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Humic substances isolated from clay soil may improve the ruminal fermentation, milk yield, and fatty acid profile: A novel approach in dairy cows

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ABSTRACT

The objective of this study was to examine the effect of clay derived-humic substances (CD-HS) on nutrient intakes, digestibility coefficients, rumen fermentation, milk yield, and milk components in Holstein-Friesian dairy cows. The experiment was designed as a 3 × 3 Latin square design using 12 dairy cows, which are allotted to basal diet without humic substance (Control), with CD-HS at 5 g/kg diet (CD-HS5), and 10 g/kg diet (CD-HS10). The experiment was conducted in 3 periods and each period consisted of 21 days with 14 days of adaptation and 7 days collection period. The humic substance supplementation did not affect the nutrient intakes and digestibility coefficients, except for crude protein, which showed a linear ($P < 0.01$) increase. The ruminal pH was similar between the treatments, but the NH_3-N content and protozoal population were decreased quadratically ($P < 0.01$) with CD-HS supplementation. Although the experimental diets did not affect the butyrate proportion, they caused a quadratic increase in total volatile fatty acids and the proportions of acetate and propionate contents. The CD-HS diets caused a linear increase ($P < 0.05$) in blood glucose and a decrease in cholesterol and blood urea nitrogen concentration. The diets did not affect ($P > 0.05$) the milk components, but they caused a linear increase ($P < 0.05$) on milk yield. Among the total fatty acids of milk, the levels of C18:0 and C24:0 and thrombogenicity index were decreased while increasing the C18:2 isomers (t9t12-C18:2 and C9,C12-C18:2) and polyunsaturated fatty acid contents. It was concluded that the clay derived-humic substances at 5 g/kg diet could cause desirable effects on nutrient intake, rumen fermentation profile, biochemical parameters, milk yield, and fatty acid profile.

Abbreviations: ADFom, acid detergent fibre; AI, atherogenicity index; ALT, alanine amino transferase; AST, aspartate amino transferase; BUN, blood urea nitrogen; C, carbon; C/H, carbon/hydrogen; C/N, carbon/nitrogen; C/O, carbon/oxygen; C/P, carbon/phosphorus; Ca, calcium; CD-HS, clay derived - humic substance; CLA, conjugated linoleic acid; CP, crude protein; DM, dry matter; EE, ether extract; FCM, fat corrected milk; Fe, iron; H, hydrogen; MUFA, monounsaturated fatty acids; N, nitrogen; NDFom, neutral detergent fibre; NH_3-N, ammonia-N; NRC, National Research Council; O, oxygen; P, phosphorus; PUFA, polyunsaturated fatty acids; SCFA, short-chain fatty acids; SNF, solid not fat; TI, thrombogenicity index; TVFA, total volatile fatty acid

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1. Introduction

Milk is a significant source of energy, high-quality protein, fat, vitamins, and both macro and micro minerals. The role of dairy cattle in providing nutrients to the world's growing population is crucial. The rapid growth of world population demands the requirement of additional protein in the form of milk or meat. Ruminal manipulation is one of the most important tools for improving milk productivity, thereby benefitting both farmer and consumer (Santra and Karim, 2003). The role of antimicrobial additives in manipulating the rumen and improving production is indispensable (Chattopadhyay et al., 2014). However, the utilization of antibiotics is facing reduced social acceptance, presumably due to the residues in livestock derived products and the consequences for public health. Because of the residue and resistance concern, the animal nutritionists have been promoting the use of natural supplements as antibiotic replacements to the ruminant diets. Among these antimicrobial feed additives, clay derived-humic substance (CD-HS) has an indispensable potentiality in increasing the performance (Terry et al., 2018b).

Humic substance, an alkali-soluble and high molecular weight molecule, is generally responsible for composting and nutrient transfer from soil to living organisms. Humic substance compounds also form coatings on clay particles, which could cause nutrient migration in water and soil. Currently, the commercial markets have been selling humic substances under different trade names for agriculture purpose. With the compound annual growth rate of 6.1%, the humic substance market is predicted to reach 624.98 million US dollars by 2026 from 388.02 million US dollars in 2018 (RAD, 2019). The humic substance-based commercial products are claimed to increase plant growth by improving the bioavailability of phytonutrients. More particularly, these substances are known to decrease the adverse effects of chemical or mineral fertilizers in the soil (Raheem et al., 2018).

The antimicrobial, detoxifying, and absorptive properties of humic substance have been tested to encourage the growth of beneficial bacteria within the soil (Islam et al., 2005). However, the extent of adoption of above-mentioned effects of humic substance on ruminal environment and production performance is questionable. Hence, the present study was conducted with a hypothesis that the clay derived-humic substance supplementation could improve the production performance of dairy cows by showing a positive effect on the nutrient intakes, digestibility coefficients, ruminal fermentation, milk yield, and fatty acid profile.

2. Materials and methods

The experimental trial was done at Noubaria experimental station, Animal Production Research Institute (APRI), Agriculture Research Center (Egypt) and the laboratory analysis was performed at the laboratory of dairy animal production, National Research Centre (Egypt). The region possesses average rainfall of 22 mm and mean annual temperature between 14 and 32 °C. All the experimental animals (cows) were maintained in accordance with the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (FASS, 2010).

2.1. Humic substances preparation

In this study, the humic substances (HS) used were extracted from brown clay soil of Experimental Farm of the Soils, Water and Environment Research Institute, Agriculture Research Center, Giza, Egypt.

