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Research Article
Effects of Humate and Probiotic on the Number of *Escherichia coli*, Blood and Antioxidant Parameters in Suckling Period of Calves

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Abstract

Objectives: This experiment investigated the effect that use of humate and probiotics as feed additives in the feeding of the calves in the milk-sucking period until the end of period cut from the milk has on the *Escherichia coli* count, blood and antioxidant parameters. **Materials and Methods:** In the experiment, 24-day-old Brown Swiss calves were used as part of the experiment, they were put in 3 different groups 8 calves each with the average weight of 42.5 kg (±2.5 kg). During the study, control group were fed dry clover grass +full-fat milk+calf starting feed (Basal diet), humate group were fed basal diet +0.15% of humate and probiotic group was fed basal diet +0.15% of probiotic. The study continued for 12 weeks. **Results:** In the 6th, 9th and 12th weeks of the study, as humate additive were decreased the number of *E. coli* ($p<0.01$), probiotic additive caused no change. It was seen that, in the 6, 9 and 12 weeks of the study, blood serum in total protein, albumin, triglycerides, HDL, AST, ALT, Ca, Fe, NO and SOD levels were not affected from humate and probiotic additives. Humate additives increased Mg and Cu MDA ($p<0.01$) levels, whereas the additives decreased glucose, phosphorus, GPx ($p>0.05$) and total cholesterol, zinc and LDL levels ($p<0.01$). **Conclusion:** In this study, humate additives had positive effect on the animals' health while it also led to oxidative stress.

Key words: Antioxidant, blood parameters, calve, *Escherichia coli*, feed additives, humate, probiotic, suckling period

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The use of productive additives such as humate and probiotics increased significantly after antibiotics became prohibited. Humic Acids (HAs) are complex organic macromolecules that comprise Humic Substances (HS), which humic, fulvic, ulmic acids and some micro minerals which originated from humus caused by materials such as phenols, amino acids, carbonhydrates, which are organic matter distributed in terrestrial soil, natural water and sediments resulting from the decay of vegetable and natural residues. MacCarthy[1] and Stevenson[2] because of their chemical properties such as solubility, pH dependence, interaction with hydrophobic groups. De Melo *et al.*[3] can transfer electrons and is a good chelating agent with many metal ions due to their properties. Additionally humic acids are used for pharmaceutical applications[4,5]. Humate compounds are reported to help suppress malignant bacteria species by providing optimum pH build in the digestive tract, help intestinal health promotion by lowering toxin level, stimulate benign in order to help microorganisms develop[6,7], decrease prevalence of diarrhea and other digestive problems[8] and act as an antiviral and anti-inflamatory[9-11] and antioxidant[12]. Another important aspect humic substances is it can be used for detoxification[13] moreover, humic acids can neutralize glyphosate and *C. botulinum* toxins[14] and which leads to an improved health of animals[15]. Although, calf's blood, biochemical and hematological parameters and rumen fermentation were changed[16,17].

Probiotics are described as natural agents which show antagonistic effects in the intestines of the given animal against pathogenic microorganisms, which also has beneficial effects on intestinal microflora, including non-pathogenic Gram-positive and facultative anaerobe, lactic acid producing live and natural intestinal bacteria, yeast cells and culture, fungus, enzymes and industrial fermentation products[18]. Probiotics have the ability to enhance intestinal health by stimulating the development of a healthy microbiota (predominated by beneficial bacteria), preventing enteric pathogens from colonizing the intestine, increasing digestive capacity, lowering the pH and improving mucosal immunity[19]. Also, it has been reported that conventional use of probiotics helps to reduce distention, repress diarrhea contraction, improve gas trointestinal health by increasing natural resistance to infections in gastrointestinal system[20]. A report on ruminants showed that the addition of live yeast culture rose rumen pH and provided the optimal conditions for fermentation[21] additionally their performance improved by Finck *et al.*[22].

In this study, the effects of using preparates as productive additives such as humate in the form Bovifarmdry™ and nutri-sacc (*Saccharomyces cereviciae*, min 3.0×10^8 CFU g^{-1}) as a probiotic, on the number of *E. coli*, blood and antioxidant. When feeding calves as feed additives from suckling period to the end of delectation period was researched and it aimed to contribute to the studies done on this subject so far.

