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Research Paper

Exposure to the environmental pollutant ammonia causes changes in gut microbiota and inflammatory markers in fattening pigs

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ABSTRACT

Ammonia (NH_3) is a major pollutant in livestock houses and atmospheric environment. It has been demonstrated that NH_3 can cause a series of damage to animals and human. However, toxicity evaluation of NH_3 on farm animals was rarely reported, especially in the intestinal microflora. Therefore, in this study, twenty-four 125-day-old fattening pigs were randomly divided into 4 groups: control group, NH_3 group (88.2 mg m^{-3} < NH_3 concentration < 90.4 mg m^{-3}), Se group (Se content: 0.5 mg kg^{-1}), and NH_3 + Se group (88.2 mg m^{-3} < NH_3 concentration < 90.4 mg m^{-3}, Se content: 0.5 mg kg^{-1}), and the effects of NH_3 and L-Selenomethionine on the microbiota composition in the jejunum and the levels of inflammatory markers in feces of fattening pigs were examined by 16S rDNA and ELISA, respectively. Our results showed that the content of Matrix metalloproteinase-9 (MMP-9), Myeloperoxidase (MPO), Lactoferrin (LTF) and Calprotectin in the ammonia group (A group) were significantly elevated compared to the control group, and the content of MMP-9, MPO, LTF and Calprotectin in the A + Se group were significantly reduced. A significant difference in microbiota composition in the phylum, class, family and genus levels was found in the A group and the NH_3 + Se group. There was a negative correlation between *Streptococcus* and Calprotectin. Our results indicated that excessive NH_3 inhalation could cause changes in inflammatory markers and beta diversity of intestinal microflora in fattening pigs. We found there was a positive correlation between MPO and *Pseudomonas*. In addition, we first proposed that L-Selenomethionine could improve the imbalance of microbial flora and the inflammatory injury caused by NH_3. Changes in intestinal microflora and inflammatory markers can be used as important indicators to evaluate NH_3 toxicity, and studying changes in intestinal microflora is also an important mechanism to reveal NH_3 toxicity.

1. Introduction

Ammonia (NH_3) is a typical air pollutant and has toxic effects on all vertebrates (Di Lorenzo et al., 2017). Up to now, the harmful effects of environmental harmful gases on animals and human beings cannot be ignored. NH_3 in environment comes from various sources such as industry, plant fertilizers (Ramanantenasoa et al., 2018), powerhouses (Bicer and Dincer, 2018), and animal husbandry (Artíñano et al., 2018). In animal husbandry, the decomposition of livestock manure is the main pathway of gaseous NH_3 production. In addition, the decomposition of secondary protein metabolite and bedding materials also enhance the NH_3 emissions. NH_3 in the atmosphere reacts with acidic gases to promote the formation of PM 2.5, resulting in serious haze (Plautz, 2018) and soil acidification. NH_3 in the environment not only deteriorates air quality, but also causes threat to animal and human health (Kearney et al., 2014). The toxic effects of NH_3 have been studied in chickens (Jing et al., 2020), rats (Dąbrowska et al., 2018), fish (Williams et al., 2017), snakes and other animals, and has attracted extensive attention in the field of toxicology.

For the health conditions of farm animals, consecutive long-term NH_3 exposure affected production performance, feed efficiency and animal welfare. And NH_3 has negative toxicological effects on multiple organs, such as spleens (An et al., 2019), liver (Xu et al., 2020), jejunum (Wang et al., 2019a) and heart (Xing et al., 2019). When gaseous NH_3 comes into blood circulation, it will lead to the increase in the blood NH_3 content, causing dysfunction in redox homeostasis to mediate the oxidative stress responses (Kim et al., 2019). Excessive NH_3 inhalation can change the mRNA level of cytokines and NLRP3 inflammasome, the content of NO and the activity of iNOS, which lead to inflammatory injury in tissues (An et al., 2019). NH_3 can also cause the changes of

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complement C3, complement C4 and immunoglobulin M, and reduce the immune function of the body (Yu et al., 2020). A recent study found that NH_3 could increase the apoptosis rate of nerve cells in broilers and showed significant neurotoxicity (Zhang et al., 2017). Accumulated evidence suggested that NH_3 was toxic to organisms, but the specific mechanism of its toxic effect was still unclear.

