



Environmental Microbiology

Ammonia determines transcriptional profile of microorganisms in anaerobic digestion



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ABSTRACT

Anaerobic digestion is important for the management of livestock manure with high ammonia level. Although ammonia effects on anaerobic digestion have been comprehensively studied, the molecular mechanism underlying ammonia inhibition still remains elusive. In this study, based on metatranscriptomic analysis, the transcriptional profile of microbial community in anaerobic digestion under low (1500 mg L⁻¹) and high NH₄⁺ (5000 mg L⁻¹) concentrations, respectively, were revealed. The results showed that high NH₄⁺ concentrations significantly inhibited methane production but facilitated the accumulations of volatile fatty acids. The expression of methanogenic pathway was significantly inhibited by high NH₄⁺ concentration but most of the other pathways were not significantly affected. Furthermore, the expressions of methanogenic genes which encode acetyl-CoA decarboxylase and methyl-coenzyme M reductase were significantly inhibited by high NH₄⁺ concentration. The inhibition of the co-expressions of the genes which encode acetyl-CoA decarboxylase was observed. Some genes involved in the pathways of aminoacyl-tRNA biosynthesis and ribosome were highly expressed under high NH₄⁺ concentration. Consequently, the ammonia inhibition on anaerobic digestion mainly focused on methanogenic process by suppressing the expressions of genes which encode acetyl-CoA decarboxylase and methyl-coenzyme M reductase. This study improved the accuracy and depth of understanding ammonia inhibition on anaerobic digestion.

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Introduction

Anaerobic digestion (AD) is a promising technology in the field of waste treatment and renewable energy production. Livestock manure has been processed increasingly by AD to reduce

pathogens and to generate bioenergy such as methane.^{1,2} Thus, the improvement of stability and efficiency of AD is crucial for the comprehensive application of this technology. Ammonia concentration is one of crucial factors regulating AD stability.³ Optimal ammonia concentrations provide sufficient buffer capacity and nutrient for microbial growth, which improves the AD stability and efficiency. However, low or

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excessive ammonia concentrations usually result in the failure of AD. It is reported that low ammonia concentration ($<500 \text{ mg L}^{-1}$) decreases methane production, biomass and the acetoclastic methanogenic activity.⁴ The high ammonia ($>4000 \text{ mg L}^{-1}$) results in the inhibition of microbial activity and accumulation of volatile fatty acids, which finally causes decreased stability and even failure of AD.^{3–6} Due to high protein content in manure, there are usually high ammonia concentrations in the AD of manure.³ Thus, AD of livestock manure has to confront the inhibition from high ammonia. It is necessary to reveal the mechanisms underlying ammonia inhibition on the AD process.

Temperature is considered as a prominent factor regulating ammonia toxicity on AD process.^{3,7,8} The thermophilic temperatures compared to mesophilic temperatures usually cause higher ammonia toxicity.^{7,8} The thermophilic temperatures enhance metabolic activities of microorganisms, which strengthens hydrolysis of substrates including proteins. This process increases ammonia concentration in the slurry, so that ammonia toxicity is undoubtedly strengthened. Thus, under mesophilic temperatures, the ammonia toxicity more depended on the initial ammonia concentration, which facilitates the understanding of the relationship between ammonia concentration and ammonia toxicity. Different methanogens are distinguishably sensitive to ammonia concentrations, which partly determines ammonia toxicity on AD process. Due to spherical *Methanosarcina* with higher ratio of volume/surface than that of rod-shaped *Methanosaeta*, the diffusion of ammonia is less into the *Methanosarcina* than *Methanosaeta*,⁹ which results in their different sensitivities to ammonia concentrations. Thus, high ammonia more probably causes failure of AD where *Methanosaeta* are dominated in the methanogens than that where *Methanosarcina* are dominated. The recovery of AD from failure depends on a reconstruction of methanogenic species,¹⁰ so that *Methanosarcina* as dominated methanogens replace *Methanosaeta*. This process probably causes the decrease of acetoclastic methanogenesis.¹¹ These studies of ammonia toxicity on AD process mainly focus on methanogens and methanogenic process.^{3,5,6,12,13}

Although the ammonia toxicity on AD process has been comprehensively revealed mainly based on methanogenic microflora,^{3,5,12–14} the transcriptional profiles of specific pathways and genes in response to ammonia have rarely been discussed. The AD process consists of AD food web (hydrolysis, acidogenesis, acetogenesis and methanogenesis), so besides methanogenesis, the other three steps of the food web also play important roles in final methane production. Thus, the transcriptional profiles of methanogenesis-related processes are necessary to be revealed based on metatranscriptomic analysis, which can provide a new sight underlying ammonia toxicity on AD process.

