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Original Article

Dietary incorporation of magnetic bentonite nanocomposite: impacts on *in vitro* fermentation pattern, nutrient digestibility, and growth performance of Baluchi male lambs

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Abstract

Background: Incorporation of bentonite into the diets of ruminants can be helpful to maximize their performance. Modifying the structure of bentonite to nano and nanocomposite has improved their chemical stability and physicochemical properties, enhancing adsorption, absorption, and cation exchange capacity. **Aims:** This study aimed to assess the effect of magnetic bentonite nanocomposite (MBNC) on *in vivo* and *in vitro* fermentation process patterns, nutrient digestibility, and growth performance of Baluchi male lambs. **Methods:** Effects of control (basal diet), natural bentonite (NB) (10 g/kg dry matter (DM)), processed bentonite (PB) (5 and 10 g/kg DM basal diet), and MBNC (5 and 10 g/kg DM basal diet) on gas production (GP), and the fermentation process were determined using *in vitro* GP technique. For the *in vivo* experiment, 20 Baluchi male lambs were used with 4 experimental treatments: control, NB (5 g/kg DM), PB (5 g/kg DM), and MBNC (5 g/kg DM) and 5 replications in a completely randomized design for 60 consecutive days. **Results:** The potential for GP and its fractional rates were significantly decreased and increased in MBNC, respectively (P<0.01). The lowest cumulative GP, and CH_4 yield were observed in MBNC (P<0.05). *In vitro*, DM and organic matter (OM) digestibility and all fermentation parameters increased with the addition of two levels of MBNC to the culture medium (P<0.01). Except for feed conversion ratio (FCR), other growth performance parameters increased with the addition of MBNC to the diet (P<0.01). The ruminal pH, total volatile fatty acids (TVFA), acetate, and propionate significantly increased when MBNC incorporated to the diet (P<0.01). The NH_3-N (P<0.001) was significantly decreased in MBNC. The bentonite supplementation decreased acetate to propionate (P=0.001) compared to the control. **Conclusion:** Adding MBNC at the 5 g/kg diet DM level can be used as a useful supplement to optimize rumen fermentation pattern, reduce methane production, and increase lamb performance.

Key words: Baluchi sheep, Gas production, *In vivo*, Nanoclay

Introduction

Supplementing ruminant rations with feed additives can optimize the ruminant's performance by regulating the fermentation process and increasing nutrient usage. Pre-gastric fermentation of organic matter by microbes in the rumen can cause inherent losses in energy (up to 15% of gross energy) and nitrogen (75-85% of nitrogen intake) (Grabherr *et al*., 2009). So, it appears that increasing the DM digestibility and mitigating CH_4 and nitrogen pollution, can enhance ruminant productivity (Connor, 2015).

Recently, the utilization of clay-based supplements to optimize ruminant performance has been developed (Al-Sudani and Al-Rubii, 2021). Bentonite is a natural clay ($(Na, Ca)(Al, Mg)(Si_4O_{10})_3(OH)_6$-$nH_2O$) containing montmorillonite as a significant constituent and characterized by colloidal properties due to its aluminosilicate structure (Maryan and Montazer, 2015; Tate *et al*., 2015). The bentonite effectively reduces the bioavailability and distribution of aflatoxin (AF) throughout the body (Maki *et al*., 2017). Due to high cation exchange capacity (CEC), natural bentonite has antibacterial properties (Banat *et al*., 2002). However, antimicrobial properties were higher in nano-bentonites compared to the natural forms (Magaña *et al*., 2008; Maryan and Montazer, 2015).

By altering the structure of bentonite through various mechanical methods and density separation procedures, the function of bentonite can be optimized (Maki *et al*., 2017). Eliminating phosphate types via a novel bentonite-alum absorptive suggested by Mahadevan *et al*. (2018). In 2017, Huang *et al*. synthesized organo-bentonite by altering the structure of bentonite with cetyl

trimethyl ammonium bromide (CTAB). The ability of the modified bentonite-alum polymer to coat ceramic substrates was demonstrated by Martinez *et al*. (2017). Pandey (2017) investigate the adsorption capacities, thermodynamics, regeneration capabilities, and kinetic behavior of the modified bentonite composites for the removal of heavy metal ions such as Cu^{2+}, Cd^{2+}, Pb^{2+}, Cr^{6+}, and Ni^{2+} from aqueous solutions. The modifications involve the use of various compounds such as nanoclays, organic-inorganic nanocomposites, and polymeric composites. The impact of nano-bentonite synthesis on *Salmonella typhimurium* mutation was investigated by Degtyareva *et al*. (2016). The investigation of the antimutagenic properties of nano-bentonite indicates that it has a moderate inhibitory impact on mutagenesis induced by mitomycin C, 2, 4-dinitrophenylhydrazine, and ethyl methane sulphonate. However, it does not hinder the genotoxic effects of hydrogen peroxide. Bama and Sundrarajan (2017) produced an antibacterial Ag/TiO_2/bentonite nanocomposite against some bacterial species.