2.1.1. Extraction of humic substances

The extraction and purification of humic substances was performed according to the procedures outlined by the International Humic Substances Society (Aiken et al., 1985). To prepare the extract containing humic and fulvic acid mixture, 1000 g air-dried soil with less than 2-mm diameter was stirred in 0.1 M alkali (NaOH) solution for 24 h at room temperature and was acidified with 6 M HCl until the pH equals 1.0. The separation of humic and fulvic acids were done by centrifuging the acidified solution at 11,200 x g for 15 min. The purification of humic acid fraction was done by suspending the humic acid precipitate with 0.1 M HCl followed by dialyzation in deionized water. Same procedure of dialyzation was performed for fulvic acids; however, the fulvic acids were purified with repeated passages through Amberlite XAD-8 no.A-6525, 1 − 0.5 mm resin (Sigma Chemical Co, St. Louis, MO, USA) and washed with 0.3 M HF before processing for dialyzation. The remnant salts, if any, were removed by passing the fulvic acids through Bio-Rad Ag MP-50, 0.05- to 0.2- mm resin (Bio-Rad Laboratories, Richmond, CA, USA) in the H form. The fractions were stored by freeze drying.

2.1.2. Characterization of humic substances

Carbon, hydrogen, and nitrogen contents (oven-dry basis) were determined for humic substances by dry combustion method as described by Mann and Saunder (1960). Oxygen was calculated by subtracting C% + H% + N% from 100. Total acidity, carboxyl groups, and phenolic − OH groups were measured on all humic substance samples using the method described by Schnitzer et al. (1982) and the obtained data are recorded in Table 1.

2.2. Milk sampling and milk composition

Twelve Holstein-Friesian dairy cows (538 ± 18 kg) with mean parity of 2.5 were blocked according to milk yield, DIM, and BW and were randomly assigned to three dietary treatments (four cows per group). The three groups were offered a basal diet without (control) or with clay derived-humic substance (CD−HS) at the rate of 5 g (CD-HS5) and 10 g (CD-HS10) per kg diet, in a 3 × 3 Latin Square design (4 replicates) with 21-day periods, in which 14 days were for adaptation and seven days for data collection. The

Table 1
Elemental analysis, total acidity and some functional groups of clay derived-humic substances.

Total nutrients (g/kg)							Atomic ratios				Ash (g/kg)
C	H	N	P	O	Ca	Fe (mg/kg)	C/H	C/O	C/N	C/P	
461.7	62.1	23.2	8.2	426.9	11.8	307.4	7.43	1.08	19.9	56.4	894
Total acidity and some functional groups (meq 100/g HA)											
Total acidity				COOH		Total - OH		Phenolic – OH		Alcoholic -OH	
573.7				257.9		447.1		314.7		133.2	

amount of supplementation used was calculated considering the pasture offered and the supply of ME for approximate milk production of 30 kg/day (NRC, 2001). Cows were fed a diet containing (per kg DM) 400 g of concentrate feed mixture, 400 g of corn silage, and 200 g of Berseem hay to meet their nutrient requirements according to NRC (2001) recommendations. The nutrient contents of feed ingredients are shown in Table 2. The weighing of cows were done by using a digital weighing platform (PS-2000, Fairmont, MN, USA) on 1st and 60th day of the experiment.

Milking was done mechanically and the milk yield was measured twice a day (07 h and 18 h), from the 15th to the 21st day of each experimental period. On the last three days of the period, four milk samples were collected during successive milkings to determine the contents of fat, protein, lactose, urea nitrogen (N-urea), total solids and non-fat solids (NFS) in the milk according to the protocols given in AOAC (2005). To preserve the 50 mL samples, a 10 mg pill of bronopol (2-bromo 2-nitropropane-1,3-diol; Egypt) was added into each container.

After that, samples were cooled at 4 °C. Milk samples for analysis of the fatty acid profile were the result of a single sample composed of four milkings of the 20th and 21st days of each experimental period, stored at -20 °C. Milk was corrected for the content of 4% fat corrected milk (FCM), according to the formula cited by the NRC (2001): FCM = 0.4 x milk production + 15 x (%fat 100^{-1}) x milk yield. The fatty acid profile of lipids in milk samples was estimated by following the procedure as used by Thanh and Suksombat (2015). Briefly, the extraction of the mixture of methanol (2:1 v/v) and dichloromethane was done by using the method proposed by Romeu-Nadal et al. (2004). The extracted contents were evaporated under a nitrogen stream followed by methylation. Approximately 30 mg of the extracted content were added with 2 mL of boron trifluoride and 1 mL of hexane standard. After thorough mixing for 30 s, the samples were methylated with 1.5 mL of NaOH in 0.5 M methanol at 90 °C for 40 min. Subsequently, the mixed samples were allowed to cool down to room temperature and added with 10 mL of deionized water. The supernatant was transferred to a centrifuge, to which 5 mL of hexane is added. Later, the tubes were centrifuged at 2000 x g at 10 °C for 20 min. 1 mL of solution is collected from top layer, which was transferred into a vial for analyzing fatty acids by GC-2014 gas chromatograph (Shimadzu Technologies, Japan) equipped with a flame-ionization detector. The injector and detector temperatures were 250 °C and 300 °C, respectively. The peaks were identified using fatty acid and fatty acid methyl ester standards (FAME, Elysia, USA; Matreya, PA, USA; and Supelco 37, Supelco Inc.). The area under fatty acid peak was used to calculate the percentage of each fatty acid. The analyzed fatty acids were reported as grams per hundred grams of FAME as per Han et al. (2014). The thrombogenicity index (TI) and atherogenicity index (AI) were calculated as per Ulbricht and Southgate (1991);

$$TI = (C14{:}0 + C16{:}0 + C18{:}0)/[(MUFA + n\text{-}6PUFA)/2 + 3(n\text{-}3\ PUFA) + (n\text{-}3\ PUFA/n\text{-}6\ PUFA)]$$

$$AI = [C12{:}0 + 4(C14{:}0) + C16{:}0]/[\text{monounsaturated fatty acids (MUFA)} + PUFA]$$

Table 2
Chemical composition of basal diet ingredients fed to Holstein dairy cows.