In this study, based on metatranscriptomic analysis, the transcriptional profiles of microbial community in response to low and high NH_4^+ concentrations, respectively, were revealed in AD. Specifically, we focused on the expressions of pathways and genes responsible for methane production under different NH_4^+ concentrations to further reveal the mechanism underlying ammonia toxicity on AD process.

Materials and methods

Setup of AD system

The AD experiment of swine manure was conducted with working volume of 2.5 L digestion sludge containing 0.75 L initial inoculum, and the total solid content was 7% (Supplement Table S1). The high ammonia treatment with NH_4^+ (5000 mg L^{-1}) (HN) and low ammonia treatment with NH_4^+ (1500 mg L^{-1}) (LN) were determined by adding NH_4Cl at the beginning of AD, which was mainly based on previous report⁶ and pre-experiments. All the treatments were conducted in triplicate at 37°C . Seed slurry was prepared by anaerobic digestion of swine manure (obtained from a pig farm in Neijiang, Sichuan Province, China) at 37°C , for one hydraulic retention time (HRT). After methane production reached the first peak (6th day) in the reactor, we performed a semi-continuous feeding mode that 500 mL of digestate was exchanged every three days with HRT of 15 days and organic loading rate of $4.5 \text{ g VS (volatile solid) L}^{-1} \text{ day}^{-1}$. The anaerobic digestion was performed for two HRT. The feeding slurry was adjusted to the corresponding NH_4^+ concentration using NH_4Cl . Details about parameters at the start of fermentation were shown in Supplement Table S1.

Sampling and analysis

At each feeding, methane content in biogas, volatile fatty acids (VFA), NH_4^+ and pH were measured to monitor AD dynamic. The methane content in biogas was measured using an Agilent 6890 gas chromatography system (Agilent Technologies, USA), equipped with a thermal conductivity detector and carrier gas of argon. The injection port, column oven, and detector were operated at 100, 70, and 150°C , respectively. The daily volume of biogas was detected by water replacement method. The VFA in the slurry was detected using Agilent 1260 Infinity liquid chromatography (Agilent Technologies, USA), equipped with a differential refraction detector (RID) and mobile phase of H_2SO_4 (0.005 M). Total solid and volatile solid were measured based on previous report.¹⁵ NH_4^+ concentration was quantified with Nessler's reagent colorimetric method.¹⁶ At the end of the second HRT, the digestate were sampled in triplicate for total RNA extraction with the RNeasy PowerMicrobiome Kit (Cat. No. 26000-50; MO BIO, USA). The quality of RNA was checked with a NanoDrop 2000 spectrophotometer (Thermo, USA). Ribosomal RNA was removed from the total RNA with the RiboMinus™ kit (Lot. No. 1539791; Invitrogen, USA). The metatranscriptomic (mRNA) sequencing was performed using an Illumina HiSeq 2000 (Illumina Inc., USA). The sequencing raw data from total 6 samples were uploaded to MG-RAST with assigned MG-RAST ID (mgs589946, mgs589949, mgs589952, mgs589955, mgs589958 and mgs589961) for further analysis.¹⁷ Prior to the analysis, the quality control pipeline in MG-RAST¹⁷ was performed to remove poor quality sequences. The annotation of functional profiles was based on the KEGG Orthologs database including 4 levels. The functional categories was presented as level 2. Level 3 reflected the KEGG pathways, and level 4 (gene expression level) showed expressions of specific



Fig. 1 – Fermentation performance under high (HN) and low (LN) NH_4^+ concentrations. All the data are presented as means \pm standard deviations ($n = 3$).

genes.¹⁸ During annotation analysis, the pipeline parameters were kept at default settings.