Intestinal tract is an important part of the digestion system and plays crucial roles in nutrient absorption and disease prevention (Wang et al., 2018a). Intestine is also called "the second brain" or "the brain in gut" (Gili et al., 2020). It interacts with brain through vagus nerve to compose the "gut-brain axis", which is regarded as the communication network between the intestine and the brain. Neurotransmitters secreted by central nervous system are responsible to control feelings and emotion, gut microbes exactly play important roles in regulating neurotransmitter or their precursor signals (Wu et al., 2020). The intestinal microflora is composed of diverse and dynamic microorganisms. Recent evidence suggested that the gut microbiota might regulate the function of multiple organs (Parker et al., 2020). Different intestinal microbiota is related to the integrity of epithelial barrier, intestinal metabolism and maintenance of immune homeostasis. It has been demonstrated that the feature and relative richness of gut microorganism community taxa could be altered by external environmental toxic agent (Zarrinpar et al., 2014). However, little is known about the relationship between changes in specific intestinal microbiota and toxicity assessment of NH_3, as well as toxicological mechanisms.

Selenium (Se) is an important essential trace nutrient for humans and animals (Helal et al., 2019) and plays an important role in antioxidants, boosting immunity, detoxifying the body and preventing cancer (Li et al., 2019). Most notably, previous researches have validated that Se can antagonize the toxic effects of environmental toxicant by reducing antioxidant levels and inflammatory response in the body (Jin et al., 2017). To our knowledge, the antagonistic effects of Se on NH_3 toxicity has not been reported. Therefore, in the present study, the microbiota composition in the jejunum of fattening pigs exposed to high levels of NH_3 was investigated by high-throughput sequencing of 16S rDNA, and the levels of inflammatory markers in feces were detected by ELISA. The aim of this study was to evaluate the effects of NH_3 on the microbiota composition and the levels of inflammatory markers in fattening pigs and the protective effects of Se, and to elaborate the relationship between intestinal flora and inflammatory response, so as to provide reference for further analysis of the toxicological mechanism of NH_3.

2. Materials and methods

2.1. Experimental design

All procedures in our study were approved by Animal Ethics Committee of the Northeast Agricultural University. Experimental treatment was in accordance with the standards described in the NIH Guide for the Care and Use of Laboratory Animals. A total of twenty-four 125-day-old fattening female pigs were selected and randomly divided into 4 groups with 6 pigs per group: control group (C group), NH_3 group (A group), Se group, and NH_3 + Se group (A + Se group), respectively. These pigs were housed in different environmentally controlled chambers (450 cm × 270 cm × 226 cm) in the same animal shed. The same treatment group were housed in an individual chamber and each chamber has 6 pigsties (135 cm × 90 cm × 115 cm). The environmental conditions were described as followed: temperature: 18.1 ± 0.2 ℃; relative humidity: 63.5% ± 1.8%; ventilation quantity: 0.35 m^3 (h kg)$^{-1}$; noise: 32.5 ± 2.5 dB; lighting time: 12 h. NH_3 concentration in the C group and the Se group was lower than 5 mg m^{-3}. The NH_3 concentration in the A group and the A + Se group was in the range of 88.2–90.4 mg m^{-3}. Exogenous NH_3 was supplemented to the chamber by the steel cylinder of liquid ammonia (Dawn Gas Co. Ltd., Harbin, China) for 8 h per day and its concentration was monitored by an Ammonia Meter/Data Logger Model ZDL-800 (Florida, USA). The pigs in the C group and the A group were feed basal diets (Table 1). Per kilogram of base diet contains 0.218 ± 0.021 mg Se. The Se content in the Se group and A + Se group was 0.5 mg kg^{-1} achieved by adding L-Selenomethionine. All experimental animals were fed with the same amount of food every day. They were placed in chambers 14 days before the formal experiment started for adapting to the chambers' environment. The chambers' temperature and humanity were ranged from 17.9 ℃ to 18.3 ℃ and from 61.7% to 65.3%, respectively. The pre-experiment was implemented for 3 days to certain the NH_3 concentrations above could not cause the death of fattening pigs. The formal experiment lasted 30 days. All the pigs had free access to feed and water throughout the experiment. At the end of the experiment, feces were collected, all the pigs were euthanized, and jejunal contents were taken. All feces samples and enteric contents were fast frozen in liquid nitrogen for 1 h and stored at − 80 ℃ for the further analysis.

