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Research Article

Effect of ZnO Nanoparticles on *In Vitro* Gas Production of Some Animal and Plant Protein Sources

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Abstract

This study was conducted to determine effect of adding ZnO nanoparticles at levels of 0, 30 and 60 ppm on *in vitro* gas production of some animal and plant protein sources. In this study, gas production at 2, 6, 12, 24, 48 and 72 h incubation were measured and 200 mg of samples were used for gas production analysis. The results showed that after 72 h of incubation, the most volume of gas production in the plant protein sources of soybean meal (SM) and in between the sources of animal protein in poultry offal meal (POM) were respectively 58.23 and 28.34 mL per 200 mg of dry matter was obtained. In related with the parameters of nutrition from incubation data, metabolizable energy (ME), for soybean meal at the levels of zero, 30 and 60 ppm ZnO nanoparticles added to the 8.55, 8.81 and 7.54 were highest and for blood meal (BM) were lowest 2.26, 2.31 and 2.01 MJ/kg dry matter (DM), respectively. The highest and the lowest amount of organic matter digestibility (DOM), short-chain fatty acids (SCFA) and microbial protein (MP) were also for SM and BM. Overall, the results showed that using levels of 0, 30 and 60 ppm of ZnO nanoparticles was no effect on *in vitro* gas production of some animal and plant protein sources but had no significant effect in some hours of incubation, gas production and nutrition parameters.

Keywords: *In vitro gas production, Nutrition parameters, Protein sources, ZnO nanoparticles*

ZnO Nanopartiküllerinin Bazı Hayvan ve Bitki Protein Kaynaklarının *İn Vitro* Gaz Üretimi Üzerine Etkisi

Özet

Bu çalışma bazı hayvan ve bitki protein kaynaklarının *in vitro* gaz üretimi üzerine 0, 30 ve 60 ppm düzeylerinde ZnO ilavesinin etkilerini belirlemek amacıyla yapılmıştır. Çalışmada 2, 6, 12, 24, 48 ve 72 saat inkübasyolarda gaz üretimi ölçülmüş ve gaz üretim analizi amacıyla 200 mg örnek kullanılmıştır. 72 saat inkübasyon sonrasında bitki kaynaklarından soya fasulyesi yemi (SM) ile hayvan kaynaklarından kanatlı sakatat yeminden (POM) elde edilen en fazla gaz üretimi 200 mg kuru maddede sırasıyla 58.23 ve 28.34 mL olarak belirlendi. İnkübasyon verilerinden elde edilen gıda parametrelerinde, soya fasulyesi yemi için metabolize edilebilir enerji 0, 30 ve 60 ppm ZnO nanopartikül ilavelerinde sırasıyla 8.55, 8.81 ve 7.54 olup en yüksek seviyede ve kanlı yem (BM) için en düşük seviyede olup sırasıyla 2.26, 2.31 ve 2.01 MJ/kg kuru madde (DM) olarak tespit edildi. En yüksek sindirilebilir organik madde (DOM) miktarı, kısa zincirli yağ asitleri (SCFA) ve mikrobiyal protein (MP) SM için belirlenirken en düşük seviyeler BM için tespit edildi. Sonuç olarak; 0, 30 ve 60 ppm düzeylerinde ZnO nanopartiküllerinin kullanımının bazı hayvansal ve bitkisel kaynaklarda *in vitro* gaz üretimi üzerine etkisinin olmadığı ve inkübasyon süreleri ile besin parametreleri üzerine anlamlı bir etkisinin bulunmadığı belirlenmiştir.

Anahtar sözcükler: *İn vitro gaz üretimi, Besin parametreleri, Protein kaynakları, ZnO nanopartikülleri*

INTRODUCTION

Zinc is as an essential trace element for almost all living organisms. This element is vital for the functionality of more than 300 enzymes and other metabolic functions such as transcription RNA, defense against free radicals and replication of DNA [1], and this is due to that zinc should be added the daily diet of ruminants [2] and for ruminant nutrition and their rumen microorganism is necessary [3]. Intake of zinc by ruminants with their rumen microbial population changes resulted in changing of the ruminal digestion and fermentation process [4].