Recent studies have shown that modifying the structure of bentonite to nano and nanocomposite has improved their chemical stability and physicochemical properties, including adsorption, absorption, and CEC (El-Nile *et al*., 2021). At the same time, the particle size and dose can affect dry matter digestibility (DMD) and reduce the methane (CH_4) and ammonia nitrogen (NH_3-N) production (El-Nile *et al*., 2021). We expect that the novel properties of the bentonite nanocomposite will enable us to regulate the ruminal fermentation process, efficiently. Therefore, the current study aimed to assess the effect of dietary supplementation with magnetic bentonite nanocomposite on *in vitro* fermentation process, nutrient digestibility, and performance in Baluchi male lambs.

Materials and Methods

Experimental feed additives

Natural aluminosilicate structured bentonite (Bentofeed™) and processed bentonite (Zarin Binder$^{plus™}$) were supplied from Vivan Trading Company, Qaen, Iran. The chemical composition and physical properties of the aluminosilicate structured bentonite used in the study are presented in Table 1.

In a typical synthesis of Fe_3O_4 bentonite nanocomposite, the 50 ml of distilled water was vigorously stirred at 80°C, causing the 2 g of $FeCl_2{\cdot}4H_2O$ and 5.2 g of $FeCl_3{\cdot}6H_2O$ to dissolve. Subsequently, 200 ml of 25% NH_4OH was gradually added to the mixture. Then, 3 g of bentonite was added to the mix. After 3 h, a magnet was used to isolate the magnetic bentonite nanocomposite particles. Synthesized bentonite nanocomposite particles were washed via ultrapure water and dried at 50°C for 24 h (Heydari *et al*., 2019).

To detect the surface morphology of the adsorbent, the scanning electron microscopy (SEM) figures (SEM, TESCAN Mira3) and Energy Dispersive X-ray (EDX) spectrum were used (Figs. 1 and 2). Fourier transformed infrared (FTIR) spectrum of a Fe_3O_4 bentonite nanocomposites particles was obtained from 400 to 4000 cm^{-1} (Perkin Elmer 1750 FTIR Spectrophotometer) (Fig. 3) (Sulaymon *et al*., 2014; Heydari *et al*., 2019).

In vitro trial

The treatments for the *in vitro* trial were comprised of a control (basal diet without supplements) (Table 2), one level of the natural bentonite (NB) (10 g/kg DM), two additional levels of the processed bentonite (PB) (5 and 10 g/kg DM basal diet), and two additional levels of the magnetic bentonite nanocomposites (MBNC) (5 and 10 g/kg DM basal diet).

Gas production procedure

The protocol outlined by Menke and Steingass (1988) was followed during the gas test. Fresh rumen fluid was collected from three male Baluchi sheep that were slaughtered and had been fed a diet formulated for

Table 1: Chemical composition and physical properties of the natural and processed forms of bentonite

Item	Natural bentonite (Bentofeed™)	Processed bentonite (Zarin Binder$^{plus™}$)
Chemical composition (%)		
TiO_2	0.22	0.22
CaO	2.65	2.65
K_2O	0.75	0.75
Na_2O	2.67	2.67
MgO	2.57	2.57
Fe_2O_3	2.34	2.34
Al_2O_3	12.7	12.7
SiO_2	64.5	64.5
Physical properties		
Water absorption capacity (%)	700-750	700-750
Swelling index (ml/2 g)	19-21	19-21
Moisture content (%)	4-8	4-8
Particle size (mesh)	50-400	37
CEC (meq/100 g)	100-110	100-110
Heavy metals (ppm)		
Pb	13	11
Cd	0.1	0.1
Hg	<0.05	<0.05
Ni	-	7
As	2.43	3
Microbial analysis (standard limit: 10^4/g)		
Added microbial population	1.80×10^2	1.80×10^2

