with a four-channel spectrophotometer (Milko-Scan, Foss Electric, Hillerød, Denmark) and somatic cell counts (SCC) using a cell counter (Fossmatic 400; Foss Electric, Hillerød, Denmark). Furthermore, urine was used to determine acetone, acetoacetate and beta hydroxybutyric acid using ELISA kits from Kamiya Biomedical Company (USA) according to manufacturer's protocol.

Statistical analysis: The experiment was conducted in Completely Randomized Design (CRD) consisting of two treatments viz., feed with diludine (T0) and feed without diludine (T1) repeated 10 times. The cows were randomized and subsequently exposed to the two treatments.

A Linear model that explains the observations according to a randomized block design was used as described below.

$Yij = \mu + \alpha i + \varepsilon ij \quad i = 1,2, \ldots a \quad j = 1,2, \ldots n \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots (1)$

Description:

Yij = observation on experimental units that received treatment diludine to cows

μ = general average, αi i = treatment effect to cows, εij = error component

Statistical hypothesis for all observations in this study are:

H0: $\alpha i = 0$

There was no additive effect of granting to the observed response

H1: at least one $\alpha i \neq 0$

Animal Ethics statement: All institutional and national guidelines for the care and use of animals were followed during the study.

Table 2: Milk Performance of cows 15 days after parturition (+15d)

Items	Treatments		*P*-value
	T0	T1	
Milk yield (kg/d)	22.76±1.88	24.11±0.81	<0.01
Milk protein (%)	3.02±0.13	3.28±0.11	0.646
Milk fat (%)	3.37±0.15	4.64±0.12	<0.01
Lactose	4.7±0.08	4.64±0.12	0.163
4% Fat Corrected Milk	19.01±0.9	20.97±0.52	0.223

T0 indicates feed without diludine; T1 indicates feed with diludine 5 g $day^{-1}cow^{-1}$.

3. Results and Discussion

Milk performance was measured and is shown in Table 2.

The level of TSOD before parturition was 32.84±5.8 for T0 and 33.7±5.1 for T1 while on the day of parturition and 15 days after parturition it was 36.47±2.3 for T0 and 46.47±2.3 for T1 and 70.52±2.1 for T0 and 78.99±2.5 for T1 respectively, which was statistically significant at P>0.05, implies that diludine affects the level of TSOD (Fig. 1).

The level of TAOC before parturition was 3.04±0.58 for T0 and 3.07±0.47 for T1; while on the day of parturition and 15 days after parturition it was 3.47±0.32 for T0 and 4.53±0.22 for T1 and 4.52±0.41 for T0 and 5.89±0.85 for T1 respectively, indicating statistically significant differences between T0 and T1 at P<0.05, hence we can confer diludine may affect the level of TAOC as shown in (Fig. 1a).

The level of MDA before parturition was 6.22±0.8 for T0 and 5.7±0.8 for T1 while on the day of parturition and 15 days after parturition it was 4.77±0.5 for T0 and 3.53±0.4 for T1 and 3.92±0.4 for T0 and 2.49±0.6 for T1 respectively, which shows statistically significant results at P<0.05 indicating diludine is affecting the level of MDA (Fig. 1b).

The level of acetone before parturition was 3.20±0.6 for T0 and 3.7±0.71 for T1 while on the day of parturition and 15 days after parturition it was 3.07±0.5 for T0 and 2.53±0.4 for T1 and 3.02±0.4 for T0 and 1.49±0.6 for T1 respectively, shows statistically significant differences between T0 and T1 at P<0.05 indicating diludine can affect the levels of acetone (Fig. 2).