Nutrients (g/kg)	Concentrate[1]	Corn silage[2]	Berseem hay
Dry matter	895.1	295.7	885.8
Crude protein	156.8	84.5	123.7
Ether extract;	31.1	25.5	22.6
Total ash	54.7	63.3	56.2
Neutral detergent fiber	326.4	617.7	588.2
Acid detergent fiber	213.8	364.3	355.7
Acid detergent lignin	47.8	53.1	61.6
Hemicellulose	112.6	253.4	232.5
Cellulose	166.0	311.2	294.1

[1] Ingredient composition of concentrate mixture (g/kg): yellow corn, 400; wheat bran, 245; sugar beet pulp, 50; soybean meal, 160; cotton seed meal, 85; molasses, 31; limestone, 17; salt, 8; and vitamin premix, 4. The vitamin premix provided per kg of diet: vitamin A, 1000 IU; vitamin D3, 250 IU; vitamin E, 7.5 IU; Ca, 1 g; P, 0.3 g; Cu, 3.25 mg; Fe, 0.2 mg; Zn, 15; mn, 3.25 mg; I, 0.2 mg; Se, 0.1 mg.
[2] Characteristics of corn silage: pH, 3.98; lactic acid (g/kg), 20.63; acetic acid (g/kg), 14.26; butyric acid (g/kg), 2.63; NH_3-N/total N, 4.68 (%).

2.3. Feed intake and nutrient digestibility

The recordings of concentrate, silage, and berseem hay were done on daily basis by subtracting the offered diets and refusals on dry matter basis. Digestibility trial was carried out at the last day of each experimental period *i.e.*, on 21^{st} day of the 21-day period by using faecal grab method. The faecal grab samples were collected directly from anus twice daily at 07:00 and 15:00 h. The collected sampls were dried at 60 °C for 48 h in hot air owen and pooled according to the treatment groups. Acid insoluble ash was used as an internal indigestibility marker for calculating the nutrient digestibility coefficients (Ferret et al., 1999).

Dried feed and faecal samples were ground to pass a 1-mm screen using a Wiley mill and analysed for dry matter (DM), crude protein (CP), ash and ether extract (EE) according to AOAC (2005; ID 920.152) official methods. Neutral detergent fibre (NDFom), acid detergent fibre (ADFom), and lignin were determined by the procedure of Van Soest et al. (1991). Concentrations (g/kg DM) of non-structural carbohydrates (NSC), cellulose, hemicellulose (HC) and organic matter (OM) were calculated by using the following equations;

NSC = 1000-(NDFom + CP + EE + ash); cellulose = NDFom − lignin; HC = NDFom − ADFom; and OM = 1000-total ash.

2.4. Sampling and analysis of rumen fluid

The sampling of ruminal contents were done at 3 h post-feeding during the last day of each experimental period to determine ammonia-N (NH_3-N) and short-chain fatty acids (SCFA). Approximately 100 mL of rumen fluid was collected from five cows of each treatment by using a stomach tube and pump, and strained through four layers of cheese cloth. From the 100 mL sample, a subsample of 5 mL was preserved in equal quantity of 0.2 M HCl for estimation of NH_3-N concentration (AOAC, 2005). Approximately 0.8 mL of strained ruminal fluid was mixed with 0.2 mL of a solution containing 250 g of metaphosphoric acid/L for SCFA analysis (Annison, 1954). Samples were stored at −20 °C until analyses. Gas-liquid chromatography (model 5890, HP, Little Falls, DE, USA) was used to measure the concentration and molar proportions of individual short chain fatty acids. A capillary column of 30 m ×0.25 mm internal diameter, and 1-m film thickness (Supelco Nukol; Sigma–Aldrich, ON, Canada) along with flame ionization detector was used for separation process. Initially, the column temperature was fixed at 100 °C. Later, the column adjustments were done in such a way that the temperature increases every minute with 20 °C interval until the temperature reaches to 140 °C. The adjustment process is continued with 8 °C interval up to 200 °C and the temperature was held for 5 min. The detector temperature was at 250 °C while the injector temperature was maintaied at 200 °C. Helium was used as the carrier gas.

2.5. Sampling and analysis of blood serum

Approximately 10 mL of blood samples were collected from the jugular vein of cows (n = 4) at 4 h post-feeding. Serum separation was done by centrifuging the blood samples at 4000× g for 20 min. The harvested serum was collected into Eppendorf tubes, which were frozen at -20 °C for further analysis. The samples were analyzed for total protein, albumin, glucose, cholesterol, serum urea nitrogen, aspartate amino transferase (AST), Alanine amino transferase (ALT) by using specific kits (Stanbio Laboratory, Boerne, Texas, USA). Globulin concentration was obtained by subtracting albumin values from their corresponding total protein values.

2.6. Statistics

The data of nutrient intakes, nutrient digestibility coefficients, and milk yield (kg/head/day) and intakes were subjected to ANCOVA while using initial collections as covariates. The confidence interval was adjusted by marking LSD. The rumen fermentation patterns, biochemical patterns, milk components, and milk fatty acid profile were fitted in multivariate procedure of general linear model (GLM). The diets were included as fixed factors and random effects were the square, period nested within square, and cow nested within square. Animal was used as an experimental unit in determining all the parameters. The values with P value below 0.05 were considered as significant and those between 0.05 and 0.1 were tended to be significant. Linear and quadratic effects for all the studied parameters were evaluated by using planned orthogonal polynomial coefficients. Entire statistical analysis was performed by using SPSS version 23.0 (SPSS, 2015).