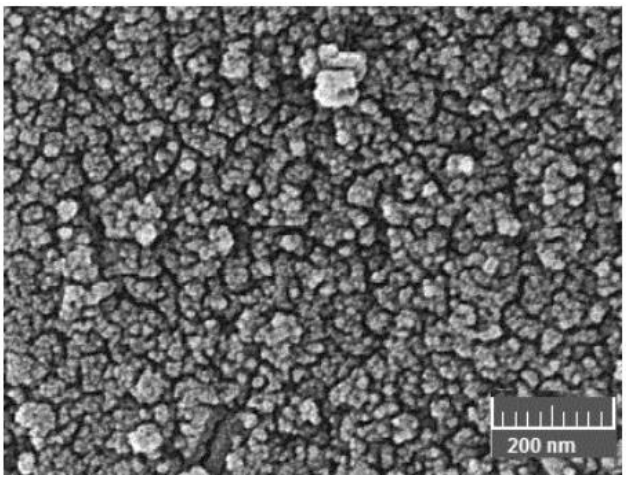

Fig. 1: SEM image of magnetic Fe_3O_4 bentonite nanoparticles

on a 200 nm scale

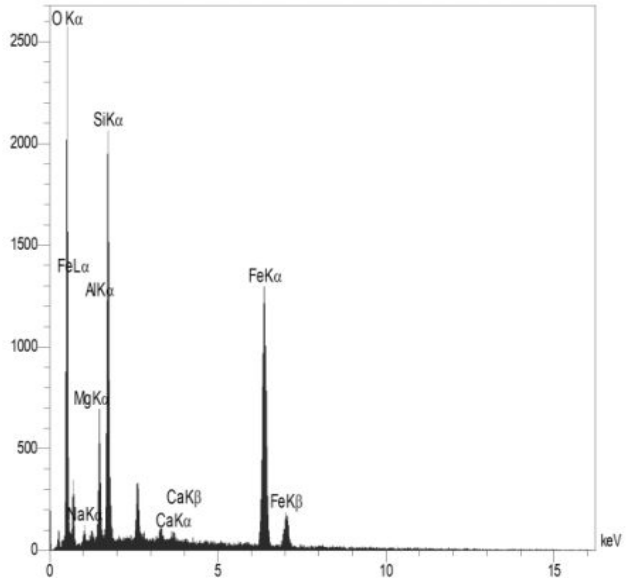

Fig. 2: Energy dispersive X-ray spectrum obtained for magnetic Fe_3O_4 bentonite nanoparticles

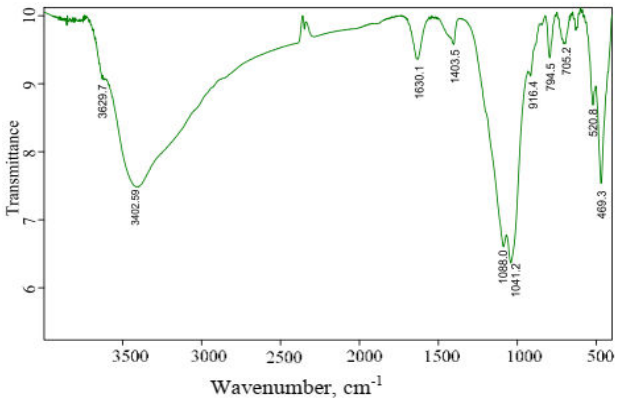

Fig. 3: Fourier transformed infrared spectrum of magnetic Fe_3O_4 bentonite nanoparticles

Table 2: Major ingredients and chemical composition of the experimental basal diet (%)

Ingredients	% Dry matter
Corn silage	38.8
Alfalfa hay	7.60
Wheat straw	3.60
Barley grain	21.0
Corn grain	12.5
Wheat bran	7.00
Soybean meal	6.75
Vitamins and minerals mixture [a]	2.50
Urea	0.25
Chemical composition (% of DM)	
Dry matter (% of fresh weight)	90.00
Metabolizable energy (Mcal/kg DM)	02.67
Crude protein	15.27
Neutral detergent fiber	31.00
Non-fiber carbohydrates	43.36
Ether extract	02.95
Ash	07.42

[a] Mineral and vitamin mixture (mg/kg): Vit E, 100 mg; Vit B, 10 mg; Vit B2, 20 mg; Vit A, 400,000 IU; Vit D, 100,000 IU; Ca, 30 g; P, 12 g; Na, 40 g; Cu, 1000 mg; I, 60 mg; Co, 60 mg; mg, 11,000; Mn, 2000 mg; Zn, 2000 mg; Fe, 3000 mg

maintenance (50:50 F:C ratio) (NRC, 2007) (Table 2). The collected rumen fluid was filtered via four layers of gauze and then stored in a warmed flask at 39°C. Four 100 ml syringes containing 200 mg of samples were used for each treatment. After adding the rumen fluid and artificial saliva (1:2, w/w) to each syringe, the syringes closed, immediately. The syringes were incubated at a temperature of 39°C for varying durations (3, 6, 9, 12, 24, 48, 72, and 96 h), and the head-space gas pressure of all syringes was recorded. Similarly, four blank syringes in each run were prepared for a gas test correction. Two incubation runs were conducted for the *in vitro* digestibility, fermentation data, and gas production parameters (four replicates in each run). The bellow equation (Ørskov and McDonald, 1979) was used to analyze *in vitro* trial data.