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Addition Zn to ruminant diet to more than needed, change their rumen fermentation and increase the ratio of propionic acid and decreases acetate to propionate ratio and it is concluded that increases the energy value of the diet [5]. Because of the unique characteristics of ZnO nanoparticles, these materials used in various industries including food, pharmaceutical, rubber, electronics and packaging and even as feed additives [6]. Reduction of particle size in the nano-scale has led to increased contact area the combination with other biomolecules and these organic molecules and inorganic chemical reactions in the body can be very different that in many materials is still unknown [7]. In relation to the effects of ZnO nanoparticles in biological systems and particularly bacteria, many researches have been done by other researchers such as antimicrobial effects of the substance on the bacteria *Pseudomonas aeruginosa, Staphylococcus aureus* and *Staphylococcus epidermises* have confirmed [8]. As for the effect of ZnO nanoparticles is very little research has been done on the performance of livestock and poultry. In a study, the use of ZnO nanoparticles at 40 mg per kg diet DM, improved poultry performancedue to essential role for body's appropriate physiological functions specially functional enzyme [9].

The *in-vitro* gas production technique have been usually used to assess feed evaluation for ruminants [10-12]. Advantages and disadvantages of *in-vitro* gas procedure are debated by Gatechew *et al*.[13]. A simple *in vitro* methodology is designated by Menke *et al*.[14] which is useful and fast, and permits a large number of samples to be ran at a stage. Makkar [15] highpoints the potential of a novel methodology using an *in-vitro* gas prodution techniques for evaluation of nutritional quality of feed resources. Recently, *in-vitro* gas production technique for feed evaluation well considered by Singh *et al.*[16]; Ayaşan *et al.*[17]; Ayaşan *et al.*[18] and Sevim *et al*.[19]. As a result, *in vitro* gas production technique is used as potentially useful technique to estimate feed intake, organic matter (OM) and dry matter digestibility, metabolizable energy (ME) of feeds and ruminal fermentation studies for ruminants [20-24]. Manipulation of ruminal fermentation with meet the needs of mineral elements for rumen microorganisms, particularly minerals such as Zinc (nano form) may be improves efficiency of protein metabolism [25].

In an experiment [26] of 0, 10 and 20 mg of zinc in ml rumen fluid *(in vitro)* used and its effect on rumen fermentation evaluated after 24 h of incubation and observed that the ruminal pH and ammonia levels were not affected by the zinc levels. Also, in an *in vitro* test using ZnO nanoparticles in the diet improve rumen bacterial growth and increasing the efficiency of energy intake in the diet [27]. In other experiment [28] amount of *in vitro* gas produced over 144 h of incubation there was no significant difference in treatments containing zinc at levels 20 and 40 ppm with control. As mentioned above, with the development of nanoparticles and its use in industries as well as due to the possibility of using it in feed livestock industry and insufficient data, however, little was known about influence of ZnO nanoparticles on ruminant nutrition, this experiment was designed. Thus, the objectives of this study were to evaluate effect of adding ZnO nanoparticles at levels of 0, 30 and 60 ppm on *in vitro* gas production of some animal and plant protein sources.

MATERIAL and METHODS

Materials

Nano-ZnO was purchased from Iranian agent of US Research Nanomaterial, Inc. Port Co., Ltd., USA. The sizes of elemental ZnO particles ranged from 10 to 30 nm, stock: US3590, in the form of white powder and Purity: 99%, APS: 10-30 nm, Color: white, Crystal Phase: single crystal, Morphology: nearly spherical, SSA: 20-60 m2/g, True Density: 5.606 g/cm3.