3. Results

3.1. Characterization of humic substances

The elemental analysis of isolated humic substances is shown in Table 2. Among the total elements, carbon is in higher amount followed by oxygen, iron, hydrogen, nitrogen, calcium, and phosphorus in descending order. Regarding the atomic rations, C/P ratio is in higher amount followed by C/N, C/H, and C/O ratios, respectively.

3.2. Nutrient intake and digestibility coefficients

The nutrient intake and digestibility coefficients of dairy cows fed varying levels of humic substances are presented in Table 3. The CD-HS supplementation did not affect ($P > 0.05$) the intake of corn silage, hay, or total dry matter; however, day of experiment showed a significant effect for intakes of corn silage and total DM. The dairy cows fed with CD-HS containing diets showed a

Table 3
Nutrient intake and digestibility coefficients of dairy cows fed diets supplemented with varying levels of clay derived-humic substances.

	Treatments			SEM	P value			
	CON	CD-HS5	CD-HS10		T-Linear	T-Quadratic	Day	D × T
Intakes (kg/day)								
Concentrate intake	7.00	7.00	7.00	–	–	–	–	–
Corn silage	22.40	22.39	22.38	0.090	0.913	0.981	0.015	0.139
Hay	2.10	2.08	2.10	0.022	0.949	0.547	0.946	0.058
Total DM Intake	16.03	16.01	16.03	0.040	0.956	0.747	0.031	0.069
Nutrient digestibility								
DM	0.640	0.642	0.649	0.006	0.443	0.683	–	–
CP	0.625	0.634	0.639	0.004	0.006	0.219	–	–
NDFom	0.439	0.450	0.458	0.005	0.074	0.207	–	–
ADFom	0.342	0.351	0.357	0.007	0.087	0.204	–	–

'- '– Not Applicable.
CON, Control; CD-HS5, Clay derived-humic substances at 5 g/kg diet; CD-HS10, Clay derived-humic substances at 10 g/kg diet; DM, Dry matter; CP, Crude protein; NDFom, Neutral detergent fiber; ADFom, Acid detergent fiber; T, Treatment; D × T, Diet × Treatment interaction.

quadratic increase ($P < 0.05$) in the digestibility coefficient of CP. The digestibility coefficients of ADFom and NDFom tended to increase ($P = 0.087$ for ADFom and 0.074 for NDFom, linear dose-effect) in calves fed CD-HS5 and CD-HS10 diets. The diets did not affect ($P > 0.05$) the digestibility coefficients of DM. No significant interactions were observed for nutrient intakes.

3.3. Rumen fermentation patterns

The effect of supplementation of CD-HS on rumen fermentation profile is shown in Table 4. The CD-HS supplementation caused a quadratic decrease ($P < 0.05$) in the concentration of NH_3-N. The cows fed CD-HS5 and CD-HS10 diets showed a linear increase ($P < 0.05$) in TVFA concentration. The individual VFA fractional analysis revealed increased acetate and propionate concentrations without affecting the butyrate and acetate to propionate ratio. Further, the protozoal concentration showed a quadratic decrease ($P < 0.05$) with supplementation of humic substances to the diets.

3.4. Serum biochemical parameters

The results of various serum biochemical parameters are presented in Table 4. The serum glucose levels showed a linear increase ($P < 0.05$) on feeding CD-HS diets. A quadratic does effect ($P < 0.05$) was noticed for serum cholesterol levels. Although the diets did not alter liver function indicators (AST and ALT), CD-HS supplementation showed a quadratic increase ($P = 0.003$) in BUN levels.

Table 4
Ruminal fermentation kinetics and biochemical parameters of dairy cows fed diets supplemented with varying levels of levels of clay derived-humic substances.

	Treatments			SEM	*P* value		
	CON	CD-HS5	CD-HS10		T-Linear	T-Quadratic	C *vs* CD-HS
Rumen fermentation patterns							
Rumen pH	6.15	6.06	6.06	0.026	0.167	0.345	0.291
NH_3-N (mg/dl)	15.45	12.24	12.37	0.490	0.008	0.011	0.003
Total volatile fatty acids (mmole/L)	10.37	12.47	12.54	0.382	0.021	0.078	0.027
Acetic acid (mol/100 mol)	52.70	54.67	54.70	0.297	0.011	0.032	0.008
Propionic acid (mol/100 mol)	22.37	23.30	23.58	0.117	0.048	0.359	0.135
Butyric acid (mol/100 mol)	11.38	11.51	11.54	0.052	0.797	0.911	0.966
Acetate: Propionate	2.36	2.35	2.32	0.080	0.535	0.957	0.825
Protozoa ($\times 10^5$/mL)	4.31	3.52	3.57	0.130	0.013	0.017	0.006
Biochemical parameters							
Glucose (mg/dl)	48.13	51.95	52.38	0.287	0.016	0.101	0.026
Cholesterol (mg/dl)	113.47	101.58	97.06	1.358	< 0.001	0.014	< 0.001
Total protein (g/dl)	6.26	6.36	6.35	0.351	0.857	0.872	0.975
Albumin (g/dl)	3.24	3.47	3.44	0.245	0.563	0.605	0.769
Globulin (g/dl)	3.02	2.89	2.91	0.178	0.655	0.693	0.857
Blood urea N (mg/dl)	18.72	16.23	15.35	0.590	0.003	0.128	0.008
AST (u/L)	61.36	61.55	61.45	0.144	0.645	0.394	0.645
ALT (u/L)	20.57	21.08	20.71	0.336	0.781	0.302	0.565

CON, Control; CD-HS5, clay derived-humic substances at 5 g/kg diet; CD-HS10, clay derived-humic substances at 10 g/kg diet; NH_3-N, Ammonia nitrogen; AST, Aspartate amino transferase; ALT, Alanine amino transferase.