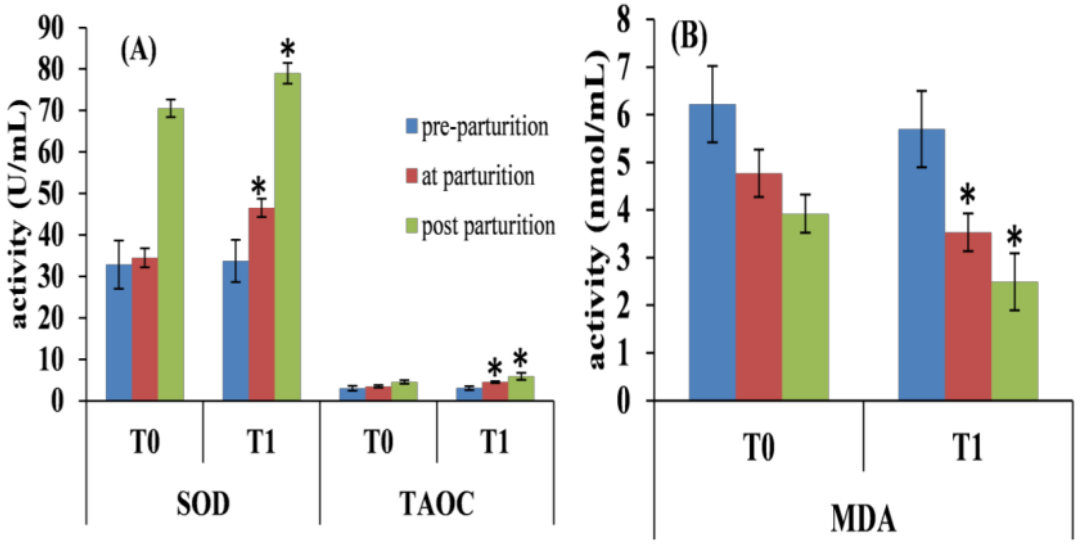

Fig. 1: Activity of SOD, TAOC and MDA a) The steady state increment of SOD and TAOC. b) The steady state of MDA. Superoxide dismutase (SOD), Total Antioxidant Capacity (TAOC), Malondialdehyde (MDA). was analyzed by spectrophotometer using determination kit at 15-days before parturition (15days before), at parturition and 15-days post parturition period.. Data represent mean±SE of three independent replicates and asterisks show significant difference (where *p* value is less than 0.05) while the bars without asterisks represent the non-significance of the data where *p* value is greater than 0.05.

Table 3: Blood profile of cows 15 days before parturition

	9 days before parturition			At the time of parturition			15 days after parturition		
	T0 (±)	T1 (±)	P Value	T0 (±)	T1 (±)	P Value	T0 (±)	T1 (±)	P Value
Glucose	3.47 (0.38)	3.58 (0.34)	*0.448*	3.95 (1.53)	2.51 (0.68)	*0.176*	4.87 (3.77)	9.6 (2.01)	***<0.01***
AKP	66.2 (11.4)	61.0 (5.3)	0.227	65.5 (5.3)	54 (3.4)	0.142	65.5 (5.3)	50.8 (1.4)	***<0.01***
ALT	28.2 (1.7)	25.4 (2.95)	0.628	21.9 (2.99)	29.7 (4.52)	0.489	32.9 (15.5)	37.7 (10.6)	0.123
AST	61.6 (4.5)	50.6 (9.3)	0.215	66.8 (7.8)	88.5 (14.2)	0.032	93.1 (9.4)	114.8 (36.8)	**<0.01**
Cholesterol	2.93 (0.8)	2.42 (0.34)	0.162	2.4 (0.37)	1.81 (0.26)	0.131	3.27 (2.35)	1.88 (0.4)	0.072
Creatine	103.8 (11.2)	90.9 (10.8)	0.161	54.1 (7.2)	60.7 (13.3)	**<0.01**	86.1 (11.4)	83.4 (11.8)	**<0.01**
Albumin	31.3 (8.8)	25.22 (8.89)	0.037	25.08 (0.89)	24.63 (2.01)	0.073	16 (1.2)	16.3 (1.8)	**<0.01**
Uric acid	24.6 (6.9)	21.1 (2.7)	0.671	50.8 (13.9)	45.1 (10.3)	0.205	38.5 (7.7)	45.8 (9.7)	**<0.01**
BUN	4.24 (0.76)	4.07 (0.35)	0.109	4.91 (1.69)	4.6 (1.03)	**<0.01**	3.51 (1.15)	3.25 (0.63)	**<0.01**
Total Protein	56.9 (7.4)	50.8 (6.09)	0.203	55.72 (4.55)	52.98 (3.31)	0.365	54.92 (5.77)	57.14 (8.2)	0.323
AGR	0.92 (0.3)	0.86 (0.17)	0.632	0.72 (0.24)	0.86 (0.81)	0.239	0.46 (0.06)	1.05 (1.0)	0.044
Globulin	30.78 (8.1)	28.24 (6.39)	0.288	29.73 (4.34)	29.03 (7.31)	0.048	36.85 (5.39)	36.13 (7.15)	**<0.01**
Total bilirubin	3.94 (1.36)	2.94 (1.4)	0.605	7.18 (2.6)	9.32 (4.9)	0.095	4.88 (2.02)	6.56 (0.59)	0.049
Direct bilirubin	2.64 (2.5)	1.99 (1.7)	0.181	2.55 (1.09)	3.73 (2.1)	0.177	2.25 (1.12)	3.36 (1.99)	**<0.01**
Indirect bilirubin	2.86 (1.19)	3.5 (1.67)	0.307	3.79 (1.7)	5.44 (3.01)	0.095	5.17 (4.01)	3.23 (1.15)	0.056
Urea	3.48 (1.1)	2.63 (0.53)	0.248	3.46 (0.37)	4.31 (0.81)	0.021	3.66 (0.33)	2.97 (0.84)	0.097

