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# Effects of supplementary nano-ZnO on in vitro ruminal fermentation, methane release, antioxidants, and microbial biomass

**Hassan RIAZI, Javad REZAEI*, Yousef ROUZBEHAN**
Department of Animal Science, Faculty of Agriculture, Tarbiat Modares University, Tehran, Iran

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**Abstract:** The effects of nano-ZnO on in vitro ruminal fermentation, methane release, total antioxidant capacity (TAC), and microbial biomass production (MBP) were assessed using an in vitro gas production technique. Treatments included a control diet and diets containing 20, 40, or 60 mg of supplemental Zn per kg dry matter (DM) as ZnO or nano-ZnO. As a result of this study, supplementation of 20 mg of Zn as nano-ZnO, similar to ZnO, decreased methane production and protozoa enumeration but improved ($P < 0.05$) TAC, MBP, digestibility and truly degraded substrate (TDS). As compared with the control treatment, adding the supplementary Zn had no effect on partitioning factor, MBP efficiency, pH, and ammonia-N ($P > 0.05$). The diets containing 40 and 60 mg of supplementary Zn, as nano-ZnO or ZnO, had no advantage over the diet containing 20 mg of Zn in terms of methane decline and TAC, TDS, and MBP increments. Overall, nano-ZnO had no adverse effect on in vitro ruminal fermentation. The addition of 20 mg of Zn as nano-ZnO per kg diet DM was enough to improve the in vitro ruminal fermentation in terms of methane release, TAC, and MBP. Thus, the higher supplementary Zn levels (40 and 60 mg/kg DM) are not recommended.

**Key words:** Zinc supplement, ZnO nanoparticles, $CH_4$, antioxidant power, in vitro fermentation

## 1. Introduction

Methane released during ruminal fermentation plays an important role in global warming [1,2]. One of the aims in ruminant nutrition is to reduce the release of methane from the rumen, without adverse effects on digestibility, animal health, and productivity [3]. Moreover, improvements of rumen microbial biomass and the antioxidant status of animals are attractive targets in feeding management. This requires the best supply of nutrients and supplementary minerals in diets. Zinc is a vital trace mineral needed for animal productivity, immunity, rumen metabolism, and the antioxidant system [3–5]. Zn insufficiency can cause the weakness of the antioxidant system [6–8] and using high levels of dietary trace minerals, such as Zn, can improve animal health and performance [9–12].

Diets are usually supplemented with Zn as inorganic (such as ZnO and $ZnSO_4$) or organic (such as Zn-amino acid complexes) sources. An organic mineral source offers further elements to animals due to its superior bioavailability [5,13]. At present, the usage of ZnO nanoparticles (nano-ZnO), with sizes of 1 to 100 nm, has increased in various fields such as mineral nutrition in livestock [4,7,14]. Nano sources of trace minerals have high bioavailability because of interesting properties such as the nano scale size, rapid and specific movement, higher area surface to volume proportion, surface activity, catalytic effectiveness, and absorption percentage [12,15,16]. Some researchers assessed the toxic impacts of Zn nanoparticles in animals [17–19]. However, there are studies supporting the beneficial effects of nanoparticles on animal performance, feed efficiency, and health as well as reduction of environmental pollution, due to the great bioavailability [15,16,20,21]. Wang et al. [12] mentioned that long-term oral Zn sulfate treatment was more toxic for animals than nano-ZnO. As reported by Singh et al. [22], feeding preruminant lambs with nano-ZnO instead of ZnO increased the Zn availability without causing toxicity. Nano-ZnO can improve villus height, crypt depth, and villus surface area in the gastrointestinal tract [12,14]. Sarker et al. [1] reported that supplementation with high levels of nano-ZnO (i.e. 500 and 1000 μg/g) decreased in vitro methane concentration compared with a control treatment (no Zn supplementation). Nanoparticles could be poisonous to certain microbes generating methane in anaerobic digestion [14,23]. Still, further studies are necessary to realize the possible helpful or harmful effects of nano-ZnO on animals [1,7,21].

* Correspondence: rezaei.j@modares.ac.ir

Based on previous studies, it was hypothesized that nano-ZnO could be absorbed by rumen microorganisms differently from ZnO due to the small size and high surface area, and they may change the rumen fermentation. Therefore, the aim of this study was to investigate the effect of increasing dietary levels of Zn as nano-ZnO, compared with ZnO, on in vitro ruminal fermentation, methane release, total antioxidant capacity (TAC), and microbial biomass production (MBP).