Statistical analysis

The ammonia effect on the general changes of gene expressions was assessed by principal coordinates analysis (PCoA) in R (<http://www.r-project.org/>), based on the Bray–Curtis dissimilarity index, using the *vegan* package. The normality and homoscedasticity of the raw data, and one way analysis of variance (ANOVA) were performed in SPSS 21 software (IBM USA). Spearman's correlation analysis, enrichment analysis and redundancy analysis (RDA) were calculated in R with the *vegan* package. The Spearman's p value was adjusted using the Benjamini and Hochberg methods.¹⁹ The taxa with average relative abundance $>0.1\%$ and significantly different expression ($p < 0.05$) between HN and LN were selected for network analysis to reveal co-response to ammonia. If the correlation p value was <0.05 , the correlation between two taxa was considered statistically robust and shown in the network. The network was visualized in Cytoscape software.²⁰

Results

Anaerobic digestion and global expressions of genes

There were significant differences of AD performances between LN and HN (Fig. 1 and Supplement Table S1). Methane production and content in the biogas were higher in LN than that in HN ($p < 0.01$). The concentrations of acetic acid and propionic acid, and pH were higher in HN. It showed that high NH_4^+ concentration inhibited methane production and resulted in some accumulations of VFA. The global expressions of genes showed obvious differences between HN and LN (Fig. 2), which well corresponded with the significant differences of performances of AD. Compared with HN, the replicates in LN clustered more closely (Fig. 2), which implied that high NH_4^+ probably caused more randomness of gene expressions in AD.

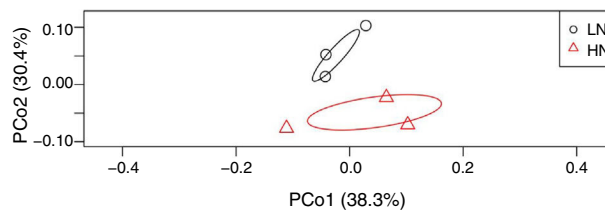


Fig. 2 – The principal coordinates analysis (PCoA) of global expressions of genes in anaerobic digestions with high NH_4^+ (HN) and low NH_4^+ (LN).

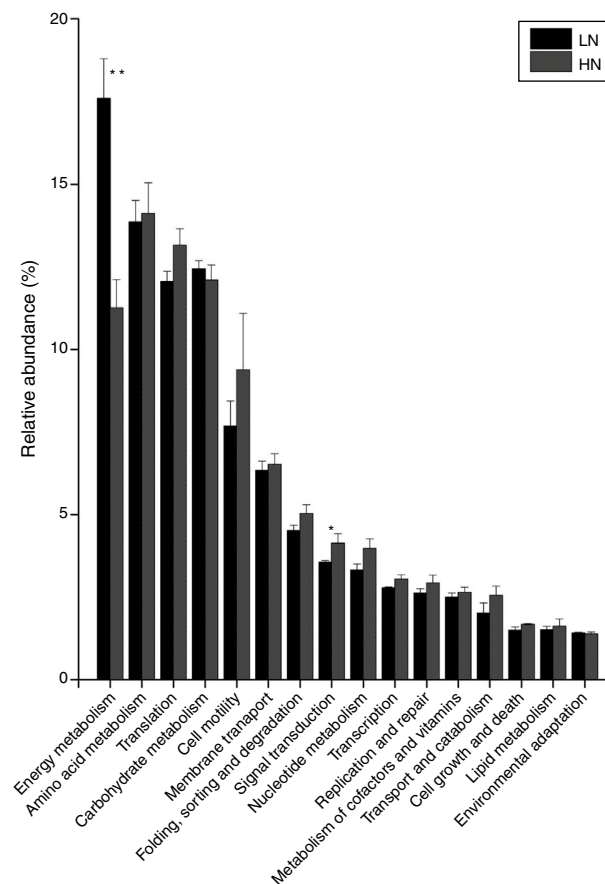


Fig. 3 – Expression profiles of level 2 at high NH_4^+ (HN) and low NH_4^+ (LN). ** significant at $p < 0.01$; * Significant at $p < 0.05$.

Differentiated expressions of metabolic pathways

In the comparison between HN and LN based on pathways (level 2), most expressions of these pathways were higher in HN, but only signal transduction was significantly higher at HN ($p < 0.05$) (Fig. 3). Surprisingly, only energy metabolism was higher in LN ($p < 0.01$). At level 3, most expressions of pathways were not significantly distinguishable between HN and LN (Table 1). The pathway of methane metabolism (ko00680) (usually indicating the methanogenesis in anaerobic digestion) has a higher expression in LN ($p < 0.01$), but the pathways of RNA polymerase (ko03020) and Valine, leucine and isoleucine degradation (ko00280) had higher expressions in HN ($p < 0.05$).

