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Response of cattle manure anaerobic digestion to zinc oxide nanoparticles: methane production, microbial community, and functions

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Abstract

The increasing use of zinc oxide nanoparticles (ZnO NPs) as feed additives has raised huge environmental concerns in the anaerobic digestion (AD) of livestock wastes. In this study the 30-day effect of ZnO NPs on AD performance of cattle manure was investigated under optimal conditions (temperature=55℃, initial pH=10, total solids contents=10%), which were obtained from response surface methodology. Results showed that ZnO NPs (5-100 mg/g TS) promoted the accumulation of SCOD, SP and SC. Removal of SP and SC or production of VFAs were not significantly decreased in the presence of 5 mg ZnO NPs/g TS, but they were negatively affected as ZnO NPs increased to 30 and 100 mg/g TS. Besides, ZnO NPs had negative effects on VFAs consumption, which led to decreased methane production with 84.55% , 92.39% , and 93.72% in the presence of 5, 30 and 100 mg ZnO NPs /g TS, respectively. The shift of microbial community demonstrated that the decrease in the abundance of functional bacteria from 72.11% to 11.24% (in families *Ruminococcaceae* and *Lachnospiraceae*), () and of methanogens from 96.82% to <1% (in genus *Methanothermobacter)* () led to poor fermentation and methanogenesis, respectively. Functional analysis indicated that ZnO NPs can enhance abundances of genes related to AD like transport and metabolism of amino acid and carbohydrate, and energy conversion. The actual genetic expression, DNA integrity, cellular membrane, and intercellular communication may contribute to the low methane yield. This study provides new insights into livestock manure reduction and reutilization in the presence of exogenous pollutants.

Keywords

Anaerobic digestion, functional analysis, livestock wastes, microbial community, zinc oxide

nanoparticles

1 Introduction

The rapidly developing intensive livestock industry in China has led to a series of environmental issues due to the occurrence of persistent pollutants (initially used as animal food additives) in livestock waste. The Chinese Government reported that the amount of chemical oxygen demand (COD) discharged from the livestock industry was increased from 41.87% in 2007 to 46.67% in 2017（Ministry of Ecological Environment of the PRC et al., 2010; Ministry of Ecological Environment of the PRC et al., 2020). Chinese production of livestock and poultry manure reached 1.64×10^{12} kg (fresh weight) in 2017 (Liu et al., 2020). , which can cause substantial environmental and public health problemsdue to its content of heavy metals (Liu et al., 2020), antibiotics (Gaballah et al., 2021), excess nitrogen and phosphorus (Li et al., 2020; Sakadevan & Nguyen, 2017), if the manure is not treated properly.

Biogas can be generated from livestock manure with high organic matter mainly through anaerobic digestion (AD) process. The potential energy generated from manure-produced biogas in China was about $5.74\text{-}6.73\times10^{12}$ MJ in 2017, which is equivalent to 4-5% of the country's energy demands (Wang et al., 2021b). Also, a high methane potential of livestock manure was estimated for Europe at 26 million m^3 biomethane/year(Scarlat et al., 2018). AD has been considered as one of the most efficient technologies for biogas generation AD performance can be affected by many factors, including the co-existence of exogenous substances that are widely used as animal food additives (Luo et al., 2020).

Zinc oxide nanoparticles (ZnO NPs) have been widely used in many fields, such as cosmetic, medication, textile and automotive, and finally led to their ubiquitous occurrence in the environment (Jin & Jin, 2019). ZnO NPs with key advantages, such as the better antibacterial and bioavailability properties, compared with conventional ZnO, were recently used as feed supplement in livestock industry (Fawzy et al., 2021). This has resulted in the presence of ZnO NPs in livestock manure with Zn concentrations ranging from 39.5 to 11379.0 mg/kg in livestock manure (Hui Wang, 2013; Liu et al., 2020). Improper application or management of ZnO NPs can even deduce an extreme case which we must take into consideration.

On the one hand, addition of zinc ions (Zn^{2+}) at 50-100 mg/L in an anaerobic co-digestion system was found to enhance COD removal rate and methanation.(Chan et al., 2019). On the other hand, it has been pointed out that ZnO NPs could significantly affect AD process by inhibiting the activity of methanogenesis due to the enhanced volatile fatty acids (VFAs) accumulation (Zhang et al., 2017b). Also, Zheng et al. investigated the short- and long- term effects of ZnO NPs concentrations (6, 30, and 150 mg/g TSS.) on AD performance, showing that inhibition of methanogenesis process could be observed even within short-term exposure, and would not mitigate over time (Zheng et al., 2015). Furthermore, the abundance of methanogenic archaea and enzyme activity could also be negatively influenced by the presence of ZnO NPs (Mu & Chen, 2011). The activities of protease, cellulase, acetated kinase, and coenzyme F-420 were adversely affected by ZnO NPs (30-150 mg/g VSS) in waste activated sludge AD system (Wang et al., 2021a). The toxic effects induced by ZnO NPs can be attributed not only to the released Zn^{2+}, which negatively impacts the substances transfer and enzyme system (Mu & Chen, 2011), but also to the intracellular reactive oxygen species (ROS) that are toxic to cytoplasmic lipids, proteins, and other intracellular intermediates (Sharma et al., 2009). Last but not least, other pollutants (e.g. norfloxacin, sulfamethazine, etc.) co-existing with ZnO

NPs in livestock manure could yield a greater impact on methane production than ZnO NPs alone (Zhao et al., 2019).

Existing literature is focusing on the effects of ZnO NPs on municipal sludge AD performance and to a lesser extent on livestock wastes. The influence of Zn on swine manure AD has been previously studied but with a focus on multi-pollutant effects and antibiotics resistance genes (Yang et al., 2020; Zhang et al., 2018; Zhang et al., 2017a). Studies illustrating the mechanisms of the effect of ZnO NPs on livestock manure are still missing elements in literature. Taking into account that municipal sludge differs a lot in terms of physical, chemical and biological characteristics from livestock wastes, as well as the extensive use of ZnO NPs in livestock industry, it is of particular importance to understand how ZnO NPs can influence livestock manure AD process.