Table 5
Milk yield and composition of dairy cows fed diets supplemented with varying levels of clay derived-humic substances.

	Treatments			SEM	*P* value		
	CON	CD-HS5	CD-HS10		T-Linear	T-Quadratic	C *vs* CD-HS
Milk yield (kg/cow/day)	17.21	18.22	18.26	0.67	0.035	0.053	0.048
Total solids (g/kg)	115.0	110.3	111.2	3.84	0.495	0.516	0.679
Fat (g/kg)	35.2	32.7	33.3	0.79	0.167	0.129	0.155
Proteins (g/kg)	33.0	30.5	30.8	1.19	0.252	0.312	0.363
SNF (g/kg)	79.8	77.7	78.0	3.09	0.672	0.714	0.873
Ash (g/kg)	7.4	7.9	8.1	0.43	0.273	0.608	0.528
Lactose (g/kg)	39.5	39.3	39.1	1.56	0.869	0.972	0.987
Fat yield (kg/day)	0.61	0.60	0.60	0.01	0.565	0.731	0.823
Protein yield (kg/day)	0.57	0.56	0.57	0.02	0.905	0.912	0.902
FCM	16.00	16.32	16.35	0.22	0.278	0.510	0.498
Energy (kcal/kg)	661.30	627.70	634.63	22.23	0.410	0.417	0.557

CON, Control; CD-HS5, clay derived-humic substances at 5 g/kg diet; CD-HS10, clay derived-humic substances at 10 g/kg diet; SNF, Solids not fat; FCM, Fat corrected milk.

Total protein, albumin, and globulin levels were not affected ($P > 0.05$) by the treatments.

3.5. Milk yield, components, and fatty acid yields

Feeding CD-HS diets caused a linear increase ($P < 0.05$) in milk yield without affecting ($P > 0.05$) the total solids, fat, protein, SNF, ash, lactose, fat yield, protein yield, FCM yield, and energy.

The milk yield and components of the cows supplemented with CD-HS diets are presented in Table 5. The total milk yield was altered ($P < 0.001$) by the day of collection with significant ($P < 0.01$) treatment and day interactions (Fig. 1). Although the diets did not affect all the milk parameters, except milk yield, they strongly modified the fatty acid proportions.

The individual fatty acid proportions of the milk are presented in Table 6. The CD-HS diets did not affect the short- and medium-chain fatty acids (< 16 carbons) in the milk fat compared to control. The CD-HS5 and CD-HS10 diets reduced ($P < 0.05$) proportion of stearic acid (C18:0) and increased ($P < 0.05$) the proportion of isomeric forms of linoleic acid (t9t12-C18:2 and C9,C12-C18:2). Interestingly, among the > 18 C fatty acid groups, the CD-HS supplementation led to a reduced ($P < 0.05$) proportion of tetracosanoic acid (C24:0).

The fatty acid groups and indices of the milk are presented in Table 7. A linear increased ($P = 0.077$) trend was observed on the unsaturated fatty acids content of the milk collected from CD-HS5 and CD-HS10 diets. Although the CD-HS diets did not affect the content of monounsaturated, omega-3, and omega-6 fatty acid groups, they caused a linear increase ($P < 0.05$) in the content of polyunsaturated fatty acids. Further, the supplementation of CD-HS at 5 or 10 g/kg diet linearly decreased ($P < 0.05$) the

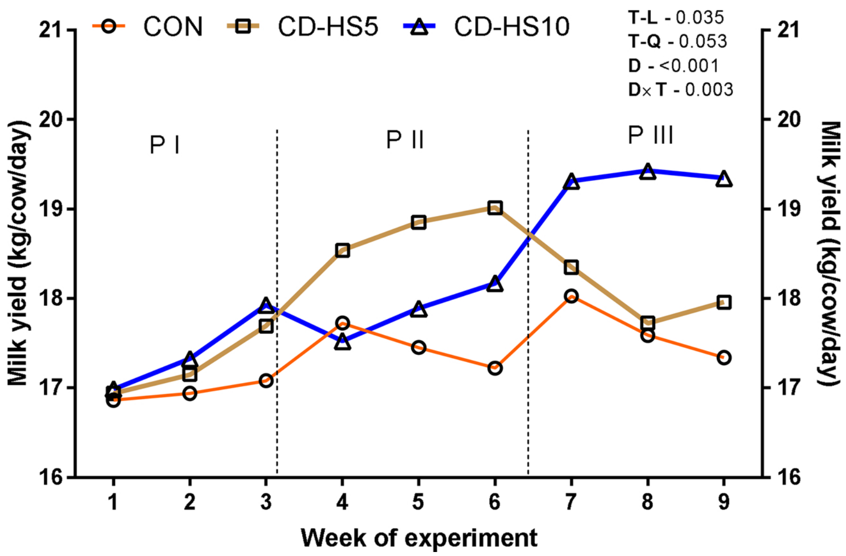

Fig. 1. Milk yield (kg/cow/day) with reference to the humic substances supplementation.
CON, Control; CD-HS5, Humic substances at 5 g/kg diet; CD-HS10, Humic substances at 10 g/kg diet; P, Period; T-L, Treatment-Linear; T-Q, Treatment-Quadratic; D, Diet; D × T, Diet × Treatment interactions.

Table 6
Individual fatty acids (g/100 g total fattyacids) of milk of dairy cows fed diets supplemented with varying levels of levels of clay derived-humic substances.