MATERIALS AND METHODS

In this study, 24 one-day-old Brown Swiss type calves at the weight of nearly 42.5 kg (±2.5 kg) were experimented in three groups having 8 calves (4 female, 4 male) in each group. During the study, the animals that were used in the experiment were fed with dry clover grass, full-fat milk, 0.15% of humate (Bovifarm dry™) and calf starter feed which includes 0.15% of probiotic (Nutri-sacc) with the levels of nutrition, material and energy as given in Table 1. The study continued for 12 weeks. During the study, the calves in the experiment groups were given milk in the amount of 10% of their live weight every week from the 1st day of the experiment on. Also they were fed with calf starter feed from the 7th day on and dry clover grass from the 28th day on. From the 3rd day of the experiment on clean potable water was always available in front of the calves.

The dry material, crude cinder and crude protein ingredients included in the samples of milk, dry clover grass and calf starter feed were determined according to the methods given in AOAC[23]. The study was conducted in three groups as control, humate and probiotic (Table1).

In the 6th, 9th and 12th weeks of the study, nearly 10 mL blood samples were taken from the calves 2 h after they were given milk and in the blood serum samples, the amounts of total protein, total cholesterol, AST, ALT, triglyceride, glucose, albumin, HDL, LDL, calcium, phosphor and ferrous were spectrophotometrically determined on Olympus Au 400 system autoanalyser. Also the determination of trace elements (Zn, Cu and Mg) of the blood serum samples were carried out on the Analyst 800 AAS model. The nitric oxide level from the parameters of antioxidant in the blood serums were spectrophotometrically analyzed with Griess reaction[24].

Superoxide dismutase was calculated by using the principle of the fact that reduction of nitroblue tetrasolium is

Table 1: Nutrition levels of the feeds used in the study (%)

Nutrients	Alfalfa hay (%)	Milk (%)	Calf starting feed (%)
Dry matter	91.70	12.15	91.40
Crude protein	7.98	4.66	19.01
Crude fat	3.85	4.10	4.67
Crude ash	8.61	0.76	8.00
Crude fiber	27.12	-	12.00
Energy	-	-	2900.00

inversely proportional with maximum adsorbance on 560 nm wavelength by studying Cu/ZnSOD enzyme activity according to the method taken from Sun *et al.*[25]. Glutathione peroxidase was spectrophotometrically analyzed and GPx enzyme activity was calculated[26]. Malondialdehyde was calculated on the basis of the principle[27] that the adsorbance of the pink-coloured complex created by thiobarbituric acide and MDA as a result of incubation on 95°C is spectrophotometrically determined on 532 nm wavelength.

Also, in order to determine the values of rumen liquid *E. coli* countin the rumen liquid that was taken with rumen catheter 2 h after the calves were given milk in the 6th, 9th and 12th weeks of the experiment was conducted with the Clinical Microbiology Diagnose Method Bilgehan[28].

The data were completed as control, humate and probiotic groups. And these groups were analyzed with the variants of gender and time by using multivariate model. The SPSS 10.0 packaged software SPSS[29] was used for the statistical analysis of the data.

Variance analysis model:

$$y_{ijkl} = \mu + a_i + b_j + c_k + d_l(\varepsilon_l)$$

where, y_{ijkl} is the calves in the milk-sucking period effect time and sex connect of humic acid and probiotic, μ is a population average, a_i is the effect of groups (Humate, probioitic, control), b_j is the effect of sex, c_k is the effect of time and d_l (ε_l) is error term.

RESULTS

The study found that the number of rumen liquid *E. coli* decreased significantly by use of humate ($p<0.01$) and over time ($p<0.05$), while decreased negligibly probiotic additives (Table 2).

Calves blood samples determined that the level of total protein, albumin, HDL, AST, ALT, calcium and iron was not influenced by humate and probiotics or gender and time. But humate does have a reducing ability on the glucose ($p<0.05$), total cholesterol ($p<0.01$), LDL ($p<0.01$) and phosphorus levels ($p<0.05$). The level of triglyceride was higher in females ($p<0.01$) and the degree of phosphorus decreased as effect by time was ($p<0.05$) (Table 3). Total protein levels in the humate group were higher for female than male ($p<0.05$), in the probiotic group it was higher in male than female ($p<0.05$) and total protein level in the control group was similar (Fig. 1a). The HDL level in terms of feed and gender was as follows. The HDL level of humate group was higher in female than male while in probiotic and control groups it was higher for male than female ($p<0.05$) (Fig. 1b).