$$Y = b (1 - e^{-ct})$$

Where,
Y: Volume of gas produced at time t
b: The potential of gas production (ml/200 mg DM)
c: The fractional rate of gas production for b (%/h)
t: The incubation time (h)

The Fievez *et al*. (2005) protocol was used for measuring the methane yield (24-hour).

After the incubation period, the contents of each syringe were filtered through a Buchner funnel with a 45-micron pore-size polyester fabric. The filtered content was then poured into a pre-weighed crucible, washed with a neutral detergent solution, and dried in an oven at 60°C (Makkar, 2010). The Menke and Steingass (1988) protocol was used for the determination of *in vitro* DM digestibility and OM digestibility:

$$\%IVDMD = (1 - wd - wb/ws) \times 100$$

Where,
wd: Weight of dry sample residue
wb: Weight of dry residues from blank
ws: Dry weight of original sample

$$\%IVOMD = 14.88 + 0.889\ GP + 0.45\ CP + 0.0651\ ash$$

Where,
GP: Cumulative gas production during incubation 24 h (ml/200 mg DM)
CP: Crude protein (%)
Ash: (% DM)

The pH of the culture medium was measured using a pH meter (Hana, Model HI 2210-01, USA). The Menke *et al*. (1979) procedure was used to determine the metabolizable energy:

$$ME\ (MJ/kg\ DM) = 2.20 + 0.136\ GP + 0.057\ CP + 0.0029\ CP^2$$

GP: Cumulative gas production during incubation 24 h (ml/200 mg DM)
CP: Crude protein (g/kg DM)

Short-chain fatty acid (SCFA) concentrations were calculated using the equation of Makkar (2005) as follows:

$$SCFA\ (mmol) = 0.0222 \times GP - 0.00425$$

Where,

GP: Cumulative gas production during incubation 24 h (ml/200 mg DM)

The Blummel *et al*. (1997) method was used to measure partitioning factor (PF), microbial biomass, and microbial biomass efficiency:

PF (mg/ml): IVDMD (mg)/GP

Where,
GP: Cumulative gas production during incubation 24 h (ml/200mg DM)

MB (mg/g DM) = IVDMD (mg) - (GP × 2.2)

Where,
GP: Cumulative gas production during incubation 24 h (ml/200 mg DM)

MBE (%) = [MB (mg/g DM)/IVDMD (mg)] × 100

In vivo trial

Twenty Baluchi male lambs [30.95 ± 2.41 kg body weight (BW), 5-6 months] were randomly assigned to four different experimental diets (n=5 per group). The dietary treatments were:
(1) Control (basal diet without supplements) (Tabel 2)
(2) Natural bentonite (NB) (5 g/kg DM)
(3) Processed bentonite (PB) (5 g/kg DM)
(4) Magnetic bentonite nanocomposites (MBNC) (5 g/kg DM)

