OPEN ACCESS
Journal of Animal Health and Production

Research Article

Impact of Bentonite, Humic Acid or Zeolite Levels with Soybean, Sunflower or Cottonseed Meals as Dietary Protein Sources on *In vitro* Rumen Fermentation

Shorouk Aladdin Helmy[1], Hossam Mahrous Ebeid[2*], Mohamed Ahmed Hanafy[1], Adel Eid Mohamed Mahmoud[1], Reham Roshdy Ali El-tanany[1]

[1]Department of Animal Production, Faculty of Agriculture, Cairo University, Giza, 12613, Egypt; [2]Dairy Science Department, National Research Centre, 33 Bohouth St. Dokki, 12622, Giza, Egypt.

Abstract | Clays such as bentonite, humic acid, and zeolite are frequently suggested to have a special buffer role in the modulating capacity of rumen cation exchange in livestock diets to reduce ammonia emissions and modulate rumen fermentation. An *in vitro* experiment was conducted to determine the effect of adding three different clay minerals in four levels of each, as follows: humic acid (0.0, 0.5, 1.0, and 1.5%); zeolite (0.0, 1.25, 2.5, and 5%); or bentonite (0.0, 1.0, 2.0, and 3.0 %) with three different protein sources, (soybeans, sunflowers, or cottonseed meals) on a dry matter basis. All rations were incubated in a rumen culture medium collected from sheep in a 3 (sources of clay), 4 (levels of clay), and 3 (protein sources) factorial design. All rations were prepared to be iso-nitrogenous. The results illustrated that humic acid addition had made a significant difference on the amount of degradable dry matter (dDM, g/kg DM), total gas production, per (ml) and (g/kg DM) for the 3 protein sources (soybean, sunflower, and cottonseed meals). All protein sources rations revealed significant linear and quadratic decreases for total gas production (TGP), ml and TGP, g/kg DM with zeolite. Moreover, zeolite has increased (P = 0.013) in dDM at 5% zeolite, but has decreased NH_3-N concentration (P = 0.009) with the cottonseed ration. Bentonite rations had quadratically increased (P = 0.01) in NH_3-N concentrations, but the TGP (ml) and TGP (g/kg DM) were linearly decreased with bentonite addition but linearly increased in soybean and sunflower rations. No significant difference was observed for cottonseeds and bentonite addition. The interaction between bentonite and the type of protein source had no significant difference either. In conclusion, addition of clay to different protein sources had different responses (positive, negative, and no effect) on dDM, pH, reducing ammonia emission and TGP in rumen fermentation.

Keywords | Clay, Bentonite, Zeolite, Humic acid, Rumen fermentation.

Received | February 21, 2022; **Accepted** | March 25, 2022; **Published** | June 01, 2022
***Correspondence** | Hossam Mahrous Ebeid, Dairy Science Department, National Research Centre, 33 Bohouth St. Dokki, 12622, Giza, Egypt; **Email:** hm.abdel-aziz@nrc.sci.eg; hossamebaid55@gmail.com
Citation | Helmy SA, Ebeid HM, Hanafy MA, Mahmoud AEM, El-Tanany RR (2022). Impact of bentonite, humic acid or zeolite levels with soybean, sunflower or cottonseed meals as dietary protein sources on *in vitro* rumen fermentation. J. Anim. Health Prod. 10(2): 158-167.
DOI | http://dx.doi.org/10.17582/journal.jahp/2022/10.2.158.167
ISSN | 2308-2801

Copyright: 2022 by the authors. Licensee ResearchersLinks Ltd, England, UK.
This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

INTRODUCTION

In intensive livestock production, protein degradation is the most single important factor affecting protein supply to the small intestine through the hydrolysis of microorganisms that count for 65% of the protein in the diet to organic acids, ammonia and CO_2 in the rumen (McDonald et al., 2011). The ammonia produced with other peptides and free amino acids is used for microbial protein synthesis by microorganisms. However, the feeding on a high percentage of rumen degraded protein sources such as soybean, sunflower, and cottonseed meals or even non protein nitrogen (NPN) is causing an increasing problem in ammonia release in the rumen and causing ammonia toxicity in the blood that might lead to losing the animal (McDonald et al., 2011). In addition to aflatoxin and mycotoxins (poisons) problems produced by the fungi and moulds that grow on feed and affect animal health through lowering immunity and causing diseases that lead to diarrhea, liver, and kidney damage, weak bones, and reduced weight gain in animals (Jaynes et al., 2007).

In the mid of the 1960s, scientists of animal nutrition introduced clay as a natural source into livestock production diets as binding agents for feeding pellets and to promote the growth and health of the animals (Sulzberger et al., 2016). The actual use of clay minerals was directed towards the production of selective sorbents applicable to both human and veterinary medicine. However, previous reports showed that eating clay (including products such as, bentonite, zeolites, or humic acid) by animals in the wild, led to detoxification of the body, alleviation of gastrointestinal infections as against rumen disorders, reduced the absorption of aflatoxin from the gastrointestinal tract (Diaz et al., 2004; Kissell et al., 2013) and reduce ammonia release in the rumen (Montalvo et al., 2012; Sulzberger et al., 2016).