	Treatment			SEM	*P* value		
	CON	CD-HS5	CD-HS10		T-Linear	T-Quadratic	C *vs* CD-HS
C4:0	2.40	2.15	2.73	0.108	0.145	0.157	0.150
C6:0	1.86	1.56	1.78	0.135	0.673	0.125	0.282
C8:0	1.14	1.18	1.36	0.124	0.213	0.760	0.425
C10:0	2.57	2.58	2.41	0.133	0.393	0.710	0.623
C11:0	0.34	0.37	0.32	0.070	0.836	0.651	0.865
C12:0	7.70	7.64	8.18	0.654	0.604	0.773	0.822
C13:0	0.35	0.32	0.31	0.078	0.677	0.923	0.920
C14:0	12.45	13.03	12.84	0.623	0.663	0.597	0.812
C14:1	1.13	1.10	1.13	0.104	0.958	0.831	0.973
C15:0	1.08	1.16	1.09	0.070	0.926	0.438	0.728
C16:0	36.85	37.08	35.50	1.442	0.509	0.691	0.716
C16:1	2.31	2.90	2.55	0.175	0.426	0.049	0.114
C17:1	0.22	0.24	0.18	0.040	0.476	0.517	0.584
C18:0	8.04	7.18	7.12	0.184	0.015	0.073	0.020
C18:1n-9	22.38	23.65	23.90	0.714	0.159	0.460	0.335
t9t12-C18:2	0.15	0.37	0.51	0.062	0.011	0.433	0.044
C9,C12-C18:2	0.90	1.40	1.40	0.125	0.035	0.099	0.046
C9,t11-CLA	0.59	0.76	0.71	0.090	0.397	0.304	0.454
T10,C12-CLA	0.03	0.03	0.05	0.014	0.463	0.761	0.709
C9,C11-CLA	0.02	0.03	0.03	0.012	0.490	0.636	0.738
T9-T11-CLA	0.08	0.11	0.10	0.013	0.219	0.256	0.295
C18:3n-3	0.15	0.16	0.17	0.022	0.355	0.943	0.666
C20:0	0.15	0.18	0.17	0.024	0.530	0.406	0.614
C20:1	0.09	0.09	0.10	0.025	0.643	0.850	0.873
C20:2	0.07	0.09	0.09	0.020	0.396	0.706	0.685
C20:3n-6	0.06	0.10	0.10	0.020	0.132	0.184	0.173
C20:4n-6	0.09	0.11	0.12	0.024	0.235	0.541	0.462
C24:0	0.07	0.06	0.03	0.012	0.037	0.872	0.112
C22:5n-3	0.01	0.01	0.01	0.000	0.854	0.592	0.856
C22:6n-3	0.01	0.01	0.01	0.000	0.714	0.881	0.919

CON, Control; CD-HS5, clay derived-humic substances at 5 g/kg diet; CD-HS10, clay derived-humic substances at 10 g/kg diet.

Table 7
Fatty acid groups (g/100 g total fattyacids) and indices of milk of dairy cows fed diets supplemented with varying levels of levels of clay-derived humic substances.

	Treatment			SEM	*P* value		
	CON	CD-HS5	CD-HS10		T-Linear	T-Quadratic	C *vs* CD-HS
Fatty acid groups							
De novo	31.02	31.09	32.14	0.942	0.422	0.777	0.678
Mixed	39.15	39.98	38.05	1.455	0.598	0.511	0.659
Preformed	33.09	34.57	34.79	0.994	0.279	0.552	0.516
C18 UFA	24.29	26.51	26.87	0.845	0.066	0.288	0.143
SFA	75.01	74.47	73.82	1.884	0.658	0.970	0.911
UFA	28.26	31.15	31.16	0.945	0.077	0.186	0.122
MUFA	26.11	27.98	27.87	0.774	0.156	0.259	0.241
PUFA	2.14	3.18	3.29	0.235	0.019	0.115	0.033
n-6	0.86	1.14	1.10	0.112	0.198	0.225	0.255
n-3	0.16	0.18	0.19	0.022	0.361	0.368	0.674
EPA + DHA	0.02	0.02	0.02	0.012	0.789	0.856	0.956
Fatty acid indices							
UFA/SFA	0.38	0.42	0.42	0.021	0.121	0.319	0.231
MUFA/SFA	0.35	0.38	0.38	0.021	0.216	0.418	0.389
PUFA/SFA	0.03	0.04	0.04	0.001	0.020	0.133	0.038
n-6/n-3	5.42	6.79	5.83	0.612	0.669	0.148	0.325

CON, Control; CD-HS5, clay derived-humic substances at 5 g/kg diet; 10, clay derived-humic substances at 10 g/kg diet.

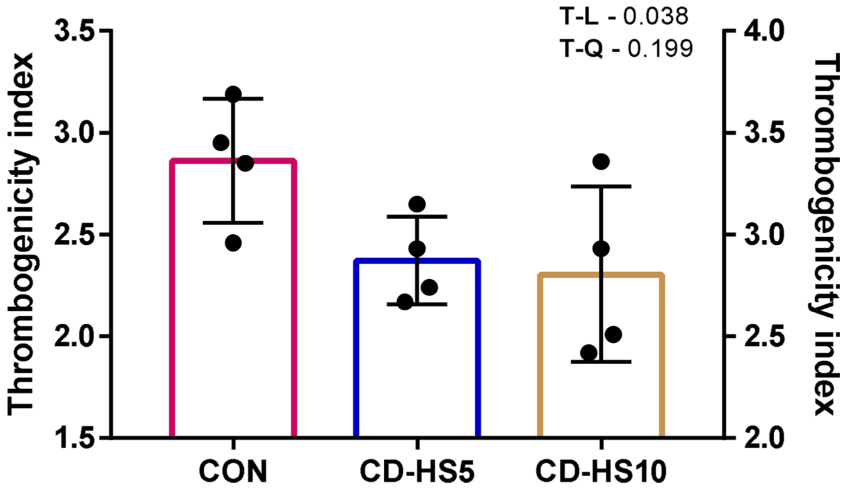

Fig. 2. Thrombogenecity index with reference to the humic substances supplementation.
CON, Control; CD-HS5, Humic substances at 5 g/kg diet; CD-HS10, Humic substances at 10 g/kg diet; T-L, Treatment-Linear; T-Q, Treatment-Quadratic