Methods

Sample Preparation, Chemical Analysis and in vitro Digestibility

This experiment was conducted at the Animal Science Laboratory of Mohaghegh Ardebili University in Iran. This experiment was conducted on sources of plant protein (SM, rapeseed meal, RM; and cottonseed meal, CM and sources of animal protein (POM, fish meal, FM and BM). The samples of SM, RM, CM, POM, FM and BM studied were obtained from feed compound manufacturers, the agricultural sector and the local slaughter house of North West Iran Ardebil Province (Meshgin, Germi and Ardabil), over the years 2014 and 2016. The prepared samples from local factories, for preventing degradation and degreasing, were used carrier materials or moisture adsorbent such as wheat bran. Therefore, some of its analyzes did not match to world feed standard analysis and their cell wall values were higher. The samples were randomly selected for the survey. Then, two local associations were randomly selected from each of the famous regions. A systematic sampling was done in each of the selected associations until total fifteen farmers or agricultural sector were selected for the study, which brings the number of farmers selected to thirty in every region. The chemical composition of the feed by conventional methods [29] and determination of *in vitro* digestibility was estimated using the equation described by Menke et al.[14]. Subsamples of protein sources were grounded through a 1 mm screen and defatting was done by extraction with petroleum ether for 6 h according to the AOAC procedure [29]. Samples of feeds were dried in a forced-air oven at 65-70ºC for24 h and DM content calculated. Ground samples (1 mm) were analysed for ash (ID 942.05) [29] and Kjeldahl N (ID 954.01) [29]. Crude protein (CP) was calculated as Kjeldahl N×6.25. Neutral-detergent fibre (NDF), acid-detergent fibre (ADF)

and acid-detergent lignin (ADL) were determined by the detergent procedures of Van Soest [30,31], with alpha amylase being added during NDF extraction. Sodium sulfite was not added. NDF was expressed without residual ash. Ether extract (EE) was determined by extracting the sample with petroleum ether using a Gerhardt Soxtherm 2000 Automatic (ID 920.39) [29].

In vitro Gas Production

Incubation was carried out at 39°C and the volume of gas production was measured at 2, 6, 12, 24, 48 and 72 h using procedures described by Menke and Steinggass [10]. Approximately, 200 mg of dried and ground (2 mm) samples were weighed and placed into 100 ml syringes. Three blanks containing 30 mL of medium only were included in the run. Average volume of gas produced from the blanks was deducted from the volume of gas produced per sample. Gas volumes obtained at varying incubation hours were fitted to the non-linear equation model of France *et al*.[32]:

$$G = A\,(1\text{-}e^{-c\,(t\text{-}L)\text{-}d\,(\sqrt{t}\,\text{-}\sqrt{L})}) \quad (1)$$

Where G is equal to the accumulation of gas produced per unit time, A is equal to the total amount of gas produced (mL), c is equal to a fixed rate of gas production (mL per hour), d is equal to a fixed rate of gas production (mL at $h^{1/2}$), L equal to the late phase, t and t ½ time equal to half of the total gas production time is cumulative.

The amount of short chain fatty acids (SCFA) [13,33], digestibility of organic matter (DOM) [14] and ME [10,15] and microbial protein [34] were estimated using the equations below.

$$ME\ (MJ/kg\ DM) = 1.06+0.1570GP+0.0084CP+0.0220EE - 0.0081CA \quad (2)$$

$$DOM\ (DM\,\%) = 9+0.99GP+0.0595CP+0.018CA \quad (3)$$

$$SCFA\ (mmol/200\ mg\ DM) = 0.0222GP\text{-}0.00425 \quad (4)$$

$$MP\ (g/kg\ OMD) = [19.3\ DOM\ (kg)]\times 6.25 \quad (5)$$

where: ME = Metabolizable energy (MJ/kg DM), GP gas is 24 h net gas production (mL/g DM), CP is crude protein (DM %), and EE is crude fat (DM %), CA= ash in g/100 g DM. As well as, DOM = OM digestibility (g/100 g DM), SCFA = Short chain fatty acid (mmoL), the microbial protein (MP) was calculated according to Czerkawski [34] formula that is shown in equation 5.

Methods of Data Analysis and Statistical Model

The results of the gas production test to repeated measures were analyzed using the SAS statistical software [35]. Comparing the average of the least significant difference (LS MEAN) was performed. Other data in a completely randomized design with 3 repeats and 3 treatments were evaluated and comparison of means using Duncan test when $P \leq 0.05$. Statistical model research design is as;

$$Y_{ij} = \mu + A_i + e_{ij} \quad (6)$$

where: Y_{ij} is the observation, μ is the population mean, A_i is the effects of experimental treatments and e_{ij} is the residual error.