## 2. Materials and methods

### 2.1. Experimental treatments

This study was conducted at Tarbiat Modares University (Tehran, Iran). A control diet free of supplementary Zn was formulated according to the nutrient requirements of growing sheep [24], with an exception for Zn level. The crude protein (CP) and metabolizable energy (ME) requirements of 8-month-old growing sheep (late maturing) with 30 kg of body weight, an average daily gain of 400 g/day, and a daily DM intake of 1.5 kg are 10.63 MJ and 133 g per kg diet DM (i.e. 3.81 MJ and 200 g per day), respectively. The Zn concentration of the control diet was 25.18 mg/kg DM. In the other treatments, the diet was supplemented with 20, 40, or 60 mg of Zn as ZnO or 20, 40, or 60 mg of Zn as nano-ZnO (>99%, 10–30 nm; US Research Nanomaterials, Inc., Houston, TX, USA) per kg DM, i.e. 7 treatments. The chemical composition of the feedstuffs used in diets is shown in Table 1. Moreover, ingredients and chemical compositions of the experimental diets are shown in Tables 2 and 3, respectively. Ash, CP, ether extract, and neutral detergent fiber of diets were measured according to the standard methods (Nos. 924.05, 988.05, 920.3, and 2002.04, respectively) of the AOAC [25]. Determinations of Zn and Ca were carried out using an atomic absorption spectrophotometer (AA-6200, Shimadzu, Japan) and P was measured by a spectrophotometric method [25].

### 2.2. In vitro 24-h gas production (GP) experiment

To assess the effect of treatments on in vitro ruminal 24-h GP and fermentation parameters, an in vitro 24-h GP experiment was conducted based on the method of Menke et al. [26]. The rumen liquid was obtained via rumen fistula from 3 adult sheep of 2 years old (body weight of 59.1 ± 1.8 kg), 30 min before the morning feeding (at 06:30). The uniform rumen fluid sample was achieved from both the liquid and fiber phases. The Guide for the Care and Use of Agricultural Animals in Research and Teaching [27] was followed in this study and all protocols were approved (No. 9530381005; Date: 28.12.2017) by the Animal Science Group of Tarbiat Modares University (Tehran, Iran). A mixed diet containing alfalfa, soybean meal, barley grain, corn grain, salt, mineral premix, and vitamin premix (at a ratio of 60:5:18.5:15:0.5:0.5:0.5 on DM basis) was offered to the animals. The rumen liquid was sucked through three layers of cheese cloth into a warm flask (39 °C) filled with $CO_2$.

The experimental diets (200 mg) were incubated in 100-mL glass syringes with buffered rumen fluid (30 mL; containing 1 volume of strained rumen fluid and 2 volumes of anaerobic minerals buffer). Seven diets were incubated at 39 °C for a period of 24 h in 3 replicates with 2 samples for each replicate, 2 different syringes per sample, and 2 separate runs in various weeks, as well as 3 syringes without diet (blank) at each run [28]. Moreover, standard hay (i.e. good quality alfalfa) was used, in triplicates, to control the quality of the rumen liquid [26].

#### 2.2.1. In vitro GP and estimated parameters

The 24-h GP was measured and the amounts of OM digestibility (OMD) and ME were predicted via the following equations [26]:

$OMD\ (\%) = 14.88 + (0.889 \times GP) + (0.45 \times CP) + (0.651 \times CA)$,

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

**Table 1.** Chemical composition (g/100 g DM or as stated) of the feedstuffs used to formulate the diets.

| Item | Alfalfa hay | Barley grain | Corn grain | Soybean meal |
|---|---|---|---|---|
| Crude protein | 14.04 | 10.17 | 9.11 | 43.35 |
| NDF | 43.61 | 23.13 | 14.39 | 14.22 |
| Ether extract | 2.53 | 1.80 | 4.01 | 2.20 |
| Ash | 8.79 | 3.11 | 2.88 | 6.21 |
| Ca | 1.30 | 0.15 | 0.20 | 0.55 |
| P | 0.22 | 0.37 | 0.30 | 0.62 |
| Zn (mg/kg DM) | 13.57 | 36.62 | 40.14 | 48.20 |
| ME (MJ/kg DM) | 8.53 | 12.68 | 13.23 | 13.86 |

NDF: Neutral detergent fiber, ME: metabolizable energy.