15 ndicates feed without diludine; T1 indicates feed with diludine 5 g day^{-1}cow^{-1}. 32

AKP: alkaline phosphatase; ALT: alkaline aminotransferase; AST: aspartate aminotransferase; GLU: glucose; AGR: albumin globulin ratio; BUN: blood urea nitrogen.

The level of acetoacetate before parturition was 4.15±0.5 for T0 and 4.24±0.61 for T1; while on day of parturition and 15 days after parturition it was 4.07±0.6 for T0 and 2.73±0.4 for T1 and 4.02±0.49 for T0 and 2.41±0.66 for T1 at P<0.05 respectively, shows T0 and T1 statistically significant implies diludine may affect the levels of acetoacetate (Fig. 2).

The level of β-HBA before parturition was 3.65±0.59 for T0 and 3.29±0.31 for T1, while on the day of parturition and 15 days after parturition it was 3.18±0.11 for T0 and 1.94±0.28 for T1 and 2.02±0.19 for T0 and 0.41±0.23 for T1 respectively, showing statistically significant differences between T0 and T1 at P<0.05 (Fig. 2). One of the parameters to know ketosis in dairy cows is determined by level β-HBA termed ketosis in dairy cows.

The level of IgA before parturition was 0.37±0.04 for T0 and 0.41±0.07 for T1 while on the day of parturition and 15 days after parturition it was 0.44±0.7 for T0 and 1.63±0.37 for T1 and 0.51±0.8 for T0 and 2.09±0.19 for T1 respectively, shows statistically significant differences between T0 and T1 at P<0.05 indicates diludine might affect the level of IgA (Fig. 3).

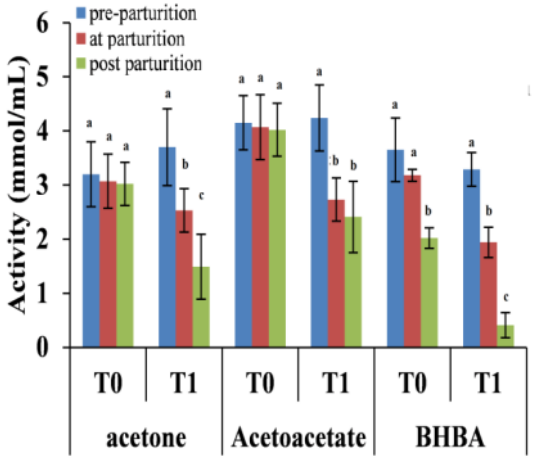

Fig. 2: Ketosis in dairy cows. The activity was measured from the samples taken 15 days parturition, at parturition (0 days) and 15 days post parturition periods. Diludine (5g day^{-1} cow^{-1}) was fed to 10 cows. Data represent mean±SE of three independent replicates and asterisks show significance while the bars without asterisks represent the non-significance of the data.