Bentonite known as Montmorillonite has an attraction to mono and divalent ions that bind proteins and nitrogenous compounds to reduce protein degradation and increase bypass protein from the rumen (Sulzberger et al., 2016; Trckova et al., 2004). On the other hand, humic acid exerts a protective action on the mucosa of the intestine as well as having antiphlogistic, adsorptive, antitoxic, and antimicrobial properties through improving the immune system. On the other hand, it plays a crucial role in reducing stress and enhancing liver function, as well as stimulating lymphocyte proliferation (Griban et al., 1991; EMEA, 1999; Kanana et al., 2019).

Most of the research on clay has been investigated by veterinarians and nutritionists. However the recommended application techniques in terms of doses, forms of addition, and types of animals along with other criteria still need more comprehensive research and experimentation. Therefore, clay's effects on rumen fermentation and dry matter digestibility have varied among studies and it is necessary to conduct more research to enhance the performance. Moreover, the recommended levels of additives were not elucidated as well as the type of ration content. So, in this study we aim to evaluate the potential effects of different clay minerals such as bentonite, zeolite, or humic acid as feed additives in different levels added to different sources of protein (soybean, sunflower, and cottonseed meals) on the mitigation of ammonia release and modulation of rumen fermentation *in vitro*.

MATERIALS AND METHODS

The *in vitro* experiment was conducted at the Dairy Sciences Department, National Research Centre, Egypt in cooperation with the Animal Production Department, Faculty of Agriculture, Cairo University, Giza, Egypt.

Clay minerals sources

Clays of bentonite and zeolites have been gotten from the Feeds Unit, Faculty of Agriculture, Cairo University, Giza, Egypt. The humic acid product was purchased from an Egyptian-Candian company for fertilizers commerce in Egypt and derived from humic lignite from the USA, containing 74% combined humic acids with 20% low molecular weight fulvic acid.

Experimental design

In vitro trials

Three protein sources (soybean meal, sunflower meal and cottonseed meal) and three clay minerals as additives (bentonite, zeolites, and humic acid) were used in this study. Each clay mineral was tested in four levels with three different mixed rations, for total of nine rations compared to three control ration groups (without addition) in a total of 12 groups for each protein source as illustrated in Table 1. The effects of different clay mineral levels on degradable dry matter (dDM), rumen fermentation (pH and ammonia concentration), and total gas production (TGP) were studied using an *in vitro* batch culture system. The experimental ration mixture was used as a substrate in the ratio of 50:50 (R:C) and its chemical composition is shown in Table (2). Each treatment was tested in 6 replicates accompanied by blank vessels (no substrate). Around 400 mg of the substrate was added to the incubation vessels of 100 mL capacity. Each vessel was filled with 40 mL of the incubation medium, consisting of rumen inoculum and buffer a ratio of 1:4 (v/v). A buffer was prepared according to Szumacher-Strabel et al. (2004) as reported by Ebeid et al. (2020a and b). All vessels containing rations of substrate were incubated at 39°C for one day before fermentation started to be warm during the injection. Rumen fluid was collected from a slaughterhouse at 5:00 pm into preheated thermos vessels and transported to the laboratory, then squeezed through 4-layers of cheesecloth into a Schott Duran® bottle (L) with under flushing with CO_2 in a water bath at 39°C where it was used as a source of inoculum. Forty mL of this rumen buffer inoculum was added to each vessel after which the headspace of each bottle was flushed with CO_2, all vessels were immediately closed with rubber stoppers followed by sealing with an aluminum cap, and the vessels were incubated at 39°C.

Sampling and analyses

After 48 hours of incubation at 39°C, the TGP was measured by the displacement of a syringe glass (100 mL), which was connected to the serum flasks. After that, the bottles were transferred directly into the fridge to stop microbial activity and fermentation. Three vessels of six were transferred into crucibles (dried, and weighted previously), and oven dried at 70 °C until completely dry to measure the dDM. The other three vessels were filtered, and pH was measured immediately after the bottles were opened (HI 9024C, HANNA Instruments, Woonsocket, Rhode Island, USA), while ammonia samples (15 mL each) were collected and stored in a freezer (-20°C) for analysis. Nessler's modified method was used to figure out how much ammonia was in the water (Szczechowiak et al., 2016).

Statistical analysis

Statistical analysis of data was carried out using the GLM procedure of SAS (2015) with three different clay minerals (humic acid, zeolite, and bentonite) in four different clay levels to three different protein sources in diets in a 3×4×3 factorial design. Differences between means of treatments for parameters (dDM, NH_3-N, pH, TGP and TGP (g/kg DM) were separated by Duncan's test (Duncan, 1955) with a P value < 0.05 to < 0.10.

The statistical model was:

Xicps= μ + αc + βps + (αβ)cps + eicps

Where: Xicps: observation icps. μ: overall mean. αc: effect of clay. βp: effect of protein source. (αβ)cp: interaction of two factors. eicps: experimental error

RESULTS AND DISCUSSION

Ingredients and rations chemical composition

The ingredients and chemical compositions of the three rations (on DM basis) were prepared to be iso-nitrogenous (15.68, 15.65, and 15.64%) CP for soybean (R1), sunflower (R2), and cottonseed rations (R3), respectively as shown in Table (2). Also, the DM content was similar among rations, but OM, EE and CF were a little bit decreased in R3 compared to R1 and R2 which could be explained by the diffractions in chemical composition among the three protein sources. The percentage of wheat bran addition was 0.5 R3 lower than in R1 and R2.