**Table 2.** Feed ingredients (g/100 g DM) of the diets containing different Zn sources.

| Zn source | Control | ZnO | | | Nano-ZnO | | |
|---|---|---|---|---|---|---|---|
| Added Zn | 0 | 20 | 40 | 60 | 20 | 40 | 60 |
| Alfalfa (14 %CP) | 45.0 | 45.0 | 45.0 | 45.0 | 45.0 | 45.0 | 45.0 |
| Barley grain | 23.0 | 23.0 | 23.0 | 23.0 | 23.0 | 23.0 | 23.0 |
| Corn grain | 25.75 | 25.75 | 25.75 | 25.75 | 25.75 | 25.75 | 25.75 |
| Soybean meal (44 %CP) | 5.0 | 5.0 | 5.0 | 5.0 | 5.0 | 5.0 | 5.0 |
| NaCl | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 |
| *Mineral premix | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| †Vitamin premix | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |

* Mineral premix contained (per kg): 120 g of Ca, 30 g of P, 60 g of Mg, 4 mg of Se, 40 mg of Co, 70 mg of Mn, 100 mg of I, and 30 mg of Cu. † Mineral premix contained (per kg): 500,000 IU of vitamin A, 100,000 IU of vitamin D3, and 8,000 IU of vitamin E.

**Table 3.** Chemical composition (g/100 g DM or as stated) of the diets containing different Zn sources.

| Zn source | Control | ZnO | | | Nano-ZnO | | |
|---|---|---|---|---|---|---|---|
| Added Zn | 0 | 20 | 40 | 60 | 20 | 40 | 60 |
| Crude protein | 13.05 | 12.98 | 13.10 | 13.09 | 13.10 | 13.07 | 13.04 |
| NDF | 30.61 | 31.00 | 30.73 | 30.59 | 31.00 | 30.85 | 30.60 |
| Ether extract | 2.69 | 2.71 | 2.67 | 2.72 | 2.68 | 2.70 | 2.63 |
| Ash | 6.59 | 6.62 | 6.57 | 6.53 | 6.69 | 6.56 | 6.71 |
| Ca | 0.692 | 0.698 | 0.694 | 0.694 | 0.700 | 0.695 | 0.691 |
| P | 0.293 | 0.285 | 0.289 | 0.290 | 0.286 | 0.291 | 0.286 |
| *Zn (mg/kg DM) | 25.18 | 43.92 | 63.19 | 84.50 | 44.11 | 63.71 | 85.03 |
| ME (MJ/kg DM) | 10.86 | 10.86 | 10.86 | 10.86 | 10.86 | 10.86 | 10.86 |

NDF: Neutral detergent fiber, ME: metabolizable energy.
* In the Zn-supplemented treatments, 20, 40, or 60 mg of Zn as ZnO or 20, 40, or 60 mg of Zn as nano-ZnO was included per kg of diet DM. The chemical composition of all the diets was determined using the standard methods of the AOAC, except ME, which was calculated from each feed's ME content.

In the above equations, OMD is OM digestibility, GP is 24-h net gas produced (mL/200 mg diet DM), CP is crude protein (%), CA is ash (%), ME is metabolizable energy, and EE is ether extract (%).

To calculate the TDS, the gas volume of 12 syringes in each treatment (3 replicates × 2 samples × 2 runs) was recorded after 24 h of incubation. Then the entire contents of each syringe were centrifuged (at 20,000 × *g* for 30 min at 4 °C) and the supernatant was removed as described by Blümmel et al. [29]. The pellet contained in the centrifuge tubes was transferred into 500-mL beakers and was boiled with neutral detergent solution (NDS) for 1 h. Thereafter, No. 2 filter crucibles [30] and hot distilled water were used to separate and wash the undissolved matters, which were dried at 60 °C to reach a constant weight, and the amount of TDS (g/kg DM) was estimated by subtracting this value (i.e. the residue after NDS treatment) from the initial weight of the incubated diet (200 mg) [29]. The MBP for each treatment was calculated as MBP (mg/g sample DM) = TDS – (mL GP × 2.2). Additionally, the 24-h partitioning factor (PF; displaying fermentation effectiveness) for each diet was obtained as TDS (mg)/GP (mL) [29].

**2.2.2. In vitro methane production**

The methane produced via fermentation of diets containing different supplementary Zn sources was measured in a separate in vitro gas test according to Fievez et al. [31].

After measuring the 24-h GP, 4 mL of NaOH solution (10 M) was injected into the syringe to absorb the produced $CO_2$. The residual gas in the syringe was $CH_4$. The methane volume was obtained from the shift in the plunger position as a result of the $CO_2$ absorption by NaOH.

**2.2.3. TAC**

The effect of the Zn supplementation on in vitro ruminal TAC was determined using the ferric reducing antioxidant power (FRAP) test as recommended by Benzie and Strain [32]. This method was established based on the reduction of ferric-tripyridyltriazine (TPTZ) to the ferrous form by the antioxidants, which leads to a blue color detected at 593 nm. Ferrous sulfate solution was used as the standard and the results were calculated as μmol $Fe^{2+}$ formed per L of rumen liquor.