AD is driven by microorganisms and highly depends on microbial structure and activity (Sasaki et al., 2011). Previous studies revealed that the shift of microbial community would be enhanced by nano-additions, and inhibition on methanogenesis contributed a lot to decreased methane production (Luo et al., 2020). However, another study found that occurrence of nano-materials could influence methane production but had no significant impact on microbial community structure during AD processes (Zhang et al., 2019). Owing to the development of proteomics and metabolomics technologies, the Clusters of Orthologous Groups of proteins (COGs) database provides a great tool of exploring underlying differences among functional proteins (Wang et al., 2019). Therefore, a comprehensive investigation of the effects of ZnO NPs on manure AD taking into consideration microbial communities as well as their functions can be carried out.

The influence of ZnO NPs on manure AD process was comprehensively investigated in this study. First, the optimal operational conditions were determined by response surface methodology (RSM) based on Box-Behnken design (BBD). Secondly, the effect of various concentrations of ZnO NPs on manure AD performance was evaluated. Also, the shift of microbial community was detected by means of high-throughput sequencing. To deeply reveal the biochemical mechanism of ZnO NPs on manure AD process, analysis of functional proteins was carried out.

2 Materials and Methods

2.1 Nanoparticles and livestock wastes

Cattle manure was collected from a manure tank in a farm in Sichuan Province, China, and then stored at -20 ºC. The main characteristics of cattle manure were analyzed as follows: total solid (TS) of 27.38 ± 5.23% (mass ratio), volatile solids (VS) of 23.97 ± 4.56% (mass ratio), total organic carbon (TOC) of 352.72 ± 25.7 mg/g TS.

Dry ZnO NPs (99.8%, metal basis) was purchased from Aladdin Reagent Co. Ltd., China, and the particle size ranged from 80 nm to 100 nm as shown by scanning electron microscopy (Figure S2). ZnO NPs suspension (10 g/L) was prepared by magnetic stirring and ultrasonication (1 h, 25 ºC, 250 W, 40 kHz) according to the methods described elsewhere (Zhang et al., 2013).

2.2 Response surface methodology design

RSM was used to evaluate the effects of temperature (*A*), initial pH (*B*), and TS (*C*) on cattle manure AD performance. The range of those factors were set as follows: temperature of 30-55 ºC, initial pH of 6-10, and TS of 6-15%. BBD was used to optimize these three independent variables (each at three levels) and 15 experiments with the actual form of three independent variables were conducted (Table S1). Methane yield (Y) was set as the response and was measured by gas bags. The statistical design of experiments s and analyses were performed using Design Expert (Version 8.0.6, Stat-Waes, Inc USA).

2.3 Anaerobic digestion batch experiment

AD was carried out using Automatic Methane Potential Test System Ⅱ (Bioprocess Control, Sweden) equipped with serum bottles, under the optimal conditions obtained from RSM. Four reactors, marked as G0, G1, G2, and G3, were set up to deeply explore the effects of ZnO NPs concentrations (0, 5, 30 and 100 mg/g TS, respectively) on cattle manure AD performance. The working volume of each reactor was 400 ± 10 mL with TS of 6%. 3 mmol NaOH was used to adjust the initial pH. Anaerobic conditions in the reactors were achieved by infusing nitrogen gas at the beginning of the batch experiments, and stratification was avoided by stirring reactors twice a day. Each AD experiment was carried out for 30 days and samples were collected for physico-chemical and microbial analyses on the 1^{st}, 6^{th}, 14^{th}, 22^{nd}, and 30^{th} day of treatment. All AD batch experiments were run in parallel in triplicates (n=3). The schematic diagram of the experimental setup is illustrated in Figure S3.

2.4 High-throughput sequencing analysis

The effect of ZnO NPs on the shift of microbial community structure in cattle manure AD process was also investigated using Illumina Miseq sequencing analysis (labeled G0, G1, G2, and G3, respectively). All samples were centrifuged for 10 min at 8000 rpm and then the Fast DNA Spin Kit for Soil (MP bio, USA) was used to extract DNA. The extracted genomic DNA was detected by 1% agarose gel electrophoresis. The total DNA was subjected to PCR amplification using the primers 338F/806R and 524F10extF/Arch958RmodR. The primer sets of 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') were used for analyzing bacterial strains, while the primers set of 524F10extF (5'-TGYCAGCCGCCGCGGTAA-3') and Arch958RmodR (5'-YCCGGCGTTGAVTCCAATT-3') were used for archaeal analysis (Liu et al., 2016; Xu et al., 2016). The processes of constructing 16S rRNA library and sequencing were conducted on the Illumina MiSeq PE 300 by Majorbio Co. (Shanghai, China) with three biological replicates.

The operational taxonomic unit (OTU) representative sequence was selected and the species information ID was obtained by comparing the Greengene database with BLASTn tool. After that, the software PICRUSt (Version 1.0.0) was used for the functional prediction of 16S rRNA gene data (Langille et al., 2013). EggNOG (http://eggnog.embl.de/) databases were employed to construct the abundance of COGs of microorganisms in manure AD in the presence of ZnO NPs concentrations.

2.5 Chemical analyses

The collected samples were centrifuged for 10 min at 8000 rpm and one part of the supernatant liquid was immediately filtered through 0.45 μm membrane to measure its soluble chemical oxygen demand (SCOD), soluble protein (SP), soluble polysaccharides (SC) and ammonia nitrogen (NH_4^+-N) content. The other part of the supernatant was filtered through a 0.22 μm membrane for VFAs determination after being acidized (to pH < 3) with 10% phosphoric acid. TS, SCOD, and NH_4^+-N were measured according to the standard methods (APHA, 1995). SP and SC were analyzed by Lowry-Folin method and phenol-sulfuric method (Lowry et al., 1951; Dubois et al., 1951), respectively. VFAs were measured by high-performance liquid chromatography (HPLC) equipped with UV detector (at 450 nm), and chromatographic column (SCR-101H, Shimadzu) based on a method developed previously (Sun et al., 2011). The total VFAs (TVFAs) concentration was obtained by summing up the concentrations of three VFAs, namely acetic acid, propionic acid, and butyric acid.

3 Results and discussion

3.1 Optimal condition for livestock manure AD

RSM was applied to evaluate the individual and interactive effects of temperature (*A*), initial pH (*B*), and total solids (*C*) on methane yield in manure AD. The BBD matrix with replication of central runs (Run 07, 10, 13) was used to estimate the error in the model and in experimental runs and the detailed information of each tested factor is listed in Table S1. Based on results from 15 designed experiments, a second order regression equation was derived using coded factors for methane yield (*Y*) (Equation 1):

$$Y=287.93\text{-}19.312A+10.58B-3.05C+0.38AB+0.32AC-0.05BC$$
$$+0.15A^2+0.12B^2+0.09C^2$$

(Equation 1)

Where *A*, *B*, *C* are the coded values of initial pH, temperature, and TS content, respectively. *Y* is predicted response of methane yield. The response surface contours and 3D figures are illustrated in Figure S1.