Effect of humic acid addition on rumen fermentation kinetics

The effect of humic acid addition at three levels (0.5, 1, and 1.5%) on protein sources is shown in Table (3). Overall significant differences were observed in the amounts of dDM (g/kg DM), TGP (ml), and TGP (g/DM) for 3 protein sources (soybean, sunflower, and cottonseed meals) when compared with 3 levels of humic acid. Moreover, the ammonia concentrations and pH values did not show a significant difference between humic levels on soybean and sunflower rations, but a significant difference was observed in the cottonseed ration. Additionally, more than 1% of humic acid had an effect on dDM and rumen fermentation kinetics in rations containing soybean. Total GP (ml or g/DM) increased at 1% humic acid addition in rations containing sunflower and cottonseed meals, but dDM was significantly decreased. The ammonia nitrogen concentrations increased at 0.5% humic acid and decreased at 1%, while it had increased again in sunflower rations without any significant difference, but the inverse trend was significantly observed in cottonseed rations.

Previous research were reported that humic acids are the fraction of humic substances that are not soluble in water under more acidic conditions (pH < 2) but are soluble at higher pH values that could be induced by the high molecular weight acids and minerals (iron, manganese, copper, and zinc) (Weber, 2002). In a study conducted *in vitro* (Shi et al., 2001), the use of humic acid in livestock was applied directly to the beef cattle manures to reduce ammonia emissions and reducing cumulative ammonia emissions by 67.6%. Additionally, Ji et al. (2006) observed that ammonia emission from manure was reduced by 3 to 18% when animals fed on various humic dietary substances at rates of 0.5 and 1 % which is consistent with our findings. However, no differences were found for ruminant ammonia concentrations or ruminant pH with beef cattle (McMurphy et al., 2011).

Recently, El-Zaiat et al. (2018) found that adding drenched humic acid at a dose of 2 g/day per goat increased ruminal pH, while ammonia concentration was decreased, which is in agreement with our research. Moreover, (Degirmencioglu, 2012) reported that humic acid is not a single molecule, but a complex mixture of many different acids containing carboxyl and phenolate groups. The phenolate group has a negative effect on ammonia and protozoa counts in the rumen (Ebeid et al., 2020a and b). In spite of the actual mechanism of humic acid's effect in animals being unclear or not yet understood, but Vaughan and Ord (1991) reported that humic substances inhibited urease activity and urease inhibition was higher in pH values. Other theories suggest that humate substances and humic binding capability may have the potential to alter rumen fermentation through slow NH_3-N release, which could cause a gradual drop and recovery of pH over a longer period of time, and result in a better utilization of degradable intake protein by rumen microbes in the rumen (McMurphy et al., 2011). Therefore, the improvement of dDM in the current study may be related to humic acid, which is thought to have a beneficial impact on ruminal and intestinal microflora stabilization, ensuring enhanced feed efficiency.

Links Researchers

Table 1: *In vitro* experimental treatments.

Protein source	Clay minerals sources levels (% from DM)		
	Bentonite	**Zeolite**	**Humic acid**
Soybean meal	0.0, Control	0.0, Control	0.0, Control
	1.0	1.25	0.5
	2.0	2.5	1.0
	3.0	5.0	1.5
Sunflower meal	0.0, Control	0.0, Control	0.0, Control
	1.0	1.25	0.5
	2.0	2.5	1.0
	3.0	5.0	1.5
Cottonseed meal	0.0, Control	0.0, Control	0.0, Control
	1.0	1.25	0.5
	2.0	2.5	1.0
	3.0	5.0	1.5

Table 2: Ingredients and chemical composition of the total mixed ration in i*n vitro* experiment

Item	Experimental rations*		
	R1	**R2**	**R3**
Ingredients			
Clover hay	50	50	50
Corn	25	25	25
Sunflower	0	9.6	0
Soybean	10	0	0
Wheat bran	13	13	12.5
Cotton seed meal	0	0	10
limestone	1.5	1.5	1.5
Salt	0.5	0.5	0.5
Urea	0	0.4	0.5
Total	100	100	100
Chemical composition (DM basis, %)			
DM	89.08	88.88	89.00
OM	93.15	92.70	92.00
Ash	6.85	7.30	8.00
CP	15.68	15.65	15.64
EE	3.32	3.29	2.96
CF	22.41	23.61	21.16
NFE	51.74	50.15	52.24

*R1: Soybean ration; R2: Sunflower ration; R3: Cotton seed ration

Table 3: Effect of humic acid addition to the experimental rations on rumen fermentation kinetics

Protein source	Levels of humic	[1]dDM, %	NH_3-N, mmol	pH	[2]TGP, ml	TGP, g/kg DM
Soybean	0.0, control	32.48^{ab}	9.93	6.41	55.00^{a}	151.29^{a}
	0.5	37.80^{a}	8.64	6.5	55.40^{a}	153.64^{a}
	1.0	31.65^{b}	7.32	6.55	58.60^{a}	162.34^{a}
	1.5	28.91^{b}	7.54	6.33	43.00^{b}	117.87^{b}
SEM		1.35	0.5	0.09	1.65	4.69