Table 1 – Relative abundances of level 3 pathways under high and low NH₄⁺ concentrations.

| Level 3 | Low NH ₄ ⁺ | High NH ₄ ⁺ | p values |
|---|----------------------------------|-----------------------------------|----------|
| ABC transporters [PATH:ko02010] | 4.66 ± 0.21 | 4.76 ± 0.37 | 0.757 |
| Alanine, aspartate and glutamate metabolism [PATH:ko00250] | 2.44 ± 0.2 | 2.7 ± 0.29 | 0.365 |
| Aminoacyl-tRNA biosynthesis [PATH:ko00970] | 3.31 ± 0.11 | 3.51 ± 0.19 | 0.270 |
| Arginine and proline metabolism [PATH:ko00330] ^a | 1.31 ± 0.08 | 1.21 ± 0.08 | 0.299 |
| Bacterial chemotaxis [PATH:ko02030] | 1.11 ± 0.12 | 1.26 ± 0.2 | 0.405 |
| Bacterial secretion system [PATH:ko03070] | 1.37 ± 0.1 | 1.4 ± 0.13 | 0.813 |
| Cell cycle-caulobacter [PATH:ko04112] ^a | 1.45 ± 0.11 | 1.64 ± 0.01 | 0.069 |
| Citrate cycle (TCA cycle) [PATH:ko00020] | 1.3 ± 0.06 | 1.39 ± 0.08 | 0.294 |
| Cysteine and methionine metabolism [PATH:ko00270] | 1.16 ± 0.18 | 1.15 ± 0.07 | 0.955 |
| DNA replication [PATH:ko03030] | 1.05 ± 0.13 | 1.21 ± 0.18 | 0.366 |
| Flagellar assembly [PATH:ko02040] | 6.65 ± 0.86 | 7.88 ± 1.69 | 0.414 |
| Glycine, serine and threonine metabolism [PATH:ko00260] | 3.27 ± 0.21 | 3.36 ± 0.26 | 0.716 |
| Glycolysis/Gluconeogenesis [PATH:ko00010] | 2.73 ± 0.09 | 2.9 ± 0.14 | 0.208 |
| HIF-1 signaling pathway [PATH:ko04066] | 1.67 ± 0.07 | 2.17 ± 0.34 | 0.109 |
| Histidine metabolism [PATH:ko00340] | 1.07 ± 0.09 | 0.95 ± 0.07 | 0.200 |
| Methane metabolism [PATH:ko00680] ^a | 10.96 ± 0.57 | 6.52 ± 0.83 | 0.003 |
| Oxidative phosphorylation [PATH:ko00190] | 6.4 ± 1.28 | 4.22 ± 0.67 | 0.100 |
| Pentose and glucuronate interconversions [PATH:ko00040] | 1.04 ± 0.06 | 0.97 ± 0.12 | 0.483 |
| Pentose phosphate pathway [PATH:ko00030] | 1.15 ± 0.08 | 1.21 ± 0.09 | 0.524 |
| Peroxisome [PATH:ko04146] | 1.97 ± 0.31 | 2.54 ± 0.31 | 0.142 |
| Plant-pathogen interaction [PATH:ko04626] | 1.43 ± 0.04 | 1.46 ± 0.05 | 0.597 |
| Purine metabolism [PATH:ko00230] | 2.42 ± 0.19 | 3.07 ± 0.3 | 0.062 |
| Pyruvate metabolism [PATH:ko00620] | 2.12 ± 0.21 | 1.69 ± 0.11 | 0.063 |
| Ribosome [PATH:ko03010] | 8.5 ± 0.26 | 9.6 ± 0.74 | 0.117 |
| RNA degradation [PATH:ko03018] | 3.64 ± 0.2 | 4.11 ± 0.18 | 0.063 |
| RNA polymerase [PATH:ko03020] ^b | 2.7 ± 0.02 | 2.98 ± 0.13 | 0.038 |
| Two-component system [PATH:ko02020] | 1.54 ± 0.1 | 1.61 ± 0.07 | 0.465 |
| Valine, leucine and isoleucine degradation [PATH:ko00280] | 1.48 ± 0.08 | 1.7 ± 0.03 | 0.027 |

Taxa with average relative abundance >1% are shown. The p values represent the significance from the comparison of the relative abundances of pathways under high and low NH₄⁺ concentrations.

^a Indicates significant correlations ($p < 0.05$) between the relative abundances of pathways with daily methane production.