The second order regression model fitted experimental data significantly with *P* value of 0.0101 and *F* of 10.10. The lack of fit was not significant with *F* of 17 and *P* value of 0.0561. It was observed that the linear and quadratic effects of temperature were significant, with its linear effect being more pronounced ($P = 0.0005$) than the quadratic effect ($P = 0.0086$) (Table S2), whereas other factors were not significant. Therefore, the effect of temperature on methane yield was found to be the most significant parameter in manure AD process. This is consistent with a previous study dealing with the co-AD of cattle manure, canola residues, and inoculum (Safari et al., 2018).

Interactions among the three factors of initial pH, temperature, and TS are shown in response surface contours and 3D figures (Figure S1). The closer the contour line is, the greater the interaction between the two factors is. Response surfaces of initial pH-temperature (TS content=10.5%) and TS-temperature (initial pH=8) suggest that the effect of temperature on methane production was enhanced when TS content or initial pH increased. Also, surfaces of TS-temperature (initial pH=8) and TS-initial pH (temperature=42.5℃) indicate that the effect of TS on methane yield was more significant when the initial pH declined or when the temperature increased. As for the initial pH, its impact on methane generation was more intense at high temperature or TS content, according to the response surfaces of initial pH-TS (temperature=42.5 ℃) and temperature-initial pH (TS=10.5%).

The optimal conditions obtained from RSM modeling were as follows: temperature of 55 °C, TS content of 6%, and initial pH of 10, which can result in 73.05 mL/g TS methane yield . This optimal initial pH (pH 10) differs from other studies that estimated optimal values at pH 7-8 (Zhai et al., 2015). This can be attributed to the fact that cattle manure, has higher buffer capacity compared to other substrates like sludge and food wastes, and was therefore easier to alleviate the negative effects created by VFAs and NH_4^+ under the initial pH of 10 (Xing et al., 2020). Besides, pH 10 could perform better in terms of decomposition of hard-degradable organic matter like lignin substances compared with other initial pH values (Ma et al., 2019). Validation of the findings was conducted by carrying out three parallel replicate experiments under the temperature of 55 °C, TS content of 6%, and initial pH of 10, that resulted in methane yield of 74.21 ± 3.34 mL/g TS, which indicates that the second order regression model (73.05 mL/g TS) fits well with the experimental data.

3.2 Effects of ZnO NPs on AD performance

3.2.1 Effects on hydrolysis products

Profiles of SCOD concentrations in the presence of different ZnO NPs amounts are shown in Fig. 1a. No obvious difference was observed among the four groups on the first day of digestion indicating that ZnO NPs had no acute inhibition on hydrolysis. As AD process progressed, the SCOD concentration in control group (G0, without ZnO NPs) was invariably lower than in the experimental groups (G1-G3, with ZnO NPs), while SCOD concentration in G1 group with a relatively low amount of ZnO NPs (5 mg/g TS) was the highest among the four groups after the 1st day of treatment. The SCOD accumulation was much faster than its utilization in G1-G3 indicating that subsequent biochemical reactions were suppressed by ZnO NPs, while SCOD derived from organics solubilization and hydrolysis was enhanced (Fig. 1a).

Variation of SP and SC concentrations, two main hydrolysis products, were also monitored and are depicted in Fig. 1b. In the first 22 days, similar variation trend of SP was observed in all four groups, while SC concentrations in the experimental groups with 5-100 mg ZnO NPs/g TS were slightly higher than that in the control group. Also, significant accumulations of SP and SC in G2 and G3 were gained in the last day of digestion. Higher concentrations of SP and SC in G1-G3 groups suggest that ZnO NPs may promote the hydrolysis of organic matter on the one hand, and may inhibit SP and SC degradations on the other hand in such dynamic processes (Chen et al., 2020). Inhibition of ZnO NPs on SP degradation was also proved by profiles of NH_4^+-N concentration illustrated in Fig. 1c which shows that concentrations of NH_4^+-N were lower in G2 and G3 than in G1 group. Besides, the lowest pH associated with SP and SC decomposition was gained in G1 (Fig. 1d). This revealed that the inhibited effects on SP and SC degradation were more severe in the presence of high ZnO NPs levels (30 and 100 mg/g TS) compared with G1 group.