***P* value**	Treatment	0.040	0.179	0.399	0.001	0.001
	Linear	0.050	0.069	0.658	0.003	0.003
	Quadratic	0.041	0.414	0.132	0.001	0.001
	Control vs. all	0.856	0.064	0.652	0.326	0.274
Sunflower	0.0, control	19.67[bc]	7.23	6.61	48.33[ab]	132.18[ab]
	0.5	23.82[ab]	9.3	6.51	40.00[b]	108.43[b]
	1.0	18.38[c]	6.56	6.45	58.00[a]	159.96[a]
	1.5	26.10[a]	8.02	6.44	51.60[ab]	142.87[ab]
SEM		1.17	0.49	0.05	2.61	7.30
***P* value**	Treatment	0.015	0.171	0.676	0.099	0.077
	Linear	0.042	0.920	0.277	0.194	0.156
	Quadratic	0.220	0.718	0.654	0.836	0.793
	Control vs. all	0.070	0.476	0.271	0.790	0.756
Cottonseed	0.0, control	25.63[ab]	7.21[b]	6.52[a]	31.75[b]	88.05[b]
	0.5	28.79[a]	6.42[b]	6.35[a]	30.75[b]	84.49[b]
	1.0	16.83[b]	8.86[a]	6.47[a]	43.66[a]	121.53[a]
	1.5	33.07[a]	7.70[ab]	6.62[a]	26.00[b]	72.27[b]
SEM		2.49	0.37	0.05	1.77	4.98
***P* value**	Treatment	0.058	0.036	0.313	0.0002	0.0003
	Linear	0.46	0.11	0.34	0.584	0.653
	Quadratic	0.080	0.680	0.136	0.001	0.001
	Control vs. all	0.862	0.415	0.748	0.424	0.446
***P* value**						
Protein source:		<.0001	0.294	0.672	<.0001	<.0001
SEM		0.950	0.373	0.048	1.44	3.99
Humic acid:		0.001	0.734	0.866	<.0001	<.0001
SEM		1.040	0.242	0.056	1.66	4.61
Protein*Treat.		0.007	0.015	0.287	0.004	0.002
SEM		1.910	0.779	0.097	2.87	7.94

Arithmetic mean in the same column within each ration with different letters differ (P<0.05).
[1]**dDM:** Degradable of dry matter; [2]**TGP**: Total gas production

Table 4: Effect of zeolite addition to the experimental rations on rumen fermentation kinetics

Protein source	Zeolite level	[1]dDM,%	NH_3-N, mmol	pH	TPG[2], ml	TGP (g /kg DM)
Soybean	0.0, control	32.48	9.93	6.41	55.00[a]	151.29[a]
	1.25	34.11	8.5	6.58	56.40[a]	155.46[a]
	2.5	26.17	9.43	6.40	55.66[a]	152.24[a]
	5	25.91	8.78	6.36	45.33[b]	123.09[b]
SEM		1.638	0.306	0.048	1.26	12.79
***P* value**	Treatment	0.151	0.330	0.447	0.007	0.020
	Linear	0.055	0.377	0.495	0.006	0.024
	Quadratic	0.729	0.535	0.323	0.008	0.126
	Control vs. all	0.276	0.157	0.733	0.275	0.196
Sunflower	0.0, control	19.67	7.23[c]	6.61[a]	48.33[a]	132.18[a]
	1.25	20.34	13.25[a]	6.44[ab]	37.80[b]	105.03[b]
	2.5	20.24	11.92[b]	6.28[b]	41.80[ab]	113.90[ab]
	5	18.8	8.425[c]	6.58[a]	43.00[ab]	118.29[ab]

SEM		0.458	0.818	0.048	1.59	4.29
***P* value**	Treatment	0.750	<0.0001	0.027	0.148	0.185
	Linear	0.581	0.248	0.429	0.425	0.426
	Quadratic	0.349	<.0001	0.007	0.071	0.077
	Control vs. all	0.915	0.0001	0.048	0.066	0.075
Cottonseed	0.0, control	25.63^{a}	7.21^{c}	6.52	31.75	88.04
	1.25	18.52^{b}	10.13^{a}	6.45	30.5	84.56
	2.5	19.06^{b}	9.30^{ab}	6.38	33.0	90.6
	5	26.74^{a}	8.11^{bc}	6.50	31.33	84.25
SEM		1.472	0.432	0.054	0.839	5.577
***P* value**	Treatment	0.013	0.009	0.853	0.770	0.867
	Linear	0.487	0.339	0.834	0.886	0.733
	Quadratic	0.002	0.003	0.455	0.910	0.630
	Control vs. all	0.033	0.007	0.609	0.947	0.652
***P* value**						
Protein source:		<.0001	0.0008	0.822	<.0001	0.003
SEM		0.868	0.227	0.048	1.09	7.77
Zeolite:		0.067	<.0001	0.212	0.065	0.132
SEM		0.987	0.271	0.056	1.36	10.93
Protein*Treat.		0.006	<.0001	0.399	0.017	0.044
SEM		1.814	0.704	0.097	2.24	17.96