^b $p < 0.01$.

(Table 1). The relative abundances of arginine and proline metabolism (ko00330) and methane metabolism showed positive correlations with daily methane production ($p < 0.05$), but those of RNA polymerase and cell cycle-caulobacter (ko04112) showed negative correlations with methane production ($p < 0.05$) (Table 1).

In redundancy analysis (RDA) of the relationship between the expressions of pathways (level 3) and the environmental variables (Fig. 4a), the expressions of the pathways including methane metabolism, arginine and proline metabolism, pentose phosphate pathway (ko00030), histidine metabolism (ko00340), oxidative phosphorylation (ko00190) and pyruvate metabolism (ko00620) positively correlated with methane production. The expressions of RNA degradation (ko03018), pentose and glucuronate interconversions (ko00040) and Citrate cycle (ko00020) positively correlated with acetic acid and pH. The expressions of DNA replication (ko03030) and peroxisome (ko04146) potentially contributed to propionic acid accumulation.

Gene differentiated expressions

Besides the overall difference of gene expressions (Fig. 2), the enrichment analysis was applied to further uncover differentiated expressions of individual genes between HN and LN. Probably attributed to significant difference of the expression

of methane metabolism (Table 1), most of these genes with significant differentiated expressions ($p < 0.05$) were involved in methane metabolism (Supplementary Table S2). These methanogenic genes mainly encode acetyl-CoA decarboxylase and methyl-coenzyme M reductase. Additionally, most of methanogenic genes showed significant positive correlations with methane production ($p < 0.05$) (Supplementary Table S2). Besides methanogenic genes, two genes involved in the pathway of pyruvate metabolism showed higher expressions in LN, but three and two genes involved in aminoacyl-tRNA biosynthesis (ko00970) and ribosome (ko03010), respectively, showed higher expressions in HN (Supplementary Table S2). In the RDA of the relationship between gene expressions and environmental variables (Fig. 4b), it indicated that besides the methanogenic genes, the genes which encode glycerol kinase, phosphate acetyltransferase, transporting ATPase and pyruvate.orthophosphate dikinase positively contributed to methane production. The genes which encode ribosomal subunits, tRNA_synthetase and phosphoribosylamine glycine ligase positively contributed to pH and acetic acid.

Although there were 19 genes (each taxon with average relative abundance >0.1%) significantly distinguishingly expressed between LN and HN (Supplementary Table S2), the co-expressions of these genes should be further verified. Based on Spearman' correlations, the co-expressions of these genes to different NH₄⁺ concentrations were further

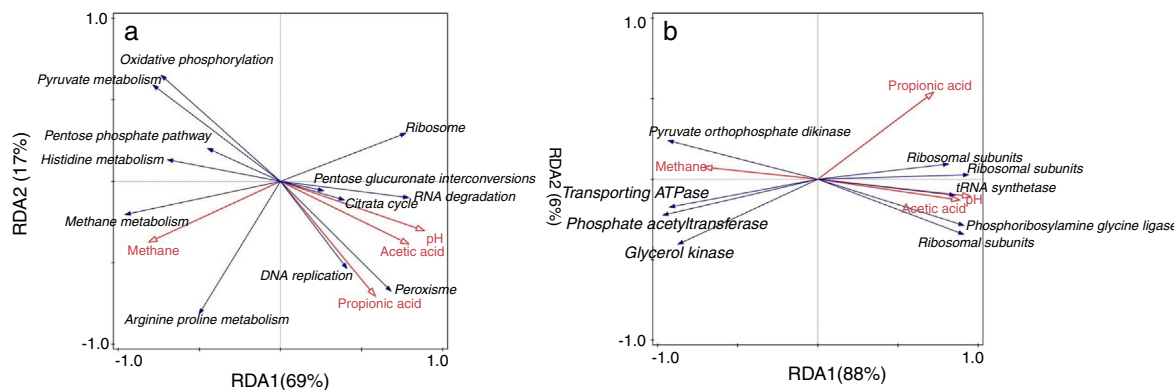


Fig. 4 – Redundancy analysis (RDA) of key pathway (a) and gene (b) expressions where the methanogenic genes mainly encoding acetyl-CoA decarboxylase and methyl-coenzyme M reductase positively contribute to methane production but are omitted to simplify the figure to be clearer.