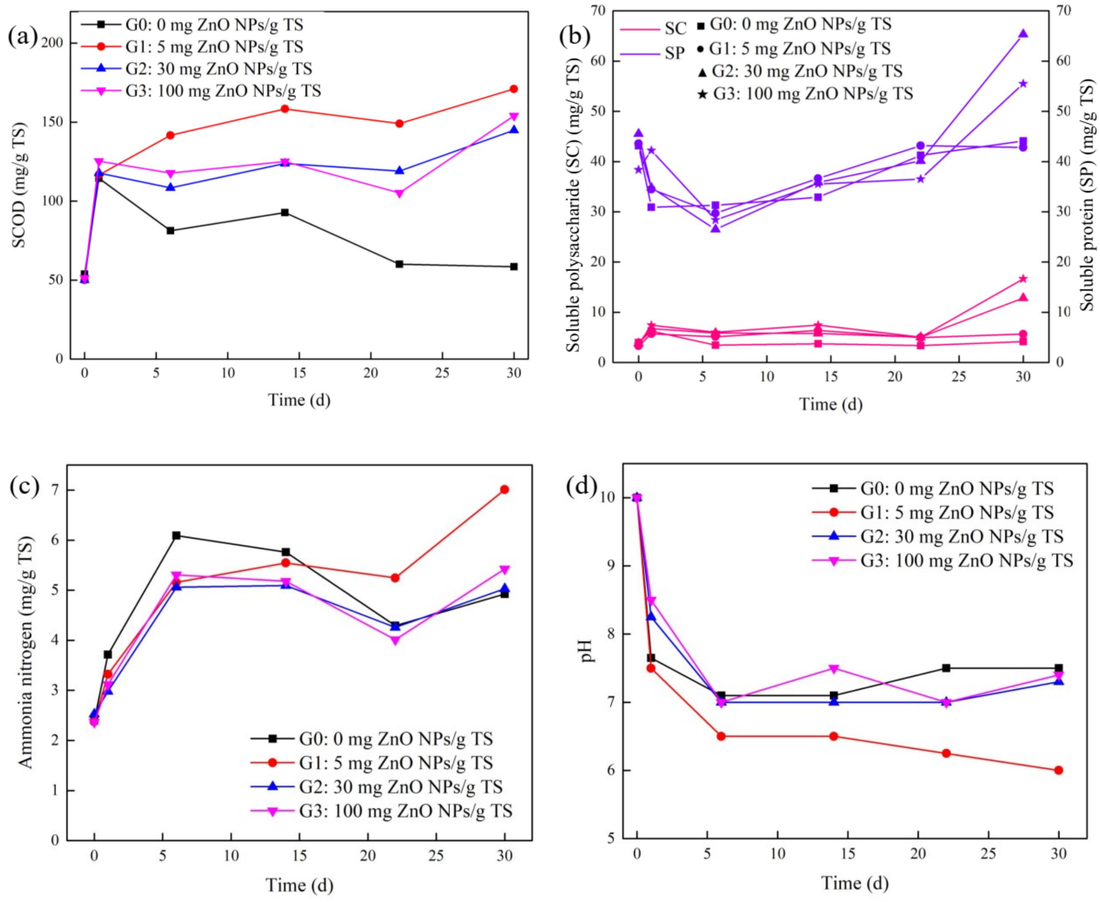


Fig. 1 Changes of SCOD (a), hydrolysis products (b), ammonia nitrogen (c), and pH (d) during AD of cattle manure with different concentrations of ZnO NPs.

3.2.2 Effects on VFAs fermentation

Since VFAs are vital substrates for methanogenesis in AD process, the variations of VFAs concentration in the four groups were also investigated, in terms of TVFAs and VFAs composition (Fig. 2). TVFAs concentration in the control group decreased after the 14^{th} day, while in experimental groups showed an upward trend as digestion went on. The highest TVFAs accumulation occurred in G1 with the growth multiple of 6.91, followed by 1.81 and 1.31 in G3 and G2, respectively, on the 30^{th} day of treatment (Fig. 2).

During the first two weeks, the TVFAs concentrations in the three experimental groups were lower than in the control group, which was probably attributed to the adverse effect on acidification led by ZnO NPs (Zheng et al., 2015). This is also in agreement with the lower SC and SP concentrations in G0. After the 14^{th} day, concentrations of TVFAs kept increasing in G1-G3 experimental groups, especially in G1, whereas a significant decrease was observed in the control group. Thisindicated that VFAs consumption might be inhibited by the presence of ZnO NPs. Besides, TVFAs accumulation was most significant in G1 among the three experimental groups along with the lowest pH (Fig.1d). Since VFAs are mainly produced from SP and SC, the lower accumulation of VFAs in G2 and G3 also indicates the inhibitory effects of ZnO NPs on SP and SC degradation (Chen et al., 2020), which also corresponded to the significant accumulation of SP and SC in groups with 30 (G2) and 100 (G3) mg/g TS of ZnO NPs (Fig. 1b).

Acetic acid is essential for methanogenesis and accounted for the largest proportion in TVFAs in all groups, followed by butyric and propionic acid (Fig. 2). Comparing the accumulation of individual acids up to the 14^{th} day of treatment, it can be seen that acetic acid was higher in G0 than in the other groups containing ZnO NPs. This indicated that there might be an inhibition resulting from ZnO NPs on accumulation of acetic acid. Besides, no significant difference in propionic acid was showed among the control and the other experimental groups during the first two weeks of treatment, which was consistent with the similar trends of SP, whose most important product is propionic acid (Feng et al., 2009). In contrast, butyric acid accumulation could be improved in G1 by the presence of ZnO NPs of 5 mg/g TS, probably due to the suppressed conversion of butyric acid, an important intermediate in producing acetic acid, at a certain concentration of ZnO NPs, and similar results were also obtained for sludge AD process (Chen et al., 2020; Zheng et al., 2019).

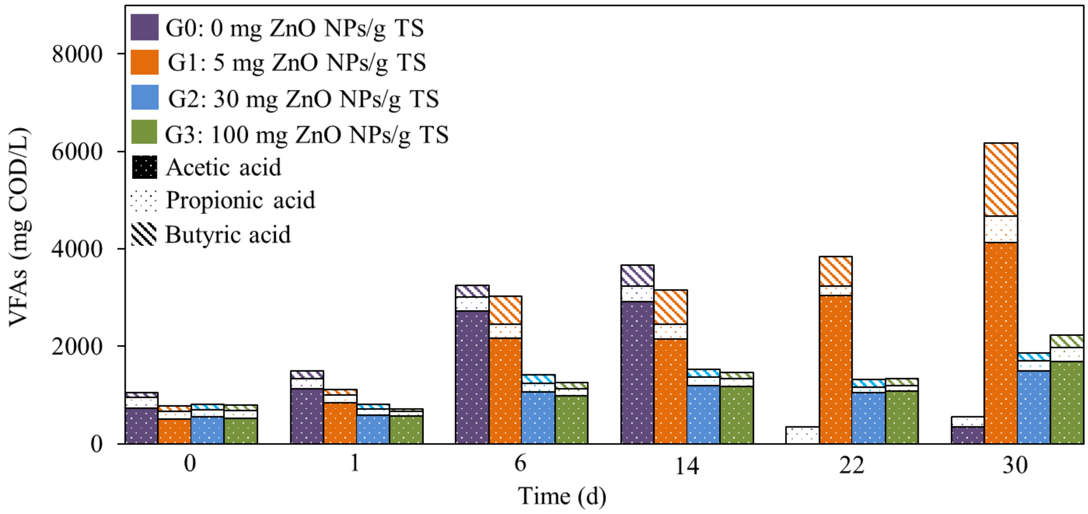


Fig. 2 Changes of TVFAs during AD of cattle manure in the presence of various concentrations of ZnO NPs

3.2.3 Effects on methane production

Cumulative methane production (CMP) and daily methane production (DMP) were monitored to evaluate the methanogenic activity and potential (Fig. 3). An inhibiting effect on CMP was highly related to ZnO NPs concentration. The final CMP was decreased by 84.55% (G1), 92.39%(G2), and 93.72% (G3), respectively, compared to the control group G0. Also, CMP remained almost constant during the first 3 days of digestion and after that inhibitory effects occurred on day 4 in G2 and G3 groups, which is earlier than day 6 when inhibition occurred for G1 group with the lowest ZnO NPs content. It can be suggested that a delayed inhibitory impact on CMP was induced by ZnO NPs, and such an inhibition showed positive correlation with ZnO NPs concentration within a certain range (0-30 mg ZnO NPs/g TS in this study).