Arithmetic mean in the same column within each ration with different letters differ (P<0.05).
[1]**dDM:** Degradable of dry matter; [2]**TGP**: Total gas production

Table 5: Effect of bentonite addition to the experimental rations on rumen fermentation kinetics

Protein source	Bentonite level	[1]dDM, %	NH_3-N, mmol	pH	[2]TPG, ml	TGP (g /kg DM)
Soybean	0.0, control	32.48	9.93^{ab}	6.41	55.00^{ab}	151.30^{a}
	1	36.28	7.93^{b}	6.56	57.33^{a}	156.79^{a}
	2	35.76	8.36^{b}	6.33	46.25^{bc}	126.90^{b}
	3	30.70	11.34^{a}	6.32	44.80^{c}	124.24^{b}
SEM		1.590	0.511	0.051	1.914	4.954
***P* value**	Treatment	0.651	0.043	0.346	0.020	0.015
	Linear	0.739	0.174	0.318	0.009	0.007
	Quadratic	0.251	0.010	0.469	0.551	0.613
	Control vs. all	0.695	0.363	0.992	0.161	0.128
Sunflower	0.0, control	19.67^{b}	7.23^{b}	6.61	48.33^{a}	132.18^{a}
	1	23.01^{ab}	12.83^{a}	6.39	32.00^{b}	88.96^{b}
	2	22.15^{b}	9.39^{b}	6.62	31.00^{b}	85.48^{b}
	3	28.43^{a}	10.35^{ab}	6.67	25.00^{b}	68.36^{b}
SEM		1.165	0.816	0.041	2.427	6.632
***P* value**	Treatment	0.060	0.033	0.054	0.0002	0.0003
	Linear	0.015	0.171	0.201	<.0001	<.0001
	Quadratic	0.420	0.043	0.063	0.043	0.065
	Control vs. all	0.038	0.017	0.473	<.0001	<.0001
Cottonseed	0.0, control	25.63^{b}	7.21	6.52	31.75	88.04
	1	28.24^{b}	7.39	6.37	29.75	82.3
	2	35.05^{a}	8.29	6.36	31.66	86.55

	3	15.44^{c}	8.18	6.39	33.75	92.86
SEM		2.713	0.357	0.051	0.783	2.145
***P* value**	Treatment	0.002	0.733	0.729	0.438	0.463
	Linear	0.018	0.356	0.446	0.302	0.375
	Quadratic	0.001	0.868	0.448	0.221	0.191
	Control vs. all	0.720	0.475	0.288	0.988	0.878
***P* value**						
Protein source:		<.0001	0.003	0.026	<.0001	0.001
SEM		1.22	0.356	0.046	1.20	7.48
Bentonite:		0.028	0.049	0.730	<.0001	0.013
SEM		1.46	0.424	0.053	1.45	9.51
Protein*TRT		0.006	0.003	0.163	0.0002	0.037
SEM		2.69	0.780	0.093	2.51	15.64

Arithmetic mean in the same column within each ration with different letters differ (P<0.05).

[1]**dDM:** Degradable of dry matter; [2]**TGP**: Total gas production.

Effect of zeolite addition on rumen fermentation kinetics

As shown in Table (4), adding of zeolite resulted in a significant linear and quadratic decrease for TGP (ml), and TGP (g/kg) with soybean ration at 5% level compared to 1.25, 2.5% zeolite, and control. The same trend was observed with dDM, NH_3-N concentration, and pH value, but with no significant effect. However, the addition of zeolite to the sunflower ration affected pH value, TGP (ml), and TGP (g/kg) quadratically compared to the control treatment, especially at a 1.25% dose.

On the other hand, the NH_3-N concentrations quadratically increased significantly at 1.25%, 2.5%, and 5% compared to control with the lowest concentration in rumen fluid solutions. Also, this trend was noticed with dDM%. Moreover, the ration containing cottonseed meal has increased significantly (P = 0.013) in terms of dDM at 5% zeolite compared to other zeolite levels (1.25 and 2.5%) but did not differ from control. Counterwise, 5% zeolite addition has a lower NH_3-N concentration (P = 0.009) than the other zeolite levels (1.25 and 2.5%), but not less than control. The values of pH, TGP (ml), and TGP (g/kg DM) in the cottonseed rations were affected by the level of zeolite in the rumen fluid without significant differences among all levels and control. In general, the current results showed that rumen fermentation kinetics for zeolite addition, different types of protein sources and the interaction had a significant effect.

Previous results found that zeolite addition in an *in vitro* study increased the NH_3-N concentration and ruminal pH (linear and quadratic effects, P<0.05) at 8 and 12 hours of incubation, but DM % was decreased insignificantly with zeolite added compared to control (Roque-Jiménez et al., 2018). This result agrees with our findings for NH_3-N concentrations with sunflower and cottonseed rations, but is inconsistent with soybean rations which have reduced NH_3 concentrations with the addition of zeolite.

Other experiments reported that adding zeolite to cows' diets significantly reduced ruminal DM digestibility (Grabherr et al., 2009; Dschaak et al., 2010; Stojkovic et al., 2012). On the other hand, no change was observed for the addition of zeolites to pH, or ammonia content in the rumen (Bosi et al., 2002). Kardaya et al. (2012) found that ruminal pH decreased with the addition of zeolite, and indicated that zeolite was able to capture NH_3 through its cation exchange capacity. However, their findings were in contrast with our observations, where ruminal pH was decreased, and ruminal NH_3 increased in zeolite addition for sunflower and cottonseed rations, and these findings inconsistent with reported by Ghoneem et al. (2022).