The diets were formulated following the NRC (2007) guidelines (50:50 forage-to-concentrate ratio). At the Torbat-e Jam animal husbandry farm, in Iran, lambs were given time to adapt to the experimental diets for 15 days before the *in vivo* trial began. After the initial period, the trial continued for a further 60 days. The lambs were kept in individual pens (2 m × 2 m) (Iranian Council on Animal Care, 1995). Lambs were fed with an unlimited supply of feed (5% refusals) twice daily (7 am and 7 pm) and had free access to fresh water. Daily dry matter intake (DMI) and orts were recorded for each lamb throughout the trial. The dietary samples were dried in a forced-air oven at 65°C for 48 h and then placed in plastic bags for subsequent chemical analysis. At the beginning of the trial, the lambs were weighed just before morning feeding, and their weights were monitored every 15 days. Daily fresh fecal samples were obtained from lambs, about one h post-feeding during a 7-days (days 53-60) using the leather bags. Fecal samples were stored for each lamb at -20°C for subsequent analysis. Fecal samples were dried for 48 h in a forced-air oven (Behdad Co., Iran) set at 65°C, then ground to pass through a 1-mm screen in a Wiley mill (Arthur H. Thomas Co., Philadelphia, PA), before being analyzed. The nutrient intake and excreted were used to determine nutrient digestibility. Rumen fluid was obtained on day 58, three h after the morning feeding, using an esophagus tube connected to a vacuum pump. The collected rumen fluid was filtered via four layers of cheesecloth, and the pH was measured right away using a pH meter (Hana, Model HI 2210- 01, USA) and then stored at -18°C for further analysis. For NH_3-N measuring, 10 ml of the mixture was mixed with 10 ml of 0.2 N HCl and stored at -18°C. The Kjeldahl method was used to determine the NH_3-N concentration (Komolong *et al*., 2001). Gas chromatography [(YL6100 GC; Young Lin Instrument, Anyang, South Korea), (50 m silica-fused: 0.32 mm ID), (column: CP-Wax Chrompack Capillary Column, Varian, Palo Alto, CA, USA), (internal standard: crotonic acid (trans-2-butenoic acid)), (carrier gas: helium) (Detector and injector temperatures: 250°C)] was used to separate and quantify the concentration of volatile fatty acids (VFAs). The AOAC protocol (2005) was used to determine the dry matter (method No. 930.15), ether extract (EE, method No. 991.36), ash (method No. 942.05), and crude protein (CP, Kjeldahl, N × 6.25, method No. 954.01) concentrations. The procedure outlined by Van Soest *et al*. (1991) and the protocol of Ankom Technology (2006) were employed for neutral detergent fiber (NDF) determination using the Ankom fiber analyzer (ANKOM, model A2001, New York, USA).

Statistical analysis

The *in vitro* and *in vivo* data were analyzed using PROC. GLM of SAS (9.4) with the following model:

$Y_{ij} = \mu + T_i + e_{ij}$

Where,
Y_{ij}: The value of each observation
μ: Overall mean
T_i: Treatment effect
e_{ij}: Experimental error

Tukey's procedure was used to measure the treatment's statistical difference ($P<0.05$).

Results

In vitro trial

The potential for GP and its fractional rates were significantly decreased and increased when MBNC was incorporated into the diet ($P<0.01$). The addition of MBNC decreased cumulative gas production during incubation times ($P_{24}=0.007$, $P_{48}=0.002$, $P_{72}<0.001$, and $P_{96}=0.001$) (except gas 12 h). In comparison with the control, NB, and PB, the lowest methane yield was observed in MBNC ($P<0.001$) (Table 3).

The addition of MBNC increased IVDMD ($P=0.05$) and IVOMD ($P=0.01$) compared to the control, NB, and PB. The pH increased ($P<0.001$) with adding doses of MBNC. Increases in the ME and SCFA were observed by MBNC supplementations ($P<0.001$). Similarly, the PF, MB, and MBE ($P<0.001$) were highest for MBNC compared to other treatments (Table 4).

In vivo trial

Except for feed conversion ratio other growth performance parameters [final BW ($P<0.001$), ADG ($P=0.002$), DMI ($P<0.001$), and nutrient digestibility ($P<0.001$)] increased with the addition of MBNC to the diet. The feed conversion ratio ($P=0.13$) did not differ among treatments (Table 5).

Table 3: Effects of natural bentonite (NB), processed bentonite (PB), and magnetic bentonite nanocomposites (MBNC) supplementation on *in vitro* gas production parameters, and methane yield

Item*	Treatments**						SEM	P-value
	Control	NB	PB		MNBC			
			Low	High	Low	High		
***In vitro* gas production parameters**								
b gas (ml/200 mg DM)	63.90[a]	61.67[a]	63.64[a]	63.73[a]	56.16[b]	57.81[c]	0.70	<0.001
c gas (/h)	0.052[ab]	0.054[ab]	0.051[a]	0.056[b]	0.066[c]	0.060[d]	0.001	<0.001
Cumulative gas production (ml/200 mg of DM)								
gas 12 h	29.58	29.20	31.85	31.10	28.83	29.30	0.23	0.10
gas 24 h	44.50[a]	44.65[a]	44.63[a]	45.00[a]	40.18[b]	40.93[b]	0.41	0.007
gas 48 h	56.43[a]	54.90[a]	55.55[a]	57.10[a]	51.30[b]	52.53[b]	0.44	0.002
gas 72 h	62.88[a]	60.53[a]	61.25[a]	62.55[a]	56.40[b]	57.53[b]	0.51	<0.001
gas 96 h	65.17[a]	63.07[a]	63.92[a]	65.72[a]	59.40[b]	60.35[b]	0.49	0.001
CH_4 24 h (ml/200 g DM)	10.69[a]	10.11[a]	9.35[b]	9.55[ab]	4.75[c]	5.21[c]	0.59	<0.001