To further investigate the effect of ZnO NPs on cattle manure AD performance, DMP variations in all experimental groups were also compared (Fig. 3b). It was found that DMP in the groups with ZnO NPs was higher than in the control group (0.35 mL/g TS) within the 1st day, with the highest methane yields being 0.69 mL/g TS, 0.55 mL/g TS, and 0.59 mL/g TS in G1, G2 and G3, respectively. This based on the profile of fermentation products in Fig. 2 might be attributed to two reasons. First, the appropriate and low amount of ZnO NPs (5 mg/g TS) could be beneficial to acetic acid formation and stimulate methanation in the short-term (within 1 day). This could be proved by the higher increasing rate of acetic acid in G1 (64.04%) than that in G0 (52.72%). Secondly, the utilization of organic substrates might be stimulated by ZnO NPs within the 1st day. Furthermore, comparison of DMP, in this study, showed that the faster inhibition of methane production could be attributed to the presence ZnO NPs at concentrations higher than 5 mg/g TS.

In addition, two DMP peaks were observed on the 5th and 21st day in G0, while there was only one DMP peak on the 5th day in G1 with 5 mg ZnO NPs/g TS. Higher VFAs generation and conversion rates contributed to these peaks when taking into account changes of SCOD, SC, SP, and TVFAs concentrations, given in Fig. 1a, 1b, and 2. The lower peak value of methane yield in G1 on the 5th day was related to the lower VFAs production resulting from the inhibitory impact of ZnO NPs on the degradation of SC and SP (Fig. 1b). Large VFAs consumption in G0 led to the formation of another peak on the 21st day. Moreover, the accumulation of VFAs was the reason for the inhibited methane yield in G1 group with 5 mg ZnO NPs/g TS, and can also be proved by Fig. 1d that shows that the pH in G1 on the 21st day was 6.25, which does not favour methane generation. In short, no significant effect on methanogenesis was induced by ZnO NPs at the short-term (1 day of digestion), but severe inhibitions on methane production did occur in the presence of high concentrations of ZnO NPs after 30 days of treatment

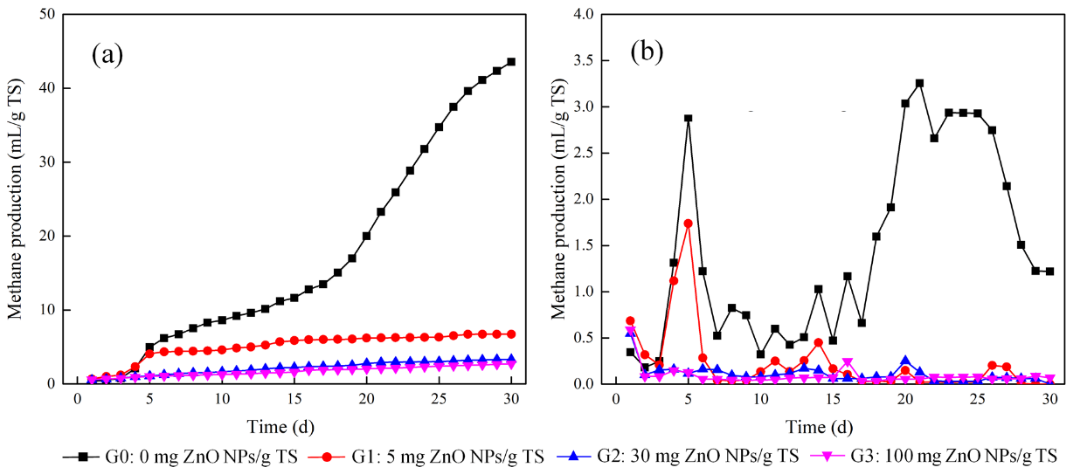


Fig. 3 Cumulative methane production (a) and daily methane production (b) during AD of cattle manure at various concentrations of ZnO NPs