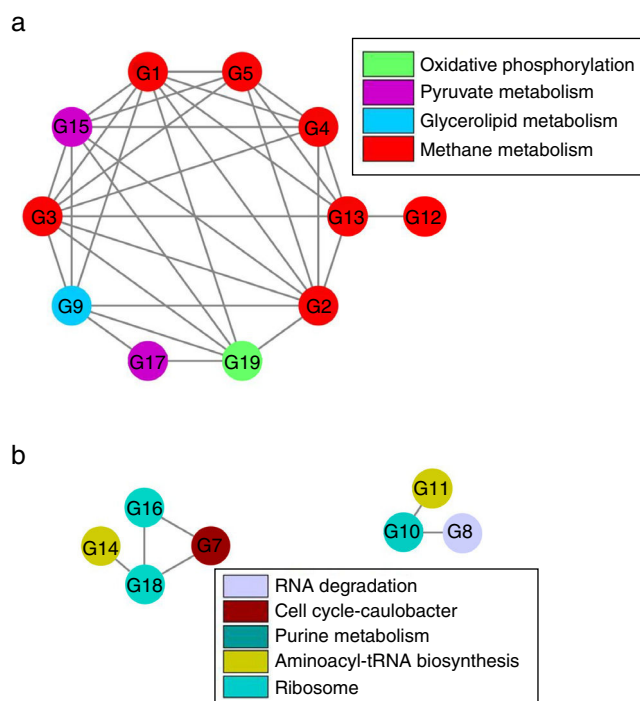


Fig. 5 – Networks of gene expressions negatively (a) and positively (b) responding to ammonia. The taxa with average relative abundance >0.1% and significant differentiated expressions between LN and HN are shown.

revealed (Fig. 5 and Supplementary Table S3). The expressions of the genes involved in methane metabolism, glycerolipid metabolism, pyruvate metabolism and oxidative phosphorylation showed a co-inhibition under the high ammonia condition. The expressions of methanogenic genes especially the genes which encode acetyl-CoA decarboxylase were comprehensively co-inhibited by high ammonia. However, the expressions of the genes involved in ribosome, aminoacyl-tRNA biosynthesis, purine metabolism, cell cycle-caulobacter and RNA degradation showed co-enhancement under the high ammonia condition. The expressions of more genes were

co-inhibited under the high ammonia condition, which supported ammonia toxicity on AD process.

Discussion

Although the hydrolysis of substrates would cause variations of ammonia concentrations during AD, in this study the operations of ammonia addition in HN and LN created distinct ammonia pressures to differentiate AD performances and microbial gene expression (Figs. 1 and 2). High ammonia significantly inhibited AD process by decreasing methane production and content in the biogas, and the accumulations of VFA. The decreased methane content in biogas not only resulted in a low methane production but also indicated a mass of byproducts. The accumulations of VFA, especially acetic acid, implied a potential inhibition of acetoclastic methanogenesis, which agreed well with previous reports.^{3,5} Although the accumulations of VFA were observed in HN, such accumulations unlikely caused excessive acidification in AD, which was further supported by the pH value (7.8 ± 0.5). Additionally, the ammonia inhibition more probably occurred under thermophilic temperatures than mesophilic condition (37°C).³ Thus, in this study, the inhibition of AD process was supposed to be directly attributed to ammonia toxicity on microbial activities, rather than other environmental factors regulated by ammonia.

The microbial activities were obviously regulated by ammonia, which was supported by the PCoA result (Fig. 2). In level 2 pathways, the expressions of energy metabolism and signal transduction were significantly influenced (Fig. 3). The AD actually is an energy metabolism process during which energy is transferred and transformed through various metabolic pathways especially the methanogenic pathways. The energy metabolism mainly including methane metabolism usually couples to actual methane production,^{1,21} so the inhibited expression of energy metabolism mainly resulted in the low methane production in HN. Signal transduction is usually involved in modulating cell behaviors in response to environmental pressures such as pH and ammonia.^{22,23} Thus, a higher expression of this pathway in HN could be explained. The obviously higher expression of