RESULTS

The chemical composition of test feed is given in *Table 1*. Highest of CP content of 59% was obtained for a blood meal. The maximum amount of crude fat 31.3% for POM and highest ash content of 20% was achieved for FM. Highest of NDF and ADF 70.6% for CM and FM and the lowest NDF and ADF were obtained 45.7 and 33.3% for SM.

Data belong to the production of gas from fermentation of plant proteins (SM, RM and CM) and animal proteins (POM, FM and BM) with or without nano ZnO at 2, 6, 12, 24, 48 and 72 h is presented in *Table 2*.

Nutritional parameters results of gas production in *Table 3* showed that between the sources of plant protein, SCFA, ME, DOM and MP of SM was obtained by adding ZnO nanoparticles levels of 0, 30 and 60 ppm respectively (0.997, 1.034 and 0.854 mmol), (8.548, 8.810 and 7.536 MJ/kg), (56.744, 58.394 and 54.873%) and (68.427, 70.437

Table 1. *Chemical composition of some plant and animal protein sources*

Protein Sources	DM %	CP (%DM)	EE (%DM)	Ash (%DM)	NDF (%DM)	ADF (%DM)
Plant						
Soybean meal	92.4	50	1.6	6.1	45.7	33.3
Rapeseed meal	91.4	37	1.2	8	51.5	46.1
Cottonseed meal	93	24	1.4	4.7	70.6	58.4
Animal						
Poultry offal meal	94.4	55	31.3	7.3	48.9	34.8
Fish meal	93.6	50	18.1	20	61.2	40.6
Blood meal	70.6	59	1.6	5	55.3	33.4

DM = dry matter (percent), CP = crude protein (% DM), EE = crude fat (% DM), Ash = ash (% DM) NDF = Neutral detergent fiber (%), ADF = Acid detergent fiber (%)

Table 2. *Effect of Zinc Nano- oxide on the amount of gas produced (mL per 200 mg DM) at different times of some plant and animal protein sources*

Protein Sources	Levels of Nano-ZnO	Incubation Hours					
		2 h	6 h	12 h	24 h	48 h	72 h
Soybean meal	0 ppm	4.667	17.670	32.000	45.110^{ab}	55.443	58.227
	30 ppm	1.667	15.003	32.000	46.777^{a}	57.110	59.783
	60 ppm	4.667	15.337	30.667	38.663^{b}	50.660	56.667
	SEM	1.2018	1.0887	2.6105	2.2068	2.0170	1.8939
	P-value	0.2063	0.2470	0.9179	0.0870	0.1416	0.5431
Rapeseed meal	0 ppm	0.667	8.330^{a}	16.000	28.663^{ab}	39.333	41.003
	30 ppm	1.000	9.663^{a}	18.667	31.997^{a}	43.667	47.337
	60 ppm	0.000	4.997^{b}	15.333	23.997^{b}	37.000	38.337
	SEM	0.5092	0.8607	1.9626	1.667	2.0994	2.5166
	P-value	0.4219	0.0214	0.4891	0.0394	0.1540	0.1042
Cottonseed meal	0 ppm	1.000	4.500	7.000	11.333^{ab}	15.417	17.750
	30 ppm	0.667	4.333	8.000	13.000^{a}	16.250	17.750
	60 ppm	1.110	3.443	6.000	9.500^{b}	16.220	18.733
	SEM	0.2026	0.4525	0.7453	0.5357	0.8053	0.5774
	P-value	0.3402	0.2818	0.2441	0.0106	0.7217	0.4326
Poultry offal meal	0 ppm	1.333	9.670	15.667	19.997	23.997	28.337
	30 ppm	0.667	10.170	16.000	19.330	22.330	28.670
	60 ppm	0.333	8.670	14.000	17.330	20.330	24.170
	SEM	0.5773	0.5000	0.6086	1.3878	1.5031	1.7821
	P-value	0.5008	0.1780	0.1190	0.4219	0.2979	0.2187
Fish meal	0 ppm	0.000	0.663^{c}	2.333^{b}	3.830^{b}	5.500^{b}	5.670^{b}
	30 ppm	0.333	1.677^{b}	4.700^{ab}	4.577^{b}	6.200^{b}	7.670^{ab}
	60 ppm	0.667	3.830^{a}	7.000^{a}	7.330^{a}	10.500^{a}	10.670^{a}
	SEM	0.4303	0.2740	0.6995	0.6917	1.0011	1.0929
	P-value	0.5787	0.0005	0.0096	0.0262	0.0246	0.0472
Blood meal	0 ppm	0	1.000^{b}	3.500^{a}	4.500	5.083	5.750
	30 ppm	0	2.500^{a}	4.500^{a}	4.333	5.750	6.083
	60 ppm	0	1.000^{b}	2.333^{b}	3.500	5.750	6.083
	SEM	0	0.1667	0.3043	0.3043	0.5092	0.6383
	P-value	0	0.0010	0.0070	0.1190	0.5927	0.9143