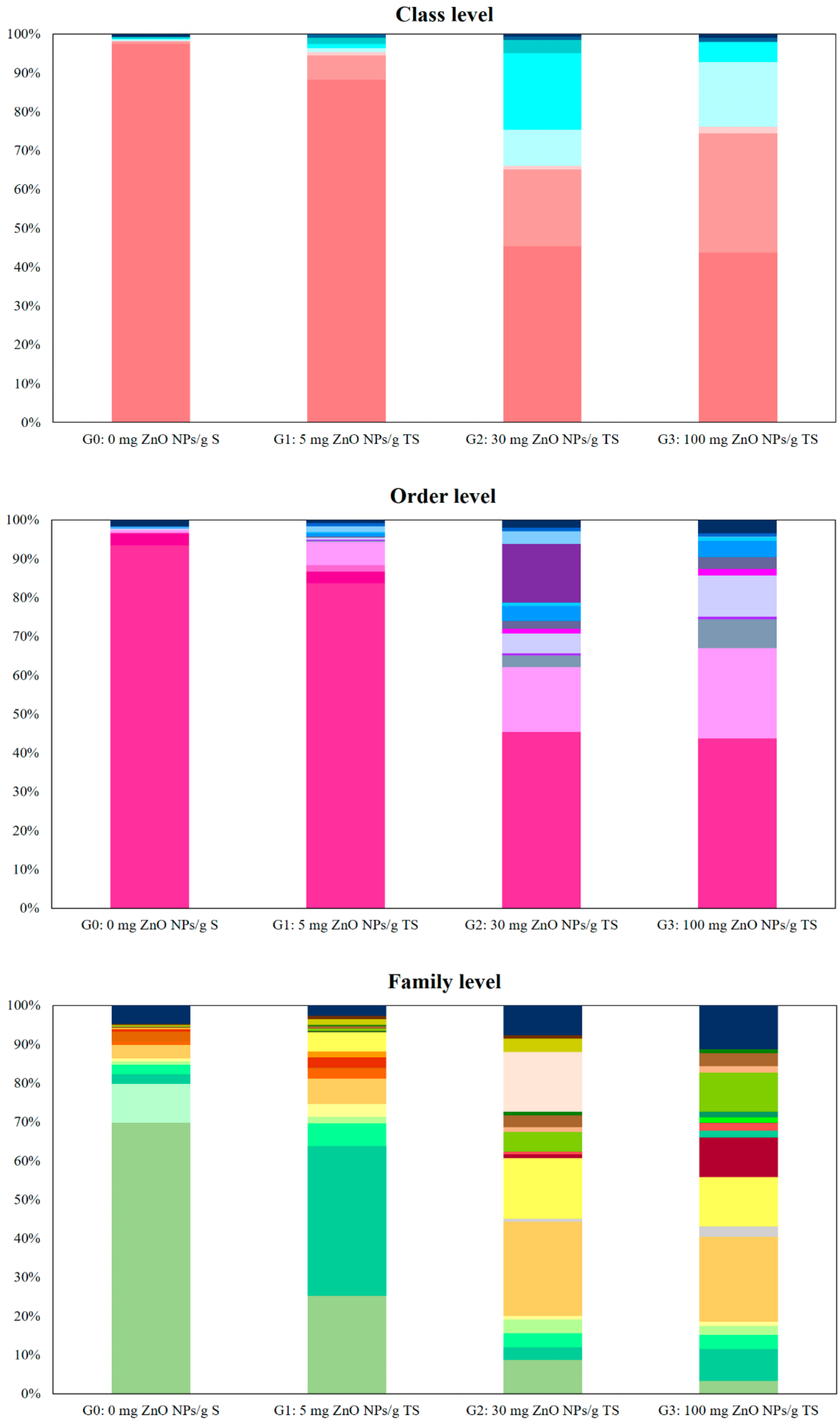
3.3 Effects on microbial community

AD process is driven by microbes, thus high throughput sequencing was employed to evaluate the influence of ZnO NPs on the microbial (bacterial and archaeal) community structure in cattle manure AD process.

3.3.1 The effects on bacterial community

The comparison of the main bacterial community responsible for hydrolysis, acidogenesis, acetogenesis, and hydrogenesis in cattle manure AD process was illustrated in Fig. 4. It was observed that bacterial diversity was enhanced in the presence of ZnO NPs. Bacteria in classes *Clostridia, Bacilli*, *Alphaproteobacteria*, and *Actinobacteria* were dominant in all experimental groups. The relative abundance of bacteria in Classes *Bacilli*, *Actinobacteria* and *Alphaproteobacteria* was promoted by ZnO NPs. In contrast, percentages of bacteria belonging to class *Clostridia* in the four groups were 97.37% (G0), 88.23% (G1), 45.38% (G2), and 43.64% (G3) of total bacterial OTUs, respectively. Strains in *Clostridia* are capable of degradation of organic compounds and acid formation during AD (Yang et al., 2015). Its negative correlation with ZnO NPs concentrations might be the reason for the inferior SP and SC degradation and VFAs formation in G1-G3 groups with ZnO NPs (Fig. 1b and 2). At the order level, bacteria in order *Clostridial*es belonging to class *Clostridia* dominated in all groups with relative abundances of 93.32% (G0), 83.52% (G1), 45.38% (G2) and 43.64% (G3) of total bacterial OTUs, respectively, suggesting a negative relationship between bacterial abundance of *Clostridiales* and increasing ZnO NPs concentrations. The obvious decrease of bacterial abundance in order *Clostridiales* was also probably the reason for lower VFAs accumulation and CMP in G1-G3 groups with ZnO NPs. A previous study has also pointed out that strains in *Clostridiales* were widely related to VFAs production, and biogas production was directly connected with VFAs generation (Straeuber et al., 2016).

Furthermore, ZnO NPs also led to the change of the composition of bacteria belonging to order *Clostridiales* at the family level. *Lachnospiraceae* in order *Clostridia* was the highest in G1 (38.27%), while proportion of family *Ruminococcaceae* was negatively related to increasing ZnO NPs concentrations. Relative abundances of family *Ruminococcaceae* in all groups were 69.68% (G0), 25.05% (G1), 8.54% (G2), and 3.17% (G3), respectively. The relative abundance of family *Family_XI_o__Clostridiale* took a large part in order *Clostridiales* in G2 and G3. Strains in families *Ruminococcaceae* and *Lachnospiraceae* can both promote degradation of cellulose and hydrogen production, which play key roles in hydrolysis and hydrogenesis in AD process (Biddle et al., 2013). The large component of *Ruminococcaceae* (25.05%) and *Lachnospiraceae* (38.27%) in G1 (5 mg ZnO NPs/g TS) supported the substantial amounts of VFAs during anaerobic fermentation which is consistent with results shown in Figure 2. In contrast, the large proportion of 23.69% and 21.44%, of family *Family_XI_o__Clostridiale*, in G3 and G4 groups, respectively, may spoil anaerobic fermentation performance with lower VFAs generation leading to SP and SC accumulation.



Class level
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
G0: 0 mg ZnO NPs/g S
G1: 5 mg ZnO NPs/g TS
G2: 30 mg ZnO NPs/g TS
G3: 100 mg ZnO NPs/g TS
Order level
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
G0: 0 mg ZnO NPs/g S
G1: 5 mg ZnO NPs/g TS
G2: 30 mg ZnO NPs/g TS
G3: 100 mg ZnO NPs/g TS
Family level
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
G0: 0 mg ZnO NPs/g S
G1: 5 mg ZnO NPs/g TS
G2: 30 mg ZnO NPs/g TS
G3: 100 mg ZnO NPs/g TS

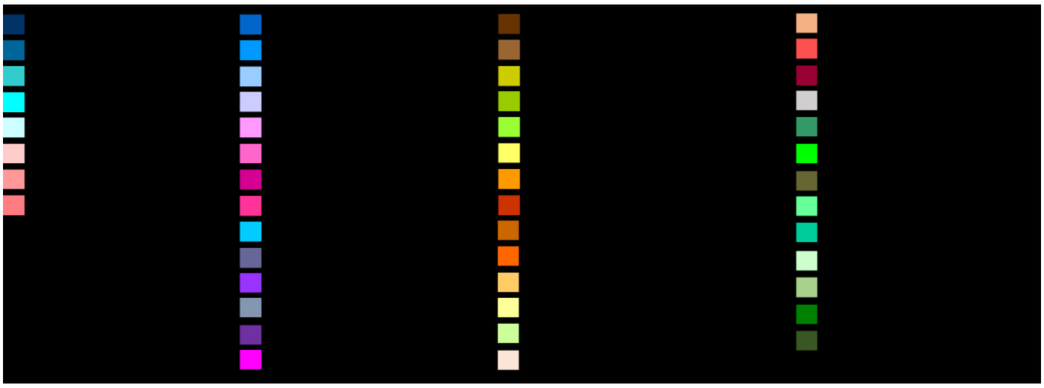


Fig. 4 Relative abundance of bacteria at the class, order and family levels in AD of cattle manure with different concentrations of ZnO NPs.