cell motility in HN coupled to that of signal transduction, which agreed with previous report.²⁴ This further supported microbial response to ammonia by behaviors or movements. Unexpectedly, in HN only the expression of energy metabolism was depressed, but other pathways had higher expressions compared to that in LN (Fig. 3). In consideration of that methanogens are not dominant microorganisms compared to bacteria such as Firmicutes, Bacteroidetes and Proteobacteria in AD,²⁵ it is reasonable to deduce that these pathways except energy metabolism were mainly conducted by bacteria. Thus, the higher expressions of these pathways in HN probably indicated high activities of bacteria under high ammonia condition. This was likely because ammonia could improve nutrient of nitrogen for bacterial growth.⁴ In level 3, the most significantly differentiated expressions between HN and LN was methane metabolism ($p=0.003$) which positively correlated with methane production (Table 1). Most of the other pathways had no significant expression differences. Interestingly, only the expressions of methane metabolism, oxidative phosphorylation and pyruvate metabolism were higher in LN and positively contributed to methane production (Fig. 4 and Table 1), but the other pathways had higher expressions in HN. This situation was similar with that of level 2. This further confirmed that high ammonia provided some improvements to substrate metabolisms mainly conducted by bacteria, but inhibited methanogenic activity. Consequently, although each step of AD food web played crucial roles in actual methane production, the ammonia toxicity on AD process mainly targeted methanogenic process to inhibit methane production.

Ammonia inhibition on methanogenic process would result in a shift in methanogenic acetate utilization from direct acetate cleavage toward syntrophic acetate oxidation.^{11,26} This was mainly attributed to the greatly decreased abundance of acetoclastic *Methanoseta* under high ammonia condition.¹¹ However, whether the expressions of genes involved in acetoclastic methanogenesis were reduced should be revealed. In methanogenic pathway, only the expressions of the genes which encode acetyl-CoA decarboxylase and methyl-coenzyme M reductase were significantly higher in LN ($p < 0.05$), and showed positive correlations with methane production ($p < 0.05$). Acetyl-CoA decarboxylase plays an important role in acetoclastic pathway²⁷ and methyl-coenzyme M reductase is the key enzyme in methanogenesis.²⁸ Thus, the higher expressions of these genes encoding the two enzymes undoubtedly improved methane production. The expressions of genes which encode acetyl-CoA decarboxylase were significantly depressed in HN, indicating that the acetoclastic methanogenesis was inhibited. Based on transcriptional profile, the ammonia toxicity on acetoclastic methanogenesis was further revealed. Additionally, ammonia inhibited methanogens' activities also probably by depressing the expressions of genes which encode methyl-coenzyme M reductase. Besides methanogenic genes, the genes involved in pyruvate metabolism had significantly higher expressions in HN, which probably improved the production of acetyl-CoA.²⁹ The genes involved in aminoacyl-tRNA biosynthesis and ribosome were significantly higher expressed in HN, indicating that ammonia potentially facilitated translation process.¹³

Although the expressions of individual key genes could generally explain the ammonia toxicity on AD process, gene

co-expressions in response to ammonia probably provided a systemic sight. The inhibition of the co-expressions of the genes involved in methane metabolism, glycerolipid metabolism, pyruvate metabolism and oxidative phosphorylation indicated that these pathways probably performed a cooperation to regulate AD efficiency that was mainly represented by methane production. The coupling relationship between methane metabolism and oxidative phosphorylation has been revealed before.¹ The glycerolipid metabolism and pyruvate metabolism probably provided potential substrates²⁹ for methanogenesis. Interestingly, nearly all the genes which encode acetyl-CoA decarboxylase showed inhibition of co-expressions under the high ammonia condition, which further demonstrated that acetoclastic methanogenesis were inhibited by high ammonia. This also showed that these genes compared to other methanogenic genes were unitedly and highly sensitive to NH_4^+ concentration. These genes could be used as potential indicators for ammonia toxicity on AD process. Additionally, the inhibition of co-expression of the genes which encode acetyl-CoA decarboxylase and methyl-coenzyme M reductase, indicating the cooperation of the two enzymes in methanogenesis.

Consequently, except methanogenesis, most of pathways were not significantly influenced by high ammonia concentration, so that they were probably not the key factors contributing to decreased methane production. Thus, ammonia toxicity on AD process mainly targeted methanogenic process by inhibiting the expressions of genes which encode acetyl-CoA decarboxylase and methyl-coenzyme M reductase to finally decrease methane production. This study revealed mechanisms underlying microbial response to ammonia pressure based on gene expressions, and further discovered ammonia inhibition only targeting on the genes encoding the above two enzymes. However, the adaptations of microbial gene expressions to ammonia pressure during longtime AD also should be considered in the future work.

Conflicts of interest

Authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bjm.2018.04.008.

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