2.2. Detection of inflammatory marker levels in feces

The collected fecal samples were diluted with cold physiological saline at a ratio of 1:9 and then centrifuged at 3,500 rpm for 10 min at 4 ℃. The supernatant was used for the subsequent analysis of inflammatory marker levels. The contents of Matrix metalloproteinase-9 (MMP-9), Myeloperoxidase (MPO), Lactoferrin (LTF) and Calprotectin were detected by corresponding ELISA assay kits (Shanghai Hengyuan biological technology Co., Ltd., Shanghai, China). All procedure was followed by the instructions of the manufacturer. The absorbance values were determined by the spectrophotometer (SpectraMax® ABS00254, California, USA). The maximum coefficient of variation presented a good reproducibility. Then, OD values were measured by enzyme-labeled instrument at 450 nm. The relative contents of all inflammatory markers were calculated based on corresponding formulas.

2.3. DNA extraction and PCR amplification

Total DNA from intestinal contents was extracted using specialized assay kits (E.Z.N.A. ®Stool DNA Kit), its quality was determined by Agarose Gel electrophoresis and its concentration was determined using ultraviolet spectrophotometer. The obtained DNA was then diluted with 50 μL ultra-pure water and mixed through a shaker. According to the V3-V4 hypervariable region of the microbial 16S rRNA gene, specific primer (341F 5′-CCTACGGGNGGCWGCAG-3′and 805R 5′-GACTACHVGGGTATCTAATCC-3′) with barcode was designed and synthesized (Sangon Biotech Co., Ltd., Shanghai, China). The designated region was amplified by commercial PCR kits in a 42.5 μL reaction system (contained 50 ng DNA) under suitable condition (98 ℃ for 30 s, 98 ℃ for 10 s, 54 ℃ for 30 s, 72 ℃ for 45 s, and 72 ℃ for 10 min). Each sample was amplified in triplicate and amplification products from the same sample were mixed together to be detected by 2% agarose gel electrophoresis, and the PCR product was then recycled through a gel recovery kit. Meanwhile, PCR product was purified with AMPure XT beads (Beckman Coulter Genomics, Danvers, MA, USA) and was eluted by Tris HCI. According to the preliminary results, all products were quantified with the

Table 1
Compositions of basal diets fed to fattening pigs.

Basic diet	Content
Soya bean cake	9%
Peanut cake	8%
Corn meal	40%
Sweet potato meal	14%
Bran	16%
Peanut seedlings	12%
Bone meal	0.5%
Salt	0.5%
Digestive energy	12.5 (MJ kg^{-1})
Crude protein	15%

spectrophotometer (Qubit®, Invitrogen, USA). Finally, each sample was mixed in the expected ratio and stored at − 20 ℃ until the following analysis.

2.4. 16S rDNA sequencing and bioinformatic analysis

Amplicon library was enriched with PCR amplification. The size and quantity of the library was identified using Agilent 2100 bioanalyzer (Agilent, USA) and the library quantification assay of Illumina (Kapa Biosciences, Woburn, MA, USA), respectively. Amplicon library was sequenced on the Illumina NovaSeq platform based on instructions of the manufacturer, which was provided by the LC-Bio. According to the unique barcode of each sample, pairing-sequences were assigned to each sample, and removed the primer sequencing and barcode. The matching-end was merged and read using FLASH. Based on the fqtrim (version 0.94), quality filtering of raw data was performed under specific filtering conditions to obtain high-quality clean tags. Chimera sequence was filtered with Vsearch software (version 2.3.4). The feature tables and feature sequences were obtained by DADA2. The clean data was assigned to multiple operational taxonomic units (OTUs) in the threshold of 97%. According to the SILVA (release 132) classifier, feature abundance was normalized using the relative abundances of each sample. Alpha diversity was used to analyze the complexity of sample species diversity by Chao1, Observed species, Goods coverage, Simpson, and Shannon, which were calculated using QIIME2 (version 2.1). Beta diversity (between sample), such as PCA, PCoA and NMDS, was calculated with QIIME2 