3.3.2 Effects on archaeal community

The comparison of the archaeal flora community among the four groups is shown in Fig. 5. Genera *Methanothermobacter*, *Methanobrevibacter, unclassified_k__noranked_d__achaea,* and *Methanosphaera* dominated in all groups. Therefore, hydrogenotrophic methanogenesis was the main pathway in thermophilic AD of cattle manure in this study. Although the diversity did not significantly change in the presence of ZnO NPs, the archaeal community were shifted in G0-G3. Strains in *Methanothermobacter* made up the main pats of archaea with 96.82% in the control group. Archaea in the presence of 5 mg ZnO NPs/g TS was mainly consisted of genera *Methanothermobacter* (22.45%), *Methanobrevibacter* (15.72%), and *unclassified_k__noranked_d__achaea* (60.11%). Strains in genus *Methanobrevibacter* were dominant in G2 (89.94%) and G3 (89.45%) with higher concentrations of ZnO NPs. Archaea in *Methanothermobacter* mainly transform H_2/CO_2 into methane, and archaea in *Methanobrevibacte* uses H_2 and/or formate as substrates (Danielsson et al., 2017; Liu et al., 2019). Therefore, the variance of archaea indicated that the conversion of H_2/CO_2 to methane might be inhibited due to the presence of ZnO NPs in this study, since H_2/CO_2 methanation was found to be the fastest methanogenic step in digested manure system (Pan et al., 2016). A vast array of methanogenic precursors were also responsible for the highest DMP in the control group (Fig. 3b). The presence of $F_{420}H_2$ oxidase in *Methanobrevibacter* species may be the reason for its prosperity in G2 and G3 (Seedorf et al., 2004). The advantages under the oxidation stress led by ZnO NPs will support a potential application in severe environment with higher amount of ZnO NPs. However, it is known that strains of *Methanobrevibacter* can be key methanogens in the AD process (Sun et al., 2021), which was inconsistent with methanogenesis performance as shown in Fig. 3. Therefore, functional analysis was conducted for further investigation.

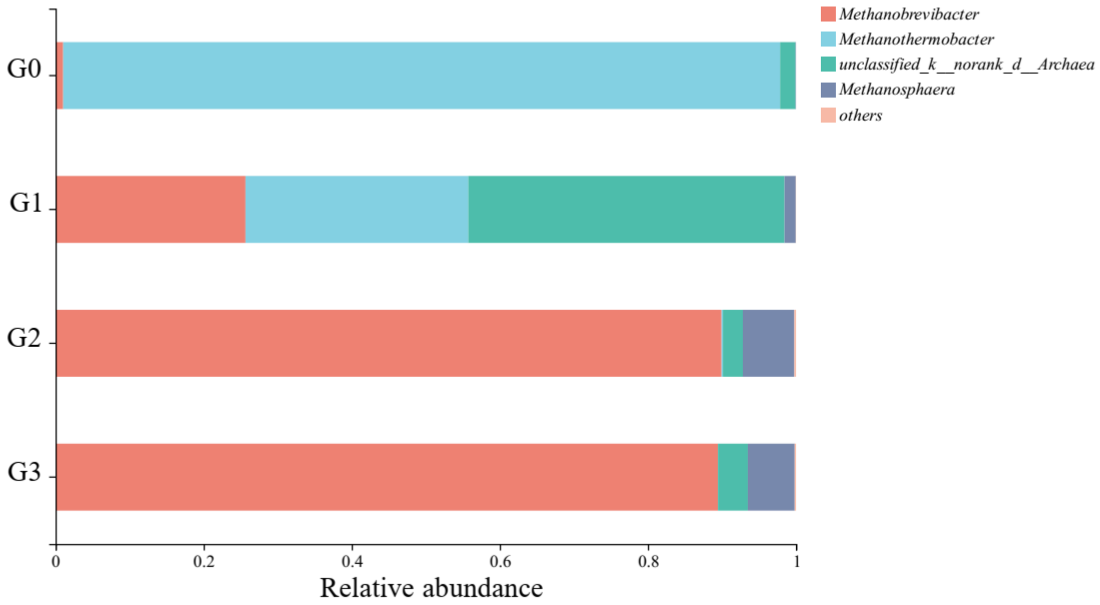


Fig. 5 Abundance of archaea at genus level in AD of cattle manure with different concentrations of ZnO NPs

3.4 Functional analysis based on 16s RNA data

The variation among the abundances of COGs with different ZnO NPs concentrations has been illustrated in Fig. 6. Functional proteins could be classified into three categories including metabolic pathways, information storage and processing, and cellular processes and signaling.

As for the bacterial COGs, amino acid transport and metabolism (AATM) and carbohydrate transport and metabolism (CTM) functions were related to SP and SC conversions. However, the variation of its abundances in the four groups was not consistent with SP and SC concentrations given in Fig. 1b. This might be attributed to the fact that although the abundances of functional proteins related to AATM and CTM could be boosted by ZnO NPs, the high ZnO NPs concentration probably inhibited its performance under exposure for 30 days. In this study the three concentration levels of ZnO NPs had similar effects on genetic abundances of AATM, while the abundance of genes coded CTM was highest in G1 with ZnO NPs of 5 mg/g TS. These results may explain the higher capacity of SC degradation compared with G2 and G3 (Fig. 1b). Also, this can indicate that ZnO NPs at 30 and 100 mg/g TS may have greater negative effects on gene expression than at 5 mg/g TS. Further investigation about response of gene expression to ZnO NPs was required.

For archaea, abundances of COGs related to energy production and conversion, AATM, and coenzyme transport and metabolism were dominant in metabolism pathways. This was attributed to the fact that methanogens need to maintain special mechanism for efficient energy conservation with unique enzymes, electron carriers, and cofactors, in reaction to the inefficient synthesis of energy in methanogenesis using H_2/CO_2, formate, methanol, and acetate as substrates (Welte & Deppenmeier, 2014). The high abundances of COGs associated with energy conservation revealed the amount of energy demand when ZnO NPs are present in cattle manure. However, similarly to bacterial COGs, the higher genetic abundances related to methanogenesis in G1-G3 were not corresponded with the lower methane yield shown in Fig. 3. Therefore, although genes conferring functional proteins related to AD were boosted, the final genetic expression may be inhibited by ZnO NPs, and gene expression in methanogens were more sensitive to ZnO NPs than bacteria. This might also be the reason that considerable relative abundances of potential functional microbes in *Methanobrevibacter* and low methane yield occurred at the same time in this study.