Different letters along the same row are significantly different according to the Tukey test. * b gas: Potential of gas production, c gas: Fractional rate of gas production, and ** Control (basal diet without supplements), one level of the NB (10 g/kg DM), two additional levels of the PB [5 (low) and 10 (high) g/kg DM basal diet], and two additional levels of the MBNC [5 (low) and 10 (high) g/kg DM basal diet]

Table 4: Effects of natural bentonite (NB), processed bentonite (PB), and magnetic bentonite nanocomposites (MBNC) supplementation on IVDMD and IVOMD, pH, ME, SCFA, PF, MB, and MBE

Item*	Treatments**						SEM	P-value
	Control	NB	PB		MNBC			
			Low	High	Low	High		
IVDMD	67.61[a]	68.55[a]	70.70[a]	69.75[a]	78.90[b]	73.90[b]	0.26	0.05
IVOMD	73.32[a]	72.90[a]	74.68[a]	72.87[a]	79.28[b]	77.10[b]	0.59	0.01
pH	6.94[a]	6.86[b]	7.14[c]	7.04[d]	7.20[e]	7.19[e]	0.03	<0.001
ME	9.88[a]	9.78[a]	9.90[a]	9.82[a]	9.59[b]	10.33[b]	0.05	<0.001
SCFA	0.88[a]	0.89[a]	0.88[a]	0.86[a]	1.00[b]	0.96[b]	0.09	<0.001
PF	2.11[a]	2.14[a]	2.10[a]	2.04[a]	2.49[b]	2.36[b]	0.04	<0.001
MB	96.01[a]	97.33[a]	95.57[a]	92.80[a]	113.47[b]	107.61[b]	1.85	<0.001
MBE	70.54[a]	71.92[a]	68.94[b]	68.62[b]	77.08[c]	75.17[d]	0.78	<0.001

Different letters along the same row are significantly different according to the Tukey test. * IVDMD: *In vitro* dry matter digestibility (%), IVOMD: *In vitro* organic matter digestibility (%), ME: Metabolizable energy (MJ/kg DM), SCFA: Short-chain fatty acids (mmol), PF: Portioning factor (mg/ml), MB: Microbial biomass (mg/g DM), MBE: Microbial biomass efficiency (%), and ** Control (basal diet without supplements), one level of the NB (10 g/kg DM), two additional levels of the PB [5 (low) and 10 (high) g/kg DM basal diet], and two additional levels of the MBNC [5 (low) and 10 (high) g/kg DM basal diet]

Table 5: Effects of natural bentonite (NB), processed bentonite (PB), and magnetic bentonite nanocomposites (MBNC) supplementation on growth performance, dry matter intake, and nutrient digestibility

Items*	Treatments**				SEM	P-value
	Control	NM	PB	MBNC		
Initial BW (kg)	30.97	30.65	31.12	31.06	0.43	0.87
Final BW (kg)	43.06[a]	44.90[b]	45.88[C]	46.29[c]	0.38	<0.001
Average daily gain (ADG) (kg/d)						
Day 15	0.143[a]	0.205[b]	0.224[b]	0.224[b]	0.013	0.006
Day 30	0.200[a]	0.250[b]	0.254[b]	0.257[b]	0.008	0.009
Day 45	0.224[a]	0.243[b]	0.251[b]	0.257[b]	0.004	0.001
Day 60	0.237[a]	0.250[b]	0.253[b]	0.256[b]	0.002	0.001
Total ADG	0.201[a]	0.237[b]	0.246[b]	0.253[c]	0.006	0.002
Total BW gain (kg)	12.09[a]	14.35[b]	14.76[b]	15.22[b]	0.380	0.002
% FCR	6.67	6.34	7.03	6.63	0.190	0.13
DMI (kg/day)	1.357[a]	1.562[b]	1.658[c]	1.851[d]	34.63	<0.001
Nutrient digestibility						
DM	0.78[a]	0.82[b]	0.83[c]	0.85[d]	0.006	<0.001
CP	0.80[a]	0.83[b]	0.84[bc]	0.86[c]	0.005	<0.001
NDF	0.45[a]	0.54[b]	0.56[bc]	0.61[c]	0.015	<0.001
OM	0.80[a]	0.83[b]	0.84[bc]	0.86[c]	0.005	<0.001

Different letters along the same row are significantly different according to the Tukey test. * BW: Body weight, FCR: Feed conversion ratio, DMI: Dry matter intake, CP: Crude protein, NDF: Neutral detergent fiber, OM: Organic matter, and **Control