This study also showed that the ratio of NO and SOD was not affected by any factor. The NO level in humate group was insignificant when it came to male and female. While in

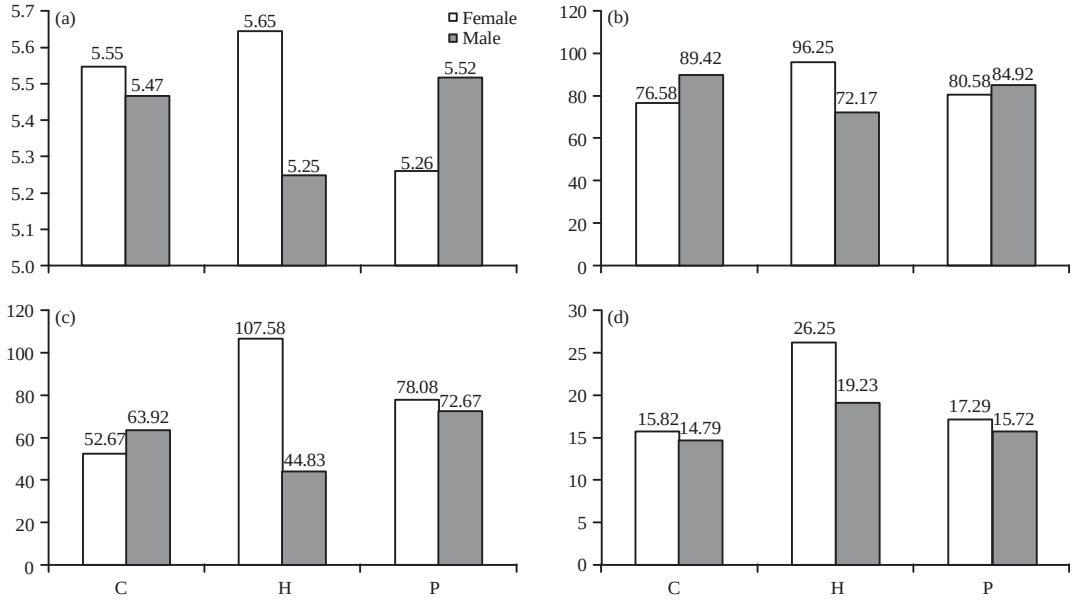

Fig. 1(a-d): Gender and ration groups impact of (a) Total protein value (g dL^{-1}), (b) HDL levels (mg dL^{-1}), (c) NO (µmol L^{-1}) and (d) MDA (µmol L^{-1}) ($p<0.05$)

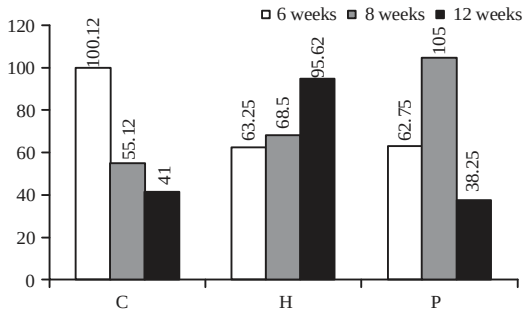

Fig. 2: Ration groups and week activity impact of levels NO (μmol L^{-1}) ($p<0.05$)

Table 2: Rumen fluid *Escherichia coli* counts (CFU g^{-1})

Factors	*E. coli*
Ration (p)	**
Control	42792[a]
Humate	29675[b]
Probiotic	35958[ab]
SE	2793
Gender (p)	-
Female	34256
Male	38028
SE	2281
Time (p)	*
6 weeks	42042[a]
9 weeks	35050[ab]
12 weeks	31333[b]
SE	2793
Overall average	36142
SE	1613

*p<0.05, **p<0.01, [a-c]Averages indicated by different letters in the same column are important differences

probiotic group it had higher NO levels in females than males ($p<0.05$) (Fig. 1c). Moreover, ratio and time interaction of NO level in humate group was the lowest in 6th week and the highest in 12th week ($p<0.05$). Probiotic group the highest was 9th week and the lowest 12th week ($p<0.05$). Control group the highest was the 6th week and the lowest 12th week ($p<0.05$) (Fig. 2). The GPx level was decreased by humate ($p<0.05$), moreover it was determined that the GPx levels in female calves were higher than in males ($p<0.05$). The level of MDA in humate group which is one of the parameters of the oxidative stress was calculated to be higher than in the other two groups ($p<0.01$). Also the MDA level of the female calves was higher than in males ($p<0.01$) (Fig. 1d). Moreover, from the 6th-12th weeks the amount of zinc increased ($p<0.05$). But the addition of both humate and probiotic additives cause a significant decrease in the content of zinc ($p<0.01$). The level of magnesium decreased significantly due to humate additive ($p<0.01$) (Table 4).

DISCUSSION

The results of the studies in which the humate and probiotic additives are separately evaluated were used due to the fact that both humate and probiotic additives are evaluated together as dietary supplement for the calves in their earliest periods.