**2.2.4. pH, VFA, ammonia, and protozoa**

These parameters were evaluated in the fermentative contents of 12 syringes per diet (3 replicates × 2 samples × 2 runs). The pH was detected using a Sartorius pH meter (Sartorius AG, Germany). The VFA concentrations were determined using the UNICAM 4600 gas chromatograph (SB Analytical, Cambridge, UK) with a capillary column (19095F-121; Agilent Technologies, Santa Clara, CA, USA) by the method of Galyean [33]. Ammonia-N was quantified by the phenol-hypochlorite method [33]. Finally, one volume of the syringe contents was mixed with one volume of 50% formalin. Thereafter, the protozoa numbers were enumerated using a hemocytometer (Neubauer Improved, Marienfeld, Germany) and a light microscope [34].

**2.3. Kinetics of GP**

In separate runs, the effect of treatments on GP kinetics was assessed using the 120-h in vitro GP experiment. The experiment was conducted based on the method of Menke et al. [26] (as described above) and the produced gas was recorded at 2, 4, 6, 8, 12, 24, 48, 72, 96, and 120 h of incubation. The kinetic variables were predicted as $y = B\ (1 - e^{-ct})$ [35]. In this exponential model, $y$, $B$, and $c$ are the gas volume detected at time $t$, asymptotic value of produced gas (mL/200 mg diet DM), and first-order fractional rate constant of produced gas (/h), respectively.

**2.4. Statistical analysis**

In this study, the data were analyzed by PROC GLM of SAS (Version 9.1, SAS Institute, Cary, NC, USA) [36] using a split-plot in a completely randomized design (7 treatments × 3 replicates × 2 samples × 2 runs). The treatment was considered as main plot and the run as subplot. The experimental unit was the syringe and the treatment effect was considered fixed. The model was $Y_{ijkl} = \mu + T_i + e_{ij} + R_k + (TR)_{ik} + e_{ijk} + e_{ijkl}$. In this model, $Y_{ijkl}$, $\mu$, $T_i$, $e_{ij}$, $R_j$, $(TR)_{ik}$, $e_{ijk}$, and $e_{ijkl}$ are the general observation, overall mean, treatment effect, treatment × replicate, run effect, treatment × run, error of split-plot, and error of sampling. The comparisons among the treatments were conducted by Duncan's multiple range test. The statistical significance of the means was defined by $P \leq 0.05$ and a trend was declared if $0.05 < P \leq 0.10$.

## 3. Results

**3.1. Methane release, TAC, and MBP**

Dietary supplementation with the different Zn sources decreased the in vitro methane production ($P < 0.05$), so the lowest methane release was observed for the diets containing the supplemental nano-ZnO (Table 4). Similar to the ZnO treatments, the in vitro ruminal TAC was improved by inclusion of nano-ZnO in the diet ($P < 0.05$). As compared with the control treatment, adding both supplemental Zn sources increased the amounts of MBP ($P < 0.05$) and MBP was maximum for the nano-ZnO treatments. However, MBP efficiency (EMBP) was not

**Table 4.** Effect of different levels of supplementary Zn (mg/kg diet DM) as nano-ZnO, compared with ZnO, on in vitro methane release, total antioxidant capacity (TAC), microbial biomass production (MBP), and MBP efficiency (EMBP).

| Zn source | Control | ZnO | | | Nano-ZnO | | | SEM | P-value | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Added Zn | 0 | 20 | 40 | 60 | 20 | 40 | 60 | | T | R | T × R |
| *$CH_4$ | 16.15$^{a}$ | 14.80$^{ab}$ | 14.65$^{ab}$ | 13.75$^{ab}$ | 12.73$^{b}$ | 11.49$^{b}$ | 11.86$^{b}$ | 0.718 | 0.025 | 0.525 | 0.894 |
| †$CH_4$ | 35.43$^{a}$ | 33.70$^{ab}$ | 33.62$^{ab}$ | 31.91$^{abc}$ | 29.17$^{bc}$ | 26.41$^{c}$ | 27.75$^{bc}$ | 1.712 | 0.034 | 0.500 | 0.978 |
| ‡$CH_4$ | 16.15$^{a}$ | 14.80$^{ab}$ | 14.65$^{ab}$ | 13.75$^{ab}$ | 12.73$^{b}$ | 11.49$^{b}$ | 11.86$^{b}$ | 0.718 | 0.025 | 0.645 | 0.905 |
| §TAC | 848$^{b}$ | 1231$^{a}$ | 1134$^{a}$ | 1212$^{a}$ | 1115$^{a}$ | 998$^{ab}$ | 1031$^{ab}$ | 61.30 | 0.031 | 0.423 | 0.896 |
| £MBP | 255$^{c}$ | 268$^{bc}$ | 272$^{bc}$ | 268$^{bc}$ | 310$^{a}$ | 283$^{ab}$ | 286$^{ab}$ | 7.117 | 0.023 | 0.769 | 0.957 |
| ¶EMBP | 346 | 348 | 354 | 343 | 379 | 357 | 356 | 10.30 | 0.363 | 0.878 | 0.962 |