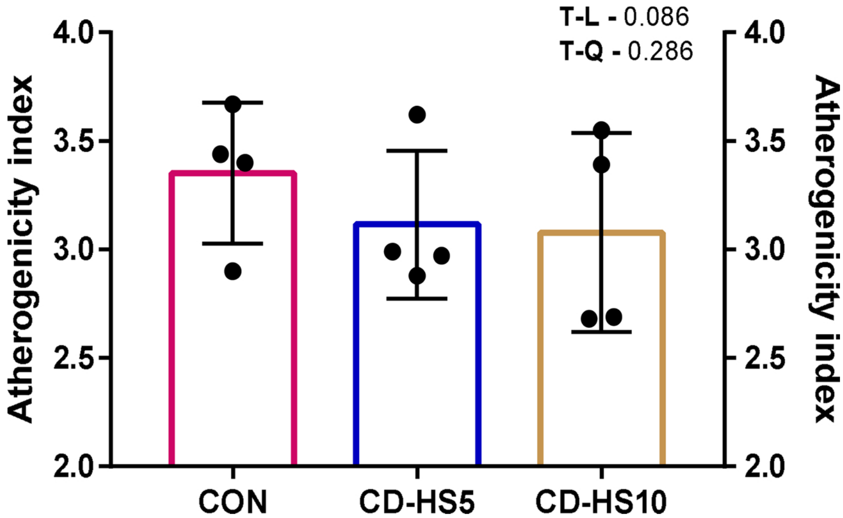

Fig. 3. Atherogenecity index with reference to the humic substances supplementation.
CON, Control; CD-HS5, Humic substances at 5 g/kg diet; CD-HS10, Humic substances at 10 g/kg diet; T-L, Treatment-Linear; T-Q, Treatment-Quadratic

thrombogenicity index (Fig. 2). Further, a linear trend ($P = 0.086$) was detected for the calculated atherogenicity index of milk (Fig. 3).

4. Discussion

4.1. Characterization of humic substances

The elemental content and functional group analysis revealed the presence of -COOH, phenolic –OH and alcoholic -OH groups. The functional structures of humic substances include phenolic hydroxyl and quinone groups apart from nitrogen and sulfur molecules, which imparts redox function to the substances (Aeschbacher et al., 2010). The elemental analysis of humic acid is similar to the values given by Sartakov et al. (2017), who studied the elemental composition of humic acid of different lakes of west Siberia. However, the aromatic core, aliphatic components, and relative contents of chemical constituents of humic substances vary with type of soils, ages, environmental conditions (Tikhova et al., 2001).

4.2. Nutrient digestibility

The supplementation of CD-HS at either 5 or 10 g/kg diet did not affect ($P > 0.05$) the nutrient intakes; however, these levels managed to increase ($P < 0.001$) the nutrient digestibility coefficients of CP. On contrary, El-Zaiat et al. (2018) reported an increased nutrient intake and unaltered CP digestibility coefficients in dairy goats supplemented with humic substances at 2 g per day. The variations in the results might be attributed to the differences in diet, species, sampling time, adaptation period, and levels of humic substances supplemented. The improved CP digestibility is because of the N binding ability of humic acid, which reduces its excretory rate. Besides, the humic substances can accelerate the oxidative processes and stimulate cell membrane metabolic activities, thereby improving the efficiency of CP absorption (El-Zaiat et al., 2018). The positive linear tendency on NDFom and ADFom digestibility coefficients could be due to the enhanced microbial activity in the rumen.

4.3. Ruminal fermentation profile

The humic substances supplementation led to a quadratic decrease ($P < 0.001$) in NH_3-N contents without affecting ruminal pH. Because of the ion-complex forming property, the humic substance bind to the ruminal-nitrogen, thus reducing the NH_3-N content (Zanin et al., 2019). Higher NH_3-N content may increase the serum NH_3-N contents subsequently increasing the N excretion through urine, thereby causing environmental pollution (Reddy et al., 2019a, 2019b). Hence, reduction of NH_3-N accumulation in the present study is a favorable outcome. The CD-HS diets may enhance the N utilization by inhibiting the urease activity in the rumen, consequently decreasing the solubility of N (Ji et al., 2006). Lower ruminal NH_3-N contents are also related to the reduced count of protozoa and microbial population shift (El-Zaiat et al., 2018). The present study noticed a quadratic increase ($P < 0.05$) in TVFA content. On the contrary, few authors reported unaltered TVFA proportion on feeding humic substance containing diets (Terry et al., 2018a).

Decreased protozoa led to lowered NH_3-N concentration. Likewise, El-zaiat et al. (2018) revealed a decreased ruminal NH_3-N contents on supplementing the humic substance at a dose of 2 g/day in goats. Similar results were also reported by few *in vitro* experiments conducted earlier (Sheng et al., 2018; Varadyova et al., 2009). The increased TVFA contents is related to the enhanced digestibility of fiber fractions. Although the acetate: propionate proportion do not vary among the groups, the humic substance supplementation increased the contents of acetic and propionic acid. The higher acetic acid and propionic acid contents in CD-HS5 and CD-HS10 could be directly related to the positive tendency in NDFom ($P = 0.074$) and ADFom ($P = 0.087$) digestibility coefficients (Brandao and Faciola, 2019). These results on individual fatty acid proportions were partially supported by the results of Newbold et al. (2015), who reported an increased acetate proportion without affecting the propionate content on defaunation. The removal of protozoal predation and decreased competition for nutrients might have increased the ruminal fungal zoospores, thereby increasing fiber degradation and acetate production (Li et al., 2018). However, several controversies exist in the ruminal protozoan-fungal relationship and need more studies to understand their interactions (Newbold et al., 2015).