It's known that NH_3-N concentration in the rumen is an indicator of the rate of ruminal N degradation. The present study results indicated that a higher inclusion of NH_3-N can be obtained in rations involving urea (sunflower and cottonseed rations) with zeolite addition. This can be added safely with no adverse effects on ruminal pH level in a normal range of 6.5 to 7, where most of the ammonia would be present in the form of NH_4+, as reported by (Abdoun et al., 2006), as a result of high ruminal NH_3 and the rapid urea hydrolysis in the rumen (Roque-Jiménez et al., 2018). This suggestion is supported by our findings in the case of soybean rations without urea supplementation compared to others involving urea in diets, where ammonia was lower in soybean rations than others.

The conflict between previous results and present data is whether there is a positive, negative, or no effect of zeolite addition on rumen fermentation especially ruminal NH_3

Links Researchers

could be due to the type of protein rations, urea supplement or not, level of NDF in the rations, type of animal, roughage to concentrate ratio, etc.

Effect of bentonite addition on rumen fermentation kinetics

According to the results shown in Table (5), the type of protein source and bentonite level significantly affected rumen DM degradability, NH_3-N concentrations, TGP (ml), and TGP (g/kg DM), while ruminal pH value was affected only by the type of protein source. No interaction effect was observed between bentonite additions and the protein sources. For soybean ration, bentonite quadratically increased (P = 0.01) NH_3-N concentrations at 3% compared to other levels. On the other hand, both TGP (ml) and TGP (g/kg DM) values were linearly decreased with bentonite addition of 2 and 3%, but linearly increased with 0 to 1% addition. For rations of sunflowers, dietary addition of bentonite at 3% showed a significant increase in apparent rumen DM degradability and quadratically increased NH_3-N concentrations at 1 and 3% of supplement levels compared to other levels.

Both of TGP (ml) and TGP (g/kg DM) were linearly decreased with bentonite addition. For rations of cottonseeds, NH3-N concentrations, ruminal pH values, TGP (ml), and TGP (g/kg DM) did not differ among levels of bentonite addition and control, but adding bentonite at 1 and 2% linearly increased the ruminal DM degradability, while adding bentonite at 3% quadratically decreased (P<0.001) the ruminal DM degradability in cottonseed rations.

An *in vitro* study by Jiang et al. (2020) found similar results between the adding of bentonite + aflatoxin B1 and control diet on NH_3-N concentration and DM degradability and attributed it to the binding of aflatoxin B1 with bentonite that has a positive effect on rumen microflora activity. However, these findings are confounded with our results, where the response of bentonite addition was affected by the type of protein source as shown in Table 5. Moreover, ammonia concentration was reduced with the bentonite addition at 2 g/day in the rumen simulation technique (Rusitec) compared to control (Wallace and Newbold, 1991). Similarly, Hristov et al. (2003) observed that ammonia and soluble protein concentrations were reduced by bentonite addition. However, the ruminal pH values, nutrient digestibility and NH_3–N concentrations were not affected by sodium bentonite addition (Chegeni et al., 2013). Interestingly, a greater increase of NH_3-N concentrations were observed with sunflower and cottonseed meal treatments with the inclusion of urea than the soybean without urea inclusion. This was probably a result of the substantial amount of soluble protein introduced into the incubation media and this suggestion agrees with that reported by (Hristov et al., 2003). Further, Wallace and Newbold (1991) reported that bentonite effects have been attributed to adsorptive interactions between the bentonite and ruminal microorganisms or their growth substrates.

Therefore, different protein sources (soybean meal, sunflower, or cottonseeds) have different rates of protein degradation in the rumen while protein sources have large quantities of soluble nitrogen that can be converted into ammonia in the rumen. Other views, a basal diet of protein type, urea supplemented with or without the addition of bentonite levels may be partly due to different responses to rumen fermentation.

CONCLUSION

The addition of clay agents such as humic acid, zeolite and bentonite to different protein sources has different effects (positive, negative, and no effect) on ruminal fermentation (dDM, pH, reducing ammonia emission and TGP). Therefore, more studies are needed to select the best level of each clay with different protein sources in rations to study *in vitro*, which could reveal whether the discovered effects on decreased ammonia emission, modulating rumen fermentation and possibly, improving feed efficiency through increasing DM degradability in rumen have a positive response or not.

ACKNOWLEDGEMENTS

The authors wish to thank Mr. Mohamed G. Embaby, Graduate student, Dept. Animal Science, Food and Nutrition Southern Illinois University, Carbondale, Illinois State, USA for his language revision and enhancing manuscript editing.

CONFLICT OF INTEREST

All authors declare that there are no present or potential conflicts of interest among the authors or with other people and organizations that could inappropriately bias their work.

NOVELTY STATEMENT

This relationship between clay minerals (bentonite, zeolite, and humic acid) as digestibility enhancers was rediscovered using different protein sources (soybean meal, sunflower meal, and cottonseed meal) via in vitro system. The data collected appeared the positive effects of clay minerals on rumen fermentation, especially gas production and reducing ammonia concentration.