Functional proteins involved in the category of information storage and processing are essential to maintain microbial vital activity (Lin et al., 2016). Total abundances of those proteins associated with transcription, translation, ribosomal structure and biogenesis, and replication, recombination and repair were relatively higher in groups with ZnO NPs, especially in G2 and G3 as shown in Fig.6. This may be attributed to DNA destruction by nanoparticles, which decreased normal performance of anaerobic fermentation and subsequent methanation (Lacerda et al., 2007).

Genes related to cellular processes and signaling mainly included cell motility, cell wall/membrane/envelope biogenesis, defense mechanism, and signal transduction metabolism. Current research has pronounced that the cell membrane transport system and the associated membrane metabolism are connected with cell recovery under ZnO NPs stress (Wu et al., 2017). Defense mechanism supports the tolerance for intercellular oxidative stress resulted from the presence of ZnO NPs (Yang et al., 2009). Signal transduction can be modified by Zn^{2+} binding sites on proteins (Maret, 2006). Up-warded abundances for these functional genes in microbes indicated cellular membrane and intercellular communication may be damaged by ZnO NPs which are also responsible for the abnormal performance of AD.

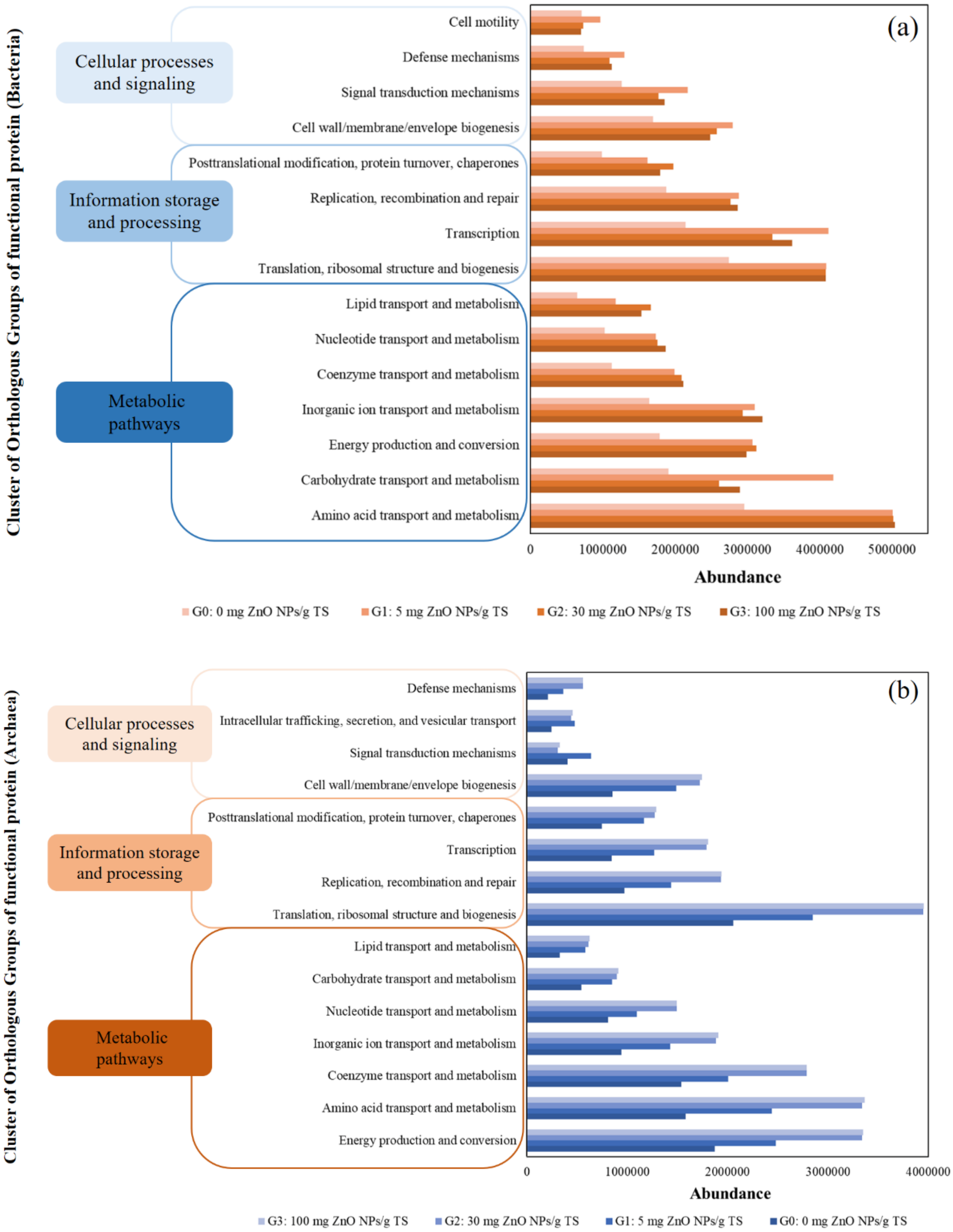


Fig. 6 Abundance of the Cluster of Orthologous Groups of functional categories in the AD of cattle manure with different concentrations of ZnO NPs. COGs identified for bacteria (a) and archaea (b) are represented separately. The abundances of unknown functions and those less than 1% were excluded.

Conclusion

The role of ZnO NPs in cattle manure AD process has been comprehensively investigated. On the one hand, ZnO NPs (5-100 mg/g TS) promoted hydrolysis and the accumulations of organic matters. On the other hand, the production of VFAs, especially acetic acids, was inhibited in the presence of 30 and 100 mg ZnO NPs/g TS, while no inhibition was observed at 5 mg ZnO NPs/g TS. As for methanogenesis, ZnO NPs (5-100 mg/g TS) exhibited severe impacts on methane production with decreasing rates of 84.55% (G1), 92.39% (G2), and 93.72% (G3). The poor performance of AD in the presence of NPs was a result of various microbial community and cellular damage. Further investigation of its actual expression should be carried out. Finally, other future work should involve the study of interactions of ZnO NPs with other pollutants, usually found in livestock wastes, and their effects on AD systems in order to resemble complexity of real-world applications.

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