Bell et al. (1997) indicated that humate substances, when added in high doses, may inhibit the protozoan populations. More recently, Terry et al. (2018a) confirmed the defaunating effect of humic substances by using rumen simulating technique (RUSITEC). As yet, no safe and practical technique to reduce protozoal count in the rumen has been developed (Newbold et al., 2015). By analyzing the beneficial response of humic substances on ruminal fermentation profile, they could be encouraged as effective defaunating agents.

4.4. Biochemical parameters

The dairy cows fed CD-HS5 and CD-HS10 diets showed a quadratic increase ($P < 0.05$) in blood glucose levels, unveiling the role of humic substance in preventing the negative energy balance and metabolic disorders. In ruminants, the higher serum glucose levels are directly associated with the gluconeogenesis in the rumen. The gluconeogenesis, in turn, is related to the rumen propionate concentration (Tirosh et al., 2019). Hence, in the present study, by analyzing the increased propionate concentration, a rise in serum glucose concentration can be easily predicted. Supplementing with CD-HS showed a quadratic decrease ($P < 0.001$) in serum cholesterol levels in dairy cows. Ho et al. (2003) concluded that CD-HS is known to release the iron from ferritin, consequently accelerating the lipid peroxidation and decreasing the blood cholesterol levels. With the increasing awareness on the blood cholesterol levels in human beings, our results stress the necessity of further studies in promoting the CD-HS based drugs for prevention of heart-related disorders.

The inclusion of humic substances caused a quadratic decrease ($P < 0.01$) in ruminal NH_3-N contents. In corroboration, Rath et al. (2006) observed a trend for blood urea N reduction in the broilers supplemented with CD-HS. The reduced BUN content in CD-HS diets is directly associated with the decreased ruminal NH_3-N content (Reddy et al., 2019a, 2019b). The CD-HS diets did not effect total protein, albumin, globulin, AST, and ALT concentrations.

4.5. Milk yield and composition

Despite the fact that humic acid can provide beneficial effects on the ruminant fermentation patterns, the role of humic substances on milk production and milk fatty acids remains unclear. No works were conducted in relation to the supplementation of humic substances and milk yield and components. Feeding CD-HS5 and CD-HS10 diets linearly enhanced ($P < 0.05$) the milk yield without affecting the percent of total solids, fat, SNF, and ash in the milk. Humic substances are known to be utilized as electron acceptors by anaerobic bacteria. Being an anaerobic vat, rumen might have provided a congenial environment for the bacteria to use humic substances, thus triggering their growth and multiplication (Coates et al., 2002). Therefore, the increased milk yield could be connected to the higher microbial protein yield. Besides, several studies demonstrated the effects of defaunation in increasing the rumen bacterial population because of the improved ecological niche for bacteria by elimination of the protozoal predation. Removal of the ciliate protozoa is known to enhance the microbial protein supply up to 30% (Newbold et al., 2015). Although the increased acetate was predicted to augment the milk fat percent, no effect was seen on any of the milk components, including fat yield. This apparent lack of correlation between milk fat percent and acetate proportions can be justified by the lowered serum cholesterol concentration.

The current study is the first to examine the effects of clay derived humic substance on the milk fatty acid composition. Hence, no literature is available to support the obtained data. The results on milk's fatty acid profile could be better attributed to the defaunating effect rather the effects of humic substance on any other processes. As explained earlier (Karnati et al., 2009), removal or reduction of protozoa from the rumen decreases the biohydrogenation of oleic, linoleic, and linolenic acids. The same is reflected in a lower proportion of stearic acid (C18:0) in the milk of cows fed CD-HS5 and CD-HS10 diets. As the protozoa do not play a direct role in biohydrogenation, the altered biohydrogenation rates among the groups could indicate the changes in the lipophilic bacterial population in CD-HS supplemented diets (Karnati et al., 2009). However, the defaunation procedure led to highly variable results, presumably because of fatty acid synthesizing ability of both protozoa and bacteria (Harfoot and Hazlewood, 1997). The thrombogenicity and atherogenicity indexes are used to predict the risk of ischaemic heart disease in humans. The significantly reduced (linear; $P < 0.05$) thrombogenicity index and the linear tendency ($P = 0.086$) in the reduction of atherogenicity index extend the use of clay derived-humic substances in low-heart risk foods' production. However, it is noteworthy that the atherogenicity index would be of interest in terms of human health only in cases of excessive fat consumption (Mensink et al., 2003; Knopp and Retzlaff, 2004).

5. Conclusions

Our research led us to conclude that humic substances derived from clay could be used at 5 g/kg diet to increase the milk yield and modify polyunsaturated fatty acids content of the milk without negatively affecting the nutrient digestibility coefficients, ruminal fermentation, and biochemical parameters. Besides, the beneficial effects of humic substances on digestibility coefficient of crude protein, rumen fermentation parameters, and serum biochemical profile project the use of clay-derived humic substances as zootechnical feed additives in ruminants. Further, the evaluation of thrombogenicity and atherogenicity indexes encourages the use of humic substances in the production of designer foods. Since the work is first study on the effect of humic substance on milk yield, milk components, and fatty acid profile, authors recommend additional research to standardize the dosages by using different levels.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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