** Dissimilar letters in each column represents is a significant difference (P<0.05)*

and 60.749 g/kg) that was compared with cottonseed meal and rapeseed meal the highest amount in which, due to crude protein of soybean meal. Also, SCFA, ME, DOM and MP of POM was (0.440, 0.425 and 0.380 mmol), (5.291, 5.186 and 4.872 MJ/kg) (32.201, 31.541 and 29.561%) and (38.842, 38.046 and 35.657 g/kg) between the sources of animal protein, respectively, that was the highest amount compared with FM and BM.

Results of the parameters predicted by the model France are presented in *Table 4*. As observed, the highest amount of potential gas production (A) in the case of ZnO nanoparticles at levels 0, 30 and 60 ppm was with 290.11, 297.76 and 273.26 mL per g DM for SM, respectively. Highest gas production rate constant (c) respectively with 0.180, 0.180, and 0.035 ml per hour related to the BM and lowest lag phase with values of 0, 0.427, and 0 was for CM.

DISCUSSION

Comparing fermentation gas production between plant and animal protein without adding nano-ZnO represents the total amount of gas produced of SM were highest compared with other investigated plant and animal protein sources. So, at hours of 2, 6 and 12 SM had highly produced gas compared to other sources of plant and animal proteins (P<0.01). For example, the fermented SM, after 6 h of incubation and 17.67 mL and RM and CM, respectively 8.33 and 4.50 mL of gas per 200 mg of DM (P<0.001). In 36, 48 and 72 h of incubation, although the difference in

Table 3. *Effect of Zinc Nano-oxide on parameters of nutritional some animal and plant protein sources*

Protein Sources	Levels of Nano-ZnO	DOM (%DM)	SCFA (mmol/200 g DM)	ME (MJ/kg DM)	MP (g/kg DOM)
Soybean meal	0 ppm	56.744	0.997^{ab}	8.548^{ab}	68.447^{ab}
	30 ppm	58.394	1.034^{a}	8.810^{a}	70.437^{a}
	60 ppm	54.873	0.854^{b}	7.536^{b}	60.749^{b}
	SEM	3.4059	0.0490	0.3465	2.6353
	P-value	0.7739	0.0870	0.0870	0.0870
Rapeseed meal	0 ppm	39.722^{ab}	0.632^{ab}	5.832^{ab}	47.915^{ab}
	30 ppm	43.022^{a}	0.706^{a}	6.356^{a}	51.896^{a}
	60 ppm	35.102^{b}	0.528^{b}	5.010^{b}	42.342^{b}
	SEM	1.650	0.0370	0.2617	1.9903
	P-value	0.0394	0.0394	0.0394	0.0394
Cottonseed meal	0 ppm	21.733^{ab}	0.247^{ab}	3.034^{ab}	26.215^{ab}
	30 ppm	23.383^{a}	0.284^{a}	3.295^{a}	28.205^{a}
	60 ppm	19.918^{b}	0.207^{b}	2.746^{b}	24.026^{b}
	SEM	0.5304	0.0119	0.0841	0.6398
	P-value	0.0106	0.0106	0.0106	0.0106
Poultry offal meal	0 ppm	32.201	0.440	5.291	38.842
	30 ppm	31.541	0.425	5.186	38.046
	60 ppm	29.561	0.380	4.872	35.657
	SEM	1.3739	0.0308	0.2179	1.6572
	P-value	0.4219	0.4219	0.4219	0.4219
Fish meal	0 ppm	16.127^{b}	0.081^{b}	2.317	19.453^{b}
	30 ppm	16.866^{ab}	0.097^{b}	2.435	20.344^{ab}
	60 ppm	19.262^{a}	0.158^{a}	2.739	23.235^{a}
	SEM	0.7107	0.0153	0.1313	0.8573
	P-value	0.0469	0.0262	0.1424	0.0469
Blood meal	0 ppm	17.055	0.096	2.257	20.573
	30 ppm	16.891	0.092	2.231	20.374
	60 ppm	16.065	0.073	2.010	19.379
	SEM	0.3012	0.0067	0.0478	0.3634
	P-value	0.1190	0.1190	0.1190	0.1190