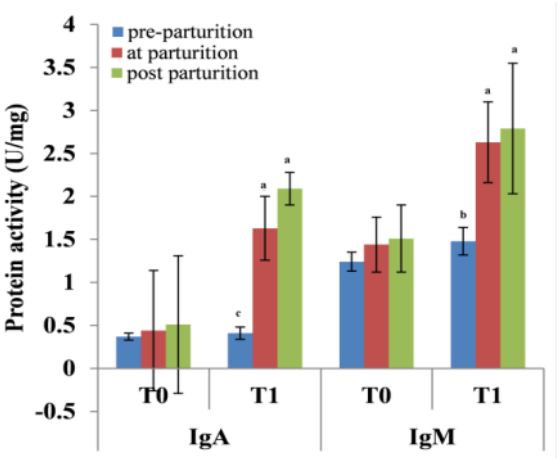

Fig. 3: IgA and IgM in response to dietary diludine in dairy cows. The protein activity was measured from samples taken 15 days prepartum , at parturition (0 days) and 15 days post parturition periods. Diludine (5g days^{-1} cow^{-1}) was fed to 10 cows. Data represent mean±SE of three independent replicates. Means with different letters are significantly differ from each toher, while the bars without letters represent the non-significance of the data.

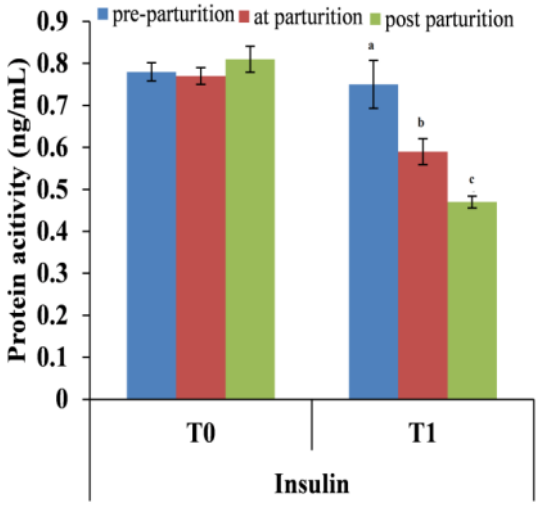

Fig. 4: Effect of diludine on Insulin level in dairy cows. Determination of Insulin was made by Gamma Radioimmunoassay Counter. The protein activity was measured from the samples collected at pre-parturition (15 days before), at parturition (0 days) and post parturition (15 days after) periods. Diludine (5g day^{-1} cow^{-1}) was fed to 10 cows. Data represent mean±SE of three independent replicates and asterisks show significance while the bars without asterisks represent the non-significance of the data.

The level of IgM before parturition was 1.24±0.11 for T0 and 1.48±0.16 for T1 while on the day of parturition and 15 days after parturition it was 1.44±0.32 for T0 and 2.63±0.47 for T1 and 1.51±0.39 for T0 and 2.79±0.76 for T1 respectively, shows statistically significant differences between T0 and T1 at P<0.05, indicates diludine might affect the level of IgM (Fig. 3).

The level of insulin before parturition was 0.78±0.022 for T0 and 0.75±0.030 for T1 while on the day of parturition and 15 days after parturition it was 0.77±0.020 for T0 and 0.59±0.023 for T1 and 0.81±0.031 for T0 and 0.47±0.021 for T1 respectively, shows no significant results at P<0.05, indicating diludine have no affect on insulin (Fig. 4).

The study of antioxidant enzymes remains important both in human and animal medicine (Sharma et al., 2011). The onset of lactation is correlated with massive metabolic changes which in turn has negative effects on animal health (Sordillo et al., 2009). The levels of SOD recorded in the present study at start of experiment i.e. 15 days before parturition (T0 32.84±5.8 and T1 33.7±5.1) indicates low activity of SOD, which is responsible for high levels of ROS causing oxidative stress in dairy cows. The increment in SOD at parturition (36.47±2.3 for T0 and 46.47±2.3 for T1) and post parturition periods (70.52±2.1 for T0 and 78.99±2.5 for T1) constitutes the first defence line against ROS consistent with the studies of Elstner et al., (1991). These results suggest diludine improves SOD activity and replenishing antioxidant enzymes which are often depleted in chronic oxidative stress, to fight ROS.