SEM: Standard error of the mean, T: treatment, R: run, *$CH_4$: % of total gas, †$CH_4$: mL/g incubated DM, ‡$CH_4$: mL/g degraded substrate, §TAC: μmol $Fe^{2+}$/L, £MBP: mg/g incubated DM, ¶EMBP: mg/g degraded substrate, $^{a-c}$ means in the same row with different superscripts differ ($P \leq 0.05$).

affected by the different Zn supplementations (P > 0.05). The run and the interaction between treatment and run did not differ for methane release, TAC, MBP, and EMBP (P > 0.05).

### 3.2. Gas volume and estimated parameters

Comparable with the ZnO treatments (Table 5), the GP, OMD, ME, and TDS of the diets containing the supplemental nano-ZnO were greater than those of the control diet (P < 0.05). Addition of the supplementary Zn sources to the diet failed to change the PF (P > 0.05). Dietary supplementation with nano-ZnO, similar to ZnO, increased *B* (P < 0.05), but had no significant effect on *c* (P > 0.05). There were not significant effects of run or treatment and run interaction on the GP and estimated parameters (P > 0.05).

### 3.3. pH, ammonia-N, VFA, and protozoa

As shown in Table 6, supplementation of the diet with nano-ZnO, like ZnO, had no effect on the in vitro ruminal

**Table 5.** Effect of different levels of supplementary Zn (mg/kg diet DM) as nano-ZnO, compared with ZnO, on in vitro gas production (GP) and estimated parameters (24-h incubation) as well as kinetics of GP (120-h incubation).

| Zn source | Control | ZnO | | | Nano-ZnO | | | SEM | P-value | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Added Zn | | 20 | 40 | 60 | 20 | 40 | 60 | | T | R | T × R |
| GP | $43.54^{b}$ | $45.59^{a}$ | $45.27^{ab}$ | $46.42^{a}$ | $45.96^{a}$ | $46.12^{a}$ | $46.92^{a}$ | 0.48 | 0.041 | 0.960 | 0.175 |
| OMD | $648^{b}$ | $666^{ab}$ | $664^{ab}$ | $674^{a}$ | $670^{a}$ | $671^{a}$ | $678^{a}$ | 3.26 | 0.024 | 0.947 | 0.488 |
| ME | $9.62^{b}$ | $9.90^{ab}$ | $9.86^{ab}$ | $10.01^{a}$ | $9.95^{a}$ | $9.97^{a}$ | $10.08^{a}$ | 0.076 | 0.047 | 0.941 | 0.531 |
| TDS | $734^{d}$ | $769^{c}$ | $770^{c}$ | $778^{bc}$ | $815^{a}$ | $790^{abc}$ | $802^{ab}$ | 7.33 | 0.018 | 0.786 | 0.820 |
| PF | 3.37 | 3.38 | 3.41 | 3.37 | 3.55 | 3.43 | 3.42 | 0.058 | 0.754 | 0.850 | 0.242 |
| *B* | $63.57^{c}$ | $66.13^{bc}$ | $66.76^{abc}$ | $67.04^{ab}$ | $69.68^{a}$ | $66.28^{bc}$ | $68.69^{ab}$ | 1.01 | 0.025 | 0.349 | 0.907 |
| *c* | 0.037 | 0.038 | 0.038 | 0.041 | 0.037 | 0.041 | 0.039 | 0.002 | 0.468 | 0.506 | 0.824 |

SEM: Standard error of the mean, T: treatment, R: run, GP: gas production (mL/200 mg DM), OMD: organic matter digestibility (g/kg), ME: metabolizable energy (MJ/kg DM), TDS: truly degraded substrate (g/kg DM), PF: partitioning factor (mg TDS/mL GP), *B*: the asymptotic value of gas production (mL/200 mg DM), *c*: the first-order fractional rate constant of gas production (/h), $^{a–c}$ means in the same row with different superscripts differ (P ≤ 0.05).