The study was conducted in harmony with this data and it was observed that the numbers of rumen liquid *E. coli* decreased when the humate and probiotic were added in the 6th, 9th and 12th weeks. Also, it was noticed that time has a significant effect on the number of rumen liquid *E. coli* and that the number of *E. coli* decreased as amount of time increased. This resulted from the fact that humate additives have antibacterial, antiviral and anti-inflammatory effects on animals Islam[30] and helps them create a defense mechanism against such pathogens as *E. coli*. It is thought that humate additives can have reductive effect on the number of *E. coli*. Also, as in the case of humates, after probiotic were added, it was reported that there was a decrease in the fecal release of *E. coli* 0157:H7 conducted by Ohya *et al*.[31,32], Tkalcic *et al*.[33] and Agazzi *et al*.[34] and the amount of *E. coli* 0157:H7 in the rumen environment[35,36].

In this study, after an analysis of the blood samples taken from the calves, it was determined that the levels of total protein, albumin, HDL, AST, ALT, Ca and ferrous were not affected by humate and probiotic or even by age and time. Also, humate had a reductive effect on glucose, total cholesterol, LDL and phosphor levels. In addition to these, triglyceride level was affected by age and time, time also affected the phosphor level. Although, it was discovered that probiotic addition decreased the blood triglyceride level while it was no effect the blood glucose and total protein level[37], in other studies conducted, it was observed that the probiotic and fulvic acids do not effect blood serum calcium, triglyceride and cholesterol levels[38] and it was noted that the humate additives do not affect the total protein, albumin, triglyceride, calcium and phosphor levels[39], chemical parameters[40] and that humate additives decreased the level of LDL and total cholesterol amount[39] but the addition of live yeast culture gave a rise to serum glucose level but the differences between groups were not observed[41]. In another study active dry yeast supplement had no significant effect on concentration of GLU, urea, TP, TC, LDL, HDL, VLDL and activity of ALT or AST but significantly ($p<0.05$) increased the blood concentrations of TG and tended to increase concentrations of Free Fatty Acids (FFA)[42].

The data gathered in this study mostly agrees with the literature data. In this study conducted, NO and SOD levels

Table 3: Blood serum values

Factors	Total protein (g dL^{-1})	Albumin (g dL^{-1})	Glucose (mg dL^{-1})	Total cholesterol (mg dL^{-1})	Triglycerides (mg dL^{-1})	HDL (mg dL^{-1})	LDL (mg dL^{-1})	AST (U L^{-1})	ALT (U L^{-1})	Calcium (mg dL^{-1})	İron (mg dL^{-1})	Phosphorus (mg dL^{-1})
Ration (p)	-	-	*	**	-	-	**	-	-	-	-	*
Control	5.17	2.62	133.21^{a}	138.67^{a}	25.79	83.00	21.62^{a}	65.37	27.00	10.21	128.58	7.79^{a}
Humate	5.12	2.79	122.42^{b}	123.37^{b}	22.58	84.21	15.04^{b}	61.12	24.42	10.46	151.37	6.71^{b}
Probiotic	5.04	2.71	136.33^{a}	141.75^{a}	26.62	82.75	23.25^{a}	71.29	26.37	10.17	135.04	7.58^{a}
SE	0.10	0.11	3.31	3.71	2.04	5.17	1.30	3.57	1.72	0.12	8.20	0.27
Gender (p)	-	-	-	-	**	-	-	-	-	-	-	-
Female	5.14	2.75	129.39	137.58	28.44	84.47	20.42	66.19	27.58	10.28	145.89	7.50
Male	5.08	2.67	131.92	131.61	21.56	82.17	19.53	65.67	24.28	10.28	130.78	7.22
SE	0.08	0.09	2.70	3.03	1.67	4.22	1.06	2.91	1.40	0.09	6.70	0.22
Time (p)	-	-	-	-	*	-	-	-	-	-	-	*
6 weeks	5.12	2.62	128.46	134.67	29.29^{a}	89.42	21.50	66.00	27.29	10.29	144.33	7.96^{a}
9 weeks	5.12	2.83	134.62	138.75	24.50^{ab}	81.92	18.83	69.92	24.79	10.46	135.04	7.08^{b}
12 weeks	5.08	2.67	128.87	130.37	21.21^{b}	78.62	19.58	61.87	25.71	10.08	128.58	7.04^{b}
SE	0.10	0.11	3.31	3.71	2.04	5.17	1.30	3.57	1.72	0.12	8.20	0.27
Overall average	5.11	2.71	130.65	134.60	25.00	83.32	19.97	65.93	25.93	10.28	138.33	7.36
SE	0.06	0.06	1.91	2.14	1.18	2.99	0.75	2.06	0.99	0.07	4.73	0.16