DOM = digestible organic matter (%DM), SCFA = short chain fatty acids (mmol/200 gDM), ME= metabolizable energy (MJ/kg DM), MP = microbial protein (g/kg DOM), SEM = standard error of mean

the amount of gas production for each feed item is clearly visible, but the gas production in samples of fermented SM was higher compared to other plant and animal sources of protein. It seems that high level of gas production by SM due to its high levels of CP (50%) and also the ADF and NDF content was typically lower than other plant sources of protein. Also, between the sources of animal protein, gas production of BM was lower due to highly crude protein (59%) and ADF lower than other animal sources of protein. Adding ZnO nanoparticles had no effect on the *in vitro* gas production after 24 h incubation all protein sources other than FM. However, some sources tend to be significantly reduced. The volume of gas production after 24 h of incubation was used as an index of energy feed value and feed digestibility [36]. According to the observations of this study, the addition of nano-ZnO on the protein source had no effect on gas production after 24 h. However, reduction and the tendency to decrease in the volume of gas production after 24 h of incubation in other study [37] are also shown. This tends to decrease with increasing the nano level, unlike results researchers [27,28], which was up high enough so that the inhibitory effect on the activity of rumen microorganisms was shown. Between sources of animal protein, POM and BM at any of the incubation times was not affected by the addition of ZnO nano-particles, but adding ZnO nanoparticles to FM except the first 2 h of incubation at other times had significantly effect on increasing gas production. In general, the results *(Table 2)* showed that the use of ZnO nanoparticles levels of 0, 30 and 60 ppm of the gas production in 24 h different

Table 4. *Effect of Zinc Nano-oxide on gas production parameters some animal and plant protein sources by France model*

Protein Sources	Levels of Nano-ZnO	A (mL)	c (mL per h	T-Lag
Soybean meal	0 ppm	290.112	0.078	0.337^{ab}
	30 ppm	297.761	0.089	0.636^{a}
	60 ppm	273.259	0.063	0^{b}
	SEM	10.5547	0.0092	0.1276
	P-value	0.3147	0.2072	0.0215
Rapeseed meal	0 ppm	209.861	0.062	0.578^{ab}
	30 ppm	239.100	0.063	0.520^{b}
	60 ppm	199.039	0.065	0.772^{a}
	SEM	14.836	0.0070	0.0610
	P-value	0.2225	0.9682	0.0606
Cottonseed meal	0 ppm	96.592	0.037	0^{ab}
	30 ppm	89.591	0.067	0.427^{a}
	60 ppm	147.306	0.012	0^{b}
	SEM	7.0106	0.0010	0.3421
	P-value	0.0022	0.0224	0.0664
Poultry offal meal	0 ppm	131.172	0.093	0.173
	30 ppm	122.044	0.123	0.540
	60 ppm	101.769	0.148	0.637
	SEM	8.5719	0.0201	0.2509
	P-value	0.1200	0.2296	0.4382
Fish meal	0 ppm	27.496^{b}	0.105	0.710
	30 ppm	36.190^{ab}	0.074	0.246
	60 ppm	50.686^{a}	0.134	0.352
	SEM	6.0372	0.0437	0.3451
	P-value	0.0872	0.6504	0.6317
Blood meal	0 ppm	25.229	0.180	0.866
	30 ppm	29.007	0.180	0.128
	60 ppm	35.114	0.035	0.074
	SEM	3.4047	0.0665	0.2995
	P-value	0.1981	0.2813	0.1941