**Table 6.** Effect of different levels of supplementary Zn (mg/kg diet DM) as nano-ZnO, compared with ZnO, on in vitro ruminal pH, ammonia-N (mg/dL), total volatile fatty acids (TVFA; mmol/L), and individual VFA (mol/100 mol).

| Zn source | Control | ZnO | | | Nano-ZnO | | | SEM | P-value | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Added Zn | 0 | 20 | 40 | 60 | 20 | 40 | 60 | | T | R | T × R |
| pH | 6.46 | 6.42 | 6.47 | 6.45 | 6.51 | 6.51 | 6.46 | 0.032 | 0.402 | 0.943 | 0.940 |
| Ammonia-N | 14.15 | 17.56 | 14.99 | 16.46 | 14.75 | 16.32 | 14.08 | 1.280 | 0.234 | 0.910 | 0.812 |
| TVFA | 55.87 | 60.95 | 61.75 | 60.96 | 60.69 | 59.99 | 60.11 | 1.776 | 0.091 | 0.957 | 0.938 |
| Acetate (A) | 64.000 | 61.118 | 62.061 | 60.828 | 60.630 | 60.033 | 61.010 | 1.814 | 0.444 | 0.831 | 0.805 |
| Propionate (P) | 22.747 | 25.464 | 24.627 | 25.220 | 25.180 | 25.018 | 25.445 | 0.932 | 0.256 | 0.739 | 0.741 |
| Butyrate | 11.113 | 10.911 | 11.273 | 11.570 | 11.846 | 12.071 | 11.000 | 0.610 | 0.458 | 0.986 | 0.970 |
| Iso-butyrate | 1.820 | 2.162 | 1.740 | 2.012 | 2.204 | 2.278 | 2.105 | 0.167 | 0.241 | 0.945 | 0.654 |
| Valerate | 0.176 | 0.196 | 0.178 | 0.202 | 0.192 | 0.201 | 0.182 | 0.271 | 0.983 | 0.753 | 0.899 |
| Iso-valerate | 0.143 | 0.159 | 0.131 | 0.160 | 0.166 | 0.171 | 0.167 | 0.013 | 0.221 | 0.901 | 0.884 |
| A:P | $2.81^{a}$ | $2.40^{b}$ | $2.52^{ab}$ | $2.41^{b}$ | $2.41^{b}$ | $2.40^{b}$ | $2.40^{b}$ | 0.095 | 0.044 | 0.892 | 0.883 |

SEM: Standard error of the mean, T: treatment, R: run, $^{a–b}$ means in the same row with different superscripts differ (P ≤ 0.05).

pH and ammonia-N concentration (P > 0.05). Compared with the control diet, the acetate to propionate ratio was lower (P < 0.05) and the total VFA concentration tended to be higher for the diets containing different supplemental Zn sources. The numbers of total protozoa, Isotrichidae, and Entodiniinae were negatively affected by dietary supplementations of ZnO and nano-ZnO (P < 0.05) and the lowest values were observed for the nano-ZnO treatments (Table 7). The subfamilies Diplodiniinae and Ophryoscolecinae were not significantly changed by dietary inclusion of the nano and inorganic Zn sources (P > 0.05). No significant effects of run or interaction between treatment and run were observed on the in vitro ruminal pH, ammonia-N, VFA, and protozoa (P > 0.05).

## 4. Discussion

### 4.1. Methane release, TAC, and MBP

The declining effect of the Zn supplements on methane production in this study could partly be associated with the lower protozoa enumeration and acetate to propionate ratio [37] for the Zn-containing diets, especially the nano-ZnO source. Methane generation can be decreased by redirecting hydrogen flow towards other electron acceptors such as propionate [38]. The decreased methane could also be due to the adverse effect of Zn supplementation on the methanogenic bacteria population [39]; in particular, the inhibitory action of nano-ZnO on methanogens has been emphasized [1,7]. It was reported that nanoparticles inhibit methane emission by reducing the population of Archaea and suppressing acetate kinase and coenzyme F420 [14]. Furthermore, methanogens are located on the external surfaces of the protozoa as symbiotics [38] and, in the present study, adhesion of methanogenic bacteria to protozoa may be reduced by the supplementary Zn, as a divalent cation, via limitation of available attachment sites on the bacterial cells [40]. Finally, as stated by Sarker et al. [2], methanogenic bacteria can convert up to 70% of acetate to methane; hence, the lower acetate proportion in the ZnO and nano-ZnO groups may be a reason for the lower methane production.

Comparable with the present study, Sarker et al. [1] showed that a diet containing nano-ZnO resulted in lower in vitro methane release compared with a diet free of supplementary Zn. Also, the reduction effect of nanoparticles (e.g., ZnO) on methane production from anaerobic codigestion of primary and excess sludge was indicated by Adegbeye et al. [14].

Although Sharma et al. [41] reported oxidative stress due to the effect of nano-ZnO, the present study showed that the Zn nanoparticles did not adversely affect the ruminal TAC. The nano-ZnO supplementation actually improved the antioxidant power, because Zn is a strong antioxidant metal decreasing the free radicals [6]. It has also been reported that nano-ZnO can increase antioxidant activity and decrease free radicals due to the increased specific surface area and thus the higher number of active sites [42,43]. Similarly, in the study conducted by Mohamed et al. [44], Zn nanoparticles improved the antioxidant capacity in sheep.