(basal diet without supplements), NB (5 g/kg DM), PB (5 g/kg DM basal diet), and MBNC (5 g/kg DM basal diet)

Table 6: Effects of natural bentonite (NB), processed bentonite (PB), and magnetic bentonite nanocomposites (MBNC) supplementation on some ruminal fermentation parameters

Items*	Treatments**				SEM	P-value
	Control	NB	PB	MBNC		
pH	6.30[a]	6.37[b]	6.46[cd]	6.52[d]	0.15	<0.001
NH_3-N (mg/dl)	15.54[a]	14.33[b]	13.68[c]	12.72[d]	0.11	<0.001
TVFA (mmol/L)	63.51[a]	62.15[a]	70.50[b]	66.35[b]	0.59	<0.001
Acetate	40.92[a]	39.88[b]	42.77[c]	42.95[c]	0.20	<0.001
Propionate	12.55[a]	13.86[b]	15.12[c]	15.66[c]	0.24	<0.001
Butyrate	10.37	10.95	10.16	10.00	0.45	0.52
Acetate/Propionate	3.26[a]	2.87[b]	2.82[b]	2.74[b]	0.04	0.001

Different letters along the same row are significantly different according to the Tukey test. * NH_3-N: Ammonia nitrogen, TVFA: Total volatile fatty acids, and ** Control (basal diet without supplements), NB (5 g/kg DM), PB (5 g/kg DM basal diet), and MBNC (5 g/kg DM basal diet)

The ruminal pH, TVFA, acetate, and propionate significantly increased when MBNC was incorporated into the diet (P<0.01). The NH_3-N (P<0.001) was significantly decreased in MBNC. The bentonite supplementation decreased acetate to propionate (P=0.001) compared to the control (Table 6).

Discussion

In vitro trial

The reduction of cumulative GP during incubation indicates that MBNC is more capable of absorbing CO_2 than other forms of bentonite. Our study's findings are in agreement with the findings of Chouikhi *et al*. (2019) and Soltan *et al*. (2021b), who found that the nano-montmorillonite had high CO_2 reversible retention capacity compared to the natural forms. This is because of increased hydrophobic surface, interlayer spacing, and the intercalation of organic cations between the -OH groups and the CO_2 molecules in nano-montmorillonite. Soltan *et al*. (2021b) reported that due to a high CEC and an increase in negative charge, the absorptive efficiency of CO_2 in nano-montmorillonite increased, which is in line with the findings of this study.

The *in vitro* results indicated that along with the CH_4 reduction, IVDMD and IVOMD increased. Other researchers' findings have indicated that bentonite supplementation may improve IVDMD and IVOMD (Khalifeh *et al*., 2012; Alhaisheh, 2015; Costa *et al*., 2019). Therefore, supplementing MBNC may alter the ruminal fermentation model towards a decrease in CH_4 with no adverse effects on DM and OM digestibility (El-Nile *et al*., 2021). Increasing the digestibility of OM in the rumen promotes H^+ production. The hydrogen is used in the CO_2 to CH_4 reduction process by ruminal microorganisms (Soltan *et al*., 2013; Patra *et al*., 2017). Hence, the lower CH_4 yield in the MBNC treatments may be associated with capturing both CO_2 and H^+. Moreover, increases in pH caused by MBNC supplementation imply the significant role of MBNC in optimizing the ruminal conditions for the cellulolytic bacteria, increasing OM digestibility, and preventing ruminal acidosis, efficiently (El-Nile *et al*., 2021; Soltan *et al*., 2021b).

Due to the high capacity of H^+ exchange, bentonite may act as an alkalinizer to increase ruminal pH (Stephenson *et al*., 1992). In line with our study, Soltan *et al*. (2021b) reported that nano-montmorillonite had the top CEC values compared with the other diets. The high CEC of nano-montmorillonite can improve the clay activity compared with its natural form (Xue *et al*., 2007; Soltan *et al*., 2021b).

The *in vitro* trial showed that the MBNC increased SCFA concentration compared to other treatments. These results might be due to the shifting of the fermentation process from CH_4 production toward synthesizing SCFAs (El-Nile *et al*., 2021). In this study, the declared increase in PF may be due to the capture of H^+, increased ruminal pH, and enhanced ruminal microbial activity associated with the CH_4 reduction and supply of adequate ATP as SCFAs for more microbial protein synthesis (Morsy *et al*., 2021; Soltan *et al*., 2021a). The increase in ruminal pH can lead to the enhancement of protein solubility and can affect the production of branched-chain fatty acids (BCFAs) (Apajalahti *et al*., 2019; Ramos *et al*., 2021). Therefore, in the present study, it seems that the activity of bentonite is more efficient in the MBNC than the other forms, and produced BCFAs can be used for more microbial protein mass (Liu *et al*., 2021; Soltan *et al*., 2021c).