** Dissimilar letters in each column represents is a significant difference (P<0.05)*
A = potential gas production (mL) c = constant rate gas production (mL per hour) T-Lag = lag phase (hours)

sources of protein except POM and BM had significantly effect that this may be due to high protein content of POM and BM than other. According to these results, adding ZnO nanoparticles at levels of 60 ppm on nutritional parameters of plant protein sources, except in the case of DOM of SM that caused a significant decrease but at level of 30 ppm had no effect on nutritional parameters these sources. Between sources of animal protein, POM and BM by ZnO nanoparticles were not influenced but adding ZnO nanoparticles on DOM, SCFA and microbial protein of FM had significant difference. This showed that 30 ppm zinc element in incubation was provided rumen micro-organisms requirement in terms of the element deficiency of a nutrient needed by rumen micro-organisms. While that the level of 60 ppm, had reduced the level of these parameters or had a tendency to decline (P<0.05). Also in trials [38] of the concentration 1142 ppm of zinc element as zinc sulfate was in the diet of Jersey bull calves and observed that the concentration of volatile fatty acids, ammoniac and rumen pH did not influence by amount zinc in the diet. They reported that these values in control groups, respectively 79.08 mM, 11.10 mg/dL and 6.69 unit and treatment with zinc supplementation 81.30 mM, 10.35 mg/dL and 6.70 unit, which is aligned with the results. In another experiment, when 430 ppm of zinc element by consumption of zinc chloride, was used in the diets Aberdeen angus cows it was observed that the concentration of total volatile fatty acids, ammoniac and rumen pH were not effect by consumption of zinc [39].

According to the results, adding ZnO nanoparticles plant protein sources other than lag phase had no significant effect on *in vitro* gas production parameters, and between the sources of animal protein also, with the exception of gas production potential (A) for FM with a tendency to significant, other sources were not effect using ZnO nanoparticles. In tests conducted by Zabuli and Aliarabi [28], the amount of gas produced over 144 h of incubation in treatments containing zinc supplementation at levels 20 and 40 ppm had been used significantly different from control groups and this shows that the amount of zinc element available along with feed ingredients used in their experiments (27.50 mg per kg of DM diet), has provided the rumen microbes requirement. With regard to the increasing levels of zinc in the rumen (due to its anti-bacterial properties) can leads to reduced bacterial growth [40], It is clear that the level of 30 ppm of zinc supplements was not enough for protein sources that had an antibacterial effect on microorganisms, therefore the addition of zinc to feed on the microorganisms that are involved in the production of gas, probably had no effect , however the amount of gas production treatments containing zinc supplementation compared to control treatment had no significant increase or decrease. But with increasing level of ZnO nanoparticles from 30 to 60 ppm decrease in the production of microbial protein was found in all sources. Thus, it is observed that the results this study confirms to the findings of other researchers. As the experiments [26] levels of 0, 10 and 20 micro gram of zinc element in ml of *in vitro* in rumen fluid of Holsteins applied and its effect after 24 h of incubation on rumen fermentation was investigated and found that levels of rumen pH and ammonia levels of zinc usage was not affected. In tests conducted by Zabuli and Aliarabi [28] indicated that the use of level 20 and 40 ppm Zn from both complementary ZnO and nano-ZnO on rumen parameters both *in vitro* and *in vivo* methods had no significant effect.

Variations in the volume of *in vitro* gas production, parameters of gas production and nutritional parameters, such as ME, SCFA, DOM and MP is caused by physical and chemical properties of protein source. Accordance with results of this research, it is concluded that among the sources of plant protein, SM and in between the sources of animal protein, POM compared to other protein sources were observed suitable for ruminants due to highly in digestibility and characteristics of fermentation and nutritional value. So it seems that the potential to be included in the diet of ruminants. It was also observed that the addition of Zinc Nano-oxide at levels 0, 30 and 60 ppm had not significant difference in the amount of gas produced, gas production parameters (fermentation) and nutritional parameters different sources of protein, especially animal protein to laboratory procedures have not been effected, and therefore zinc element concentrations used in this study treatments were not high enough which could affect the fermentation process and population of microorganisms.

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