*p<0.05, **p<0.01, $^{a-c}$Averages indicated by different letters in the same column are important differences

Table 4: Values of Antioxidant parameters

Factors	Magnesium (mg L^{-1})	Zinc (mg L^{-1})	Copper(mg L^{-1})	NO (µmol L^{-1})	SOD (U mL^{-1})	GPx (U L^{-1})	MDA (µmol L^{-1})
Ration (p)	**	**	**	-	-	*	**
Control	17.38^{a}	225.00^{a}	68.17^{b}	65.42	0.58	-335.51^{a}	14.87^{a}
Humate	22.04^{b}	64.75^{c}	79.21^{a}	75.79	0.08	-413.80^{b}	22.42^{b}
Probiotic	18.42^{a}	196.83^{b}	58.92^{c}	68.67	0.00	-382.72^{ab}	16.00^{a}
SE	0.39	6.56	2.70	11.81	0.23	18.53	0.80
Gender (p)	-	-	-	-	-	*	**
Female	18.86	165.42	69.14	79.44	0.08	-403.91	19.50
Male	19.69	158.97	68.39	60.47	0.36	-350.82	16.03
SE	0.32	5.35	2.20	9.65	0.19	15.13	0.65
Time (p)	-	*	-	-	-	-	-
6 weeks	19.33	150.87^{a}	73.00	75.37	0.25	-359.64	17.71
9 weeks	19.38	159.25^{ab}	65.42	76.21	0.33	-385.61	18.21
12 weeks	19.13	176.46^{b}	67.87	58.29	0.08	-386.82	17.37
SE	0.39	6.56	2.70	11.81	0.23	18.53	0.80
Overall average	19.28	162.19	68.76	69.96	0.22	-377.31	17.76
SE	0.23	3.78	1.56	6.82	0.13	10.71	0.46

*p<0.05, **p<0.01, $^{a-c}$Averages indicated by different letters in the same column are important differences

were not affected by any factors, however, it was observed that humate had a reductive effect on GPX level. It was detected that GPX levels were lower in female calves than in male ones. Also it was observed that MDA level from oxidative stress parameters were significantly higher in the humate added groups compared to the other two groups where it was detected that MDA level in female calves was higher than in the male ones. Additionally the zinc level increased from 6th-12th weeks. Yet, in a study conducted on broiler, it was reported that broiler did not have a significant difference when it came to the zinc level in liver, kidney, leg muscles and blood serum when a combination of humic acid and zinc was used, in spite of the fact that zinc level decreased in significant amounts when humate and probiotic were added[43]. In order to determine how stress factors into the health status of animals, blood anti-oxidants are generally used. Especially MDA level is used as an indicator of an oxidative in the organism[44]. In the limited studies conducted, it has been reported that the humate addition leads to an increase in the level of MDA and NO in the blood for the calves, a decrease in the activities of SOD and GPx[45] and that the probiotic addition increases the SOD and GPx levels and decreases the MDA level in swines[46]. And in another study conducted, it was concluded that humic acid addition spoiled oxidative balance and led to oxidative stress in rats[47] and in a study conducted on Japanese quails, it was reported that a high-level humic acid additive (600 mg kg^{-1}) increases oxidative stress and therefore decreased the antioxidant capacity and low concentration of humic acid additive does not affect total antioxidant capacity[48]. The results from the previous data demonstrated that the effect of humate on antioxidant parameters is related to the rate of its ration and decreases the MDA level by

causing humate liquid peroxidation[49] and this leads to spoiling of the balance between oxidants and anti-oxidants in calves and thus leads to oxidative stress.

CONCLUSION

This study has shown the effects of humate additives on the rumen fluid and blood parameter is greater when compared with probiotic additives. Humate additives decreased not only the number of *E. coli* which is closely related to the animal's health and also to the blood LDL level. Moreover, it was found that humate additives lead to oxidative stress resulting in an increase in the MDA ($p<0.01$) levels and a decrease in the activities of GPx (Glutathione peroxidase) ($p>0.05$) in the blood. Therefore, it was found from the gathered data that at times it agreed with and sometimes it was in conflict with the literature data. The reason for this, stems from animal breeds, age, environmental conditions and ration, humate quantities and characteristics used in the studies. For this reason, further scientific studies should be carried out and its the only possible way to reach definite conclusion.

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