AUTHORS CONTRIBUTION

AEM, HME and MAH contributed to the study conception and design. MAH contributed to supervision and validation. HME and SHAH prepared materials, investigation, collected data and formal analyses. HME prepared the first draft of the manuscript. MAH, AEM and RRAE revised the manuscript. All authors read and approved the final manuscript.

REFERENCES

Abdoun K, Stumpff F, Martens H (2006). Ammonia and urea transport across the rumen epithelium: a review. Anim. Health Res. Rev. 7(1-2): 43 - 59. https://doi.org/10.1017/S1466252307001156

Bosi P, Creston D, Casini L (2002). Production performance of dairy cows after the dietary addition of clinoptilolite. Ital. J. Anim. Sci. 1(3): 187 - 195. https://doi.org/10.4081/ijas.2002.187

Chegeni A, Li YL, Deng KD, Jiang CG, Diao QY (2013). Effect of dietary polymer-coated urea and sodium bentonite on digestibility, rumen fermentation, and microbial protein yield in sheep fed high levels of corn stalk. Livest. Sci. 157(1): 141 - 150. https://doi.org/10.1016/j.livsci.2013.07.001

Degirmencioglu T (2012). Possibilities of using humic acid in diets for Saanen goats. Mljekarstvo: časopis za unaprjeđenje proizvodnje i prerade mlijeka, 62(4): 278 - 283.

Diaz DE, Hagler WM, Blackwelder JT, Eve JA, Hopkins BA, Anderson KL, Whitlow LW (2004). Aflatoxin binders II: Reduction of aflatoxin M1 in milk by sequestering agents of cows consuming aflatoxin in feed. Mycopathologia 157(2): 233 - 241. https://doi.org/10.1023/B:MYCO.0000020587.93872.59

Dschaak CM, Eun JS, Young AJ, Stott RD, Peterson S (2010). Effects of supplementation of natural zeolite on intake, digestion, ruminal fermentation, and lactational performance of dairy cows. Prof. Anim. Sci. 26(6): 647 - 654. https://doi.org/10.15232/S1080-7446(15)30662-8

Duncan, D. B. (1955). Multiple range and multiple F tests. Biometrics, 11(1): 1 - 42. https://doi.org/10.2307/3001478

Ebeid HM, Hassan FU, Li M, Peng L, Peng K, Liang X, Yang C (2020a). Camelina sativa L. oil mitigates enteric in vitro methane production, modulates ruminal fermentation, and ruminal bacterial diversity in buffaloes. Front. Vet. Sci., 7: 550 - 564. https://doi.org/10.3389/fvets.2020.00550

Ebeid HM, Mengwei L, Kholif AE, Hassan FU, Lijuan P, Xin L, Chengjian Y (2020b). Moringa oleifera oil modulates rumen microflora to mediate in vitro fermentation kinetics and methanogenesis in total mix rations. Curr. Microbiol. 77(7): 1271 - 1282. https://doi.org/10.1007/s00284-020-01935-2

El-Zaiat HM, Morsy AS, El-Wakeel EA, Anwer MM, Sallam SM (2018). Impact of humic acid as an organic additive on ruminal fermentation constituents, blood parameters and milk production in goats and their kids growth rate. J. Anim. Feed Sci. 27(2): 105 - 113. https://doi.org/10.22358/jafs/92074/2018

EMEA (1999). Committee for veterinary medicinal products. Humic acids and their sodium salts. Available at: www.emea.eu.int/pdfs/vet/mrls/055499en.pdf. Last modified April 21, 2008. Accessed February 1999

Ghoneem WM, El-Tanany RR, Mahmoud AE (2022). Effect of natural zeolite as a rumen buffer on growth performance and nitrogen utilization of Barki lambs. Pakistan J. Zool. 54(3): 1199 - 1207. https://doi.org/10.17582/journal.pjz/20191207121206

Grabherr H, Spolders M, Fürll M, Flachowsky G (2009). Effect of several doses of zeolite A on feed intake, energy metabolism and on mineral metabolism in dairy cows around calving. J. Anim. Physiol. Anim. Nutr. 93(2): 221 - 236. https://doi.org/10.1111/j.1439-0396.2008.00808.x

Griban VG, Baranahenko VA, Kasyan SS, Verlos SV (1991). Use of hydrohumate (sodium salt of humic acid) for enhancing the natural resistance of cows with subclinical nutritional disorders. Vet. Moskova 12: 54 - 56.