In vivo trial

Consistent with our findings, Al-Dbaisi (2019) reported that bentonite (2 and 4%) increased total body weight gain (BWG) compared with the control. Similarly, Muhammad *et al*. (2018) observed an enhancement in total BWG and nutrient digestibility following the supplementation of 20 g bentonite/lamb/day. This enchantment can be attributed to the improvement in ruminal pH via adding bentonite, maintaining the amount of water inside the gut, and reducing ammonia toxicity (Aazami *et al*., 2017). Additionally, stimulating and growing cellulolytic bacteria via eliminating or reducing the side effects of mycotoxins and improving the rumen environment can increase organic matter degradation and nutrient

utilization (EFSA, 2011). Large effective surfaces in nanoclays might increase chemical catalysis due to a larger area of mass compared to volume (Hamadi *et al*., 2015). This is why supplemented MBNC is superior compared to the other groups. Moreover, nanomaterials display distinct size-dependent mechanical properties compared to general materials (Wu *et al*., 2020).

In some experiments, the pH enhanced for both natural and nanoclay forms (El-Nile *et al*., 2021; Soltan *et al*., 2021b), which agrees with our study. Natural bentonite, a 2:1 phyllosilicate clay, possesses a crystal lattice structure consisting of an alumina octahedral sheet situated between two silica tetrahedral sheets. The space between its layers holds water molecules and inorganic cations. So, compared to other 1:1 phyllosilicate clay, natural bentonite is a 2:1 with a high CEC and surface area (Magaña *et al*., 2008). Increased pH values caused by bentonite addition may be due to the high CEC of bentonite forms to improve ruminal fermentation. However, interlayer collapse due to manipulation of the natural bentonite structure can influence the swelling capacity and surface charge (Magaña *et al*., 2008). The higher CEC of MBNC could supply optimized conditions for the incorporation of metal hydrolysates and ions into its interlayer space (Xue *et al*., 2007). Therefore, MBNC was the most effective treatment for increasing pH.

In the current study, lower NH_3-N concentration in the MBNC group was agreed with El-Nile *et al*. (2021) and Soltan *et al*. (2021b), who found a lower NH_3-N concentration in nanoclay forms. It can be suggested that the ability of bentonite forms as cation exchangers to retain and exchange the saliva ammonium ion might be the reason for the decreased NH_3-N concentration in the rumen (El-Nile *et al*., 2021). However, in comparison with the control treatment, the nano-bentonite forms show greater CEC (Soltan *et al*., 2021b). Based on previous studies (Sweeney *et al*., 1984; Marden *et al*., 2008), a stable ruminal pH creates a more favorable environment for increased microbial growth. In this study, the decrease in NH_3-N concentration that was observed may be attributed to the stable rumen pH within the normal range, which is linked to the availability of energy (as SCFAs production) and nitrogen (as sufficient NH_3-N concentration) for more microbial protein synthesis (El-Nile *et al*., 2021).

The VFA concentration depended on DMI, nutrient digestibility, and substrate availability for ruminal fermentation (Firkins *et al*., 1986; Robinson *et al*., 1986). The findings of the *in vivo* trial indicated that the addition of MBNC to the diet increased the concentration of propionate. Moreover, the observed results show a decrease in the acetate-to-propionate ratio. This decrease may be the result of a change in the pattern of SCFA production from acetate to greater production of propionate. This change could explain why the fermentation process occurred more efficiently. It's possible that ruminal microbes used more hydrogen ions (H+) to synthesize SCFAs, particularly propionate (El-Nile *et al*., 2021). The VFAs pattern indicates that nano-bentonite has a different effect on rumen fermentation than its natural form. These findings support our initial hypothesis that modifying bentonite structure to nanocomposite form improves chemical stability, physicochemical properties, and efficiency as feed additives.

In conclusion, magnetic bentonite nanocomposite (MBNC) as a dietary supplement had useful effects on the fermentation pattern, nutrient digestibility, and performance of Baluchi male lambs. These findings show that MBNC at the level of 5 g/kg can be used as a useful supplement to optimize rumen fermentation patterns, reduce methane production, and increase lamb performance.

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Conflict of interest

The authors declare that there was no conflict of interest.

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