The higher MBP for the diets containing Zn supplements could be related to the better adhesion of the ruminal microbes to the feed, which improves the colonization and activity of microbial populations [45,46]. The improved MBP could also be due to the lower energy loss as methane, which may lead to better synchrony between energy and nitrogen sources. The promoting effect of metal nanoparticles (as prebiotics and probiotics) on the growth of beneficial bacteria in digesta was suggested by Adegbeye et al. [14]. Bąkowski et al. [42] also mentioned increased ruminal microorganisms as an effect of Zn nanoparticles.

**Table 7.** Effect of different levels of supplementary Zn (mg/kg diet DM) as nano-ZnO, compared with ZnO, on in vitro ruminal protozoa enumeration ($\times 10^5$/mL digesta).

| Zn source | Control | ZnO | | | Nano-ZnO | | | SEM | P value | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Added Zn | 0 | 20 | 40 | 60 | 20 | 40 | 60 | | T | R | T × R |
| Total protozoa | $15.82^{a}$ | $13.82^{b}$ | $14.01^{b}$ | $13.76^{b}$ | $13.39^{b}$ | $13.30^{b}$ | $13.41^{b}$ | 0.411 | 0.034 | 0.852 | 0.944 |
| Isotrichidae | $2.98^{a}$ | $2.47^{ab}$ | $2.39^{ab}$ | $2.21^{b}$ | $2.50^{ab}$ | $2.40^{ab}$ | $2.30^{ab}$ | 0.215 | 0.045 | 0.920 | 0.700 |
| Ophryoscolecidae | | | | | | | | | | | |
| Entodiniinae | $8.97^{a}$ | $7.91^{ab}$ | $7.75^{b}$ | $7.81^{ab}$ | $7.37^{b}$ | $7.50^{b}$ | $7.33^{b}$ | 0.305 | 0.039 | 0.769 | 0.815 |
| Diplodiniinae | 3.01 | 2.49 | 2.81 | 2.86 | 2.43 | 2.42 | 2.73 | 0.279 | 0.460 | 0.960 | 0.839 |
| Ophryoscolecinae | 0.963 | 0.953 | 1.06 | 0.880 | 0.991 | 0.982 | 0.953 | 0.071 | 0.354 | 0.945 | 0.895 |

SEM: Standard error of the mean, T: treatment, R: run, $^{a–b}$ means in the same row with different superscripts differ (P ≤ 0.05).

**4.2. Gas volume and estimated parameters**

Unlike studies that focused on the toxic effects of Zn nanoparticles on animals [17–19], nano-ZnO in the current experiment had no adverse effect on the dietary energy availability. Similar to ZnO, the increasing effect of supplementary nano-ZnO on the in vitro GP, OMD, ME, and TDS could be caused by the positive influence of Zn on the ruminal microbial growth and the role of Zn as a bivalent cation in the better attachment between the microbes and feed particles [46]. Moreover, it is mentioned that nanoparticles demonstrate nanocatalyst activity and supplementing Zn nanoparticles can increase the activity of some digestive enzymes (protease, amylase, and lipase), resulting in higher diet digestibility [14]. The positive effect observed on the diet fermentation indicated that the Zn requirements of the ruminal microbes were better met by dietary supplementation of the Zn sources, especially nano-ZnO. In the study conducted by Sarker et al. [1], dietary inclusion of nano-ZnO had no positive or negative effects on in vitro ruminal GP. Adegbeye et al. [14], however, reported higher diet digestibility with dietary supplementation with nanoparticles. On the other hand, the effect of nano-ZnO in improvement of the TDS was slightly higher than that of ZnO, probably due to the greater surface activity and stronger absorbing capability of the former, encouraging more growth of fiber-degrading microorganisms [47]. The improving effect of other bivalent cation nanoparticles (such as Ca) on the cellulase activity, and thereby higher fiber degradation, was observed by Yousef et al. [48]. They related it to ionic strength, which increases the bacterial adhesion to the substrate.

Similar to the present study, the diet digestibility was affected positively by Zn nanoparticles in in vivo [44] and in vitro [47] studies. However, other researchers reported that feeding nano-ZnO instead of ZnO failed to change the digestibility of diets in piglets [9].

In the present study, the PF values for all treatments were within the usual physiological range (2.7 to 4.4) reported in common nutritional conditions [29]. The ineffectiveness of the experimental treatments on PF was due to parallel alterations in the amounts of TDS and GP. Also, a feedstuff with greater PF means that a higher proportion of the TDS is incorporated into the microbial biomass, i.e. the EMBP is higher [29,30]. No effect of Zn supplementations on PF in the present study was consistent with the similar EMBP in different treatments.