Hristov AN, Ivan M, Neill L, McAllister TA (2003). Evaluation of several potential bioactive agents for reducing protozoal activity in vitro. Anim. Feed Sci. Technol.105(1-4): 163 - 184. https://doi.org/10.1016/S0377-8401(03)00060-9

Jaynes WF, Zartman RE, Hudnall WH (2007). Aflatoxin B1 adsorption by clays from water and corn meal. Appl. Clay Sci. 1; 36(1-3): 197 - 205. https://doi.org/10.1016/j.clay.2006.06.012

Ji F, McGlone JJ, Kim SW (2006). Effects of dietary humic substances on pig growth performance, carcass characteristics, and ammonia emission. J. Anim. Sci. 84(9): 2482 - 2490. https://doi.org/10.2527/jas.2005-206

Jiang Y, Ogunade IM, Arriola KG, Pech-Cervantes AA, Kim DH, Li X, Adesogan AT (2020). Effects of a physiologically relevant concentration of aflatoxin B1 with or without sequestering agents on in vitro rumen fermentation of a dairy cow diet. J. Dairy Sci. 103(2): 1559-1565. https://doi.org/10.3168/jds.2019-17318

Kanana HA, Elwakeel EA, Elkomy AG, Sallam SMA (2019). Effect of humic acid and selenium supplementation on immunity and performance of newborn calves. Egyptian J. Nutr. and Feeds 22(2): 251 - 263. https://doi.org/10.21608/ejnf.2019.79390

Kardaya D, Sudrajat D, Dihansih E (2012). Efficacy of dietary urea-impregnated zeolite in improving rumen fermentation characteristics of local lamb. Media Peternakan, 35(3): 207 - 207. https://doi.org/10.5398/medpet.2012.35.3.207

Kissell L, Davidson S, Hopkins BA, Smith GW, Whitlow LW (2013). Effect of experimental feed additives on aflatoxin in milk of dairy cows fed aflatoxin-contaminated diets. J. Anim. Physiol. Anim. Nutr. 97(4): 694 - 700. https://doi.org/10.1111/j.1439-0396.2012.01311.x

McDonald P, Edwards R, Greenhalgh J, Morgan C, Sinclair L, Wilkinson R (2011). Animal Nutrition 7th edition England UK. Harlow, England: Pearson Education Limited

McMurphy CP, Duff GC, Sanders SR, Cuneo SP, Chirase NK (2011). Effects of supplementing humates on rumen fermentation in Holstein steers. S. Afr. J. Anim. Sci. 41(2): 134 - 140. https://doi.org/10.4314/sajas.v41i2.71017

Montalvo S, Guerrero L, Borja R, Sánchez E, Milán Z, Cortés I, De La La Rubia MA (2012). Application of natural zeolites in anaerobic digestion processes: A review. Appl. Clay Sci. 58: 125 - 133. https://doi.org/10.1016/j.clay.2012.01.013

Roque-Jiménez JA, Pinos-Rodríguez JM, Rojo-Rub R, Mendoza GD, Vazquez A, De Jesus JC, Lee-Rangel HA (2018). Effect of natural zeolite on live weight changes, ruminal fermentation and nitrogen metabolism of ewe lambs. S. Afr. J. Anim. Sci. 48(6): 1148 - 1155. https://doi.org/10.4314/

sajas.v48i6.19

SAS Institute. (2015). SAS 9.4 SQL Procedure User's Guide. SAS Institute., Cary, NC

Shi Y, Parker DB, Cole NA, Auvermann BW, Mehlhorn JE (2001). Surface amendments to minimize ammonia emissions from beef cattle feedlots. Transactions of the ASAE 44(3): 677-682. https://doi.org/10.13031/2013.6105

Stojkovic J, Ilic Z, Milenkovic M, Jasovic B, Cilev G (2012). The effect of natural zeolite on fattening lambs production results. J. Hyg. Eng. Des. 1: 302 - 304.

Sulzberger SA, Kalebich CC, Melnichenko S, Cardoso FC (2016). Effects of clay after a grain challenge on milk composition and on ruminal, blood, and fecal pH in Holstein cows. J. Dairy Sci. 99(10): 8028 - 8040. https://doi.org/10.3168/jds.2016-11030

Szczechowiak J, Szumacher-Strabel M, El-Sherbiny M, Pers-Kamczyc E, Pawlak P, Cieslak A (2016). Rumen fermentation, methane concentration and fatty acid proportion in the rumen and milk of dairy cows fed condensed tannin and/or fish-soybean oils blend. Anim. Feed Sci. Technol. 216: 93 - 107. https://doi.org/10.1016/j.anifeedsci.2016.03.014

Szumacher-Strabel M, Martin SA, Potkanski A, Cieslak A, Kowalczyk J (2004). Changes in fermentation processes as the effect of vegetable oil supplementation in in vitro studies. J. Anim. Feed Sci. 13: 215 - 218. https://doi.org/10.22358/jafs/73843/2004

Trckova M, Matlova L, Dvorska L, Pavlik I (2004). Kaolin, bentonite, and zeolites as feed supplements for animals: health advantages and risks. Vet Med (Praha), 49(10): 389. https://doi.org/10.17221/5728-VETMED

Vaughan D, Ord BG (1991). Influence of natural and synthetic humic substances on the activity of urease. J. Soil Sci. 42(1): 17 - 23. https://doi.org/10.1111/j.1365-2389.1991.tb00087.x

Wallace RJ, Newbold CJ (1991). Effects of bentonite on fermentation in the rumen simulation technique (Rusitec) and on rumen ciliate protozoa. J. Agric. Sci. 116(1): 163 - 168. https://doi.org/10.1017/S0021859600076279

Weber J (2002). Definition of soil organic matter. Humintech: Humic acids based products.https://www.humintech.com/fileadmin/content_images/agriculture/information/articles_pdf/DEFINITION_OF_SOIL_ORGANIC_MATTER.pdf