The high levels of MDA (62 μmols/l) at the first week of parturition indicate oxidative stress in dairy cows during transition periods (Adela et al., 2006). In the present study the decrease in levels of MDA at parturition (4.77±0.5 for T0 and 3.53±0.4 for T1) and post parturition periods (3.92±0.4 for T0 and 2.49±0.6) with diludine as dietary additive points towards improved correlation with high activity of antioxidant enzymes (SOD).

The oxidative stress index has been used in several oxidant stress conditions with consistent results (Erel, 2004, Miyazawa, 1989). In the present study the levels of TAOC at parturition (.47±0.32 for T0 and 4.53±0.22 for T1) and post parturition (4.52±0.41 for T0 and 5.89±0.85 for T1) significantly increased when using diludine as a dietary additive.

The chemical substances like acetone, beta hydroxybutyric acid and acetoacetate are called ketone bodies (Singh et al., 2011). The level of acetoacetate is considered to be a good marker of ketosis disease in dairy cows. Urine samples were collected to check the levels of different ketone bodies including acetone, acetoacetate and beta-hydroxybutyric acid. The decrement in the levels of ketone bodies at parturition time indicates a probable positive effect on ketosis in dairy cows.

The accumulation of the total immunoglobulin produced in the entire body is 75% (Macpherson and Slack, 2007). Most IgA is produced in the mucosa-associated tissue by large numbers of plasma cells in the mucosal sub epithelium (Conley and Delacroix, 2007; Novak et al., 2001; Novak et al., 2001). The mucosal surfaces all together make a huge surface area (~400 m^2 in the human adult) and the importance for such an intensive IgA generation at the mucosa might reflect a critical requirement for immune protection of mucosal sites (Childers et al., 2009). The level of IgA at parturition (0.44±0.7 for T0 and 1.63±0.37 for T1) and post parturition period is (0.51±0.8 for T0 and 2.09±0.19 for T1). The results indicate an increment to IgA levels which shows that diludine might improve innate immune response of dairy cows to various viral and bacterial pathogens.

Immunoglubulin M (IgM) is another antibody which provides innate immunity to dairy cows. In the

present study with diludine as a dietary additive, levels of IgM increased at the parturition (1.44±0.32 for T0 and 2.63±0.47 for T1), and post parturition period (1.51±0.39 for T0 and 2.79±0.76 for T1) which indicates a better immune response of dairy cows to fight several pathogens. Conclusively, diludine is shown to have an enhancing effect on IgM levels during parturition periods. The immune function may increase when the immune system is at normal or risk state with the addition of dietary diludine.

Insulin is an important hormone, playing a crucial role in the metabolism of carbohydrates, proteins and fats. Low insulin level causes insulin dependent Type 1 diabetes resulting in high blood level sugars and dehydration. On the other hand, high insulin levels cause low blood sugar level resulting in weakness, anxiety and convulsions. The results in the present study show a constant state of insulin level for healthy dairy cow (0.5ng/ml) with a minor insignificant increase with diludine as a dietary additive. This indicates diludine has no negative effects on insulin levels.

4. Conclusion

Diludine exerts a positive effect on overall animal health and reduces oxidative stress as shown by increased levels of immune indicators and high levels of SOD and TAOC while decreased MDA levels. The proper formulation of dairy feed with adequate quantity of diludine might improves the animal resistance to adverse conditions including heat and disease, Further studies are required to validate the role of diludine in dairy feed as antioxidative agent.

List of Abbreviations: ALT: alanine aminotransferase; MDA: Malondialdehyde; SOD: superoxide dismutase; TOAC: total antioxidant capacity.

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Competing Interests: The authors declare that they have no conflict of interest.

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