**4.3. pH, ammonia-N, VFA, and protozoa**

The in vitro ruminal pH (6.42 to 6.51) of the treatments was within the typical range (5.5 to 6.8) in a normal ruminal fermentation circumstance [3]. Ruminal pH being unaffected by the dietary nano-ZnO supplementation was similar to the in vitro report of Sarker et al. [1].

The ammonia-N levels in all treatments (14.08 to 17.56 mg/dL) were within the normal biological range (8.5 to 30 mg/dL) [49]. The similar concentration of ammonia-N among the treatments may show that the proteolysis and deamination of amino acids were accompanied by more assimilation of ammonia in microbial biomass, as reflected in the higher MBP. Moreover, it may be an indication of the same activity of the ammonia-producing bacteria [3]. However, Adegbeye et al. [14] mentioned lower ruminal ammonia-N concentrations by dietary nanomaterial supplementation in sheep. Arelovich et al. [50] also reported a reduction of ruminal ammonia by addition of Zn, probably due to the declined proteolysis or the better utilization of ammonia by ruminal microbes.

These discrepancies among the studies may be related to factors such as the type of basal diets, the level of Zn (deficiency or adequacy) in basal diets, the purity of Zn sources, and the content or availability of other minerals [13,51]. For example, the dissimilar action of dietary Zn supplementation may be due to different interferences from various concentrations of other minerals (e.g., Ca, Fe, Cu, and P) in different studies. Moreover, in a Zn supplement of higher purity, there are fewer interfering factors for Zn action [51]. On the other hand, both rumen bacteria and feed particles have negative surface charges; thus, the optimum concentration of cations, such as Zn, creates an attraction between the microbe and feed surface [52]. However, high Zn levels (in the form of heavy metal salts) in some studies may denature and inactivate soluble proteins including feed-degrading enzymes or may limit available attachment sites on the bacterial cell [40]. Also, it was noted that at a sufficient dietary level of Zn, the supplementary Zn bioavailability may be less important than the circumstances of limited dietary levels of Zn [53]. It seems that in our research, the concentration of Zn in the control diet was not sufficient for proper microbial activity and optimal production of microbial biomass, but the Zn supplementations improved these parameters.

The slightly higher in vitro ruminal VFA for the diets including Zn supplements could be related to the higher TDS [3] of these diets compared with the control. The improved TDS and hence the higher VFA production are indications of the positive effect of the nanoparticles on the activity of the microbial population [14]. Another probable reason could be the higher energy utilization by the rumen microorganisms, for producing higher microbial biomass, causing more total VFA production in ZnO- and nano-ZnO-supplemented groups, as mentioned by Sarker et al. [1]. On the other hand, the lower acetate to propionate ratio of the nano-ZnO groups, similar to ZnO, was in parallel with their lower methane production as compared with the control diet, due to redirecting the hydrogen flow towards propionate [38]. In another study,

Adegbeye et al. [14] mentioned that dietary nanoparticle supplementation improved the diet fermentation and increased the propionate concentration. Contrary to the present study, Sarker et al. [1] reported a decreased in vitro total VFA concentration by inclusion of nano-ZnO in the diet compared with the control treatment. They noted that high levels of nano-ZnO may sometimes kill higher amounts of methanogenic bacteria, which results in a greater amount of unconverted total VFA [1]. Swain et al. [54], however, showed that feeding goats with different levels of nano-ZnO instead of ZnO failed to change individual and total rumen VFA.

In the present study, the lowest protozoa enumeration was observed for the nano-ZnO-supplemented groups, which was in parallel with the results on methane release, so that the lowest methane was detected for the nano-ZnO treatments. The decreased in vitro ruminal protozoa count with the Zn supplementations may possibly be due to the physiological changes in the cell membrane integrity, endocytosis rate, cell proliferation, grazing capacity, and metabolic activity [55]. Antiprotozoal activity of other nanoparticles (e.g., silver) was suggested by Bąkowski et al. [42]. Contrary to the present work, no effect of Zn sources on protozoa enumeration was reported by Kumar [47].

In conclusion, dietary Zn supplementation with nano-ZnO had no adverse effect on in vitro ruminal fermentation and digestibility. Addition of 20 mg of Zn supplement as nano-ZnO per kg diet DM, similar to ZnO, was sufficient to improve the in vitro ruminal fermentation in terms of the amounts of methane release, TAC, and MBP. Thus, higher supplementary Zn levels (i.e. 40 and 60 mg/kg DM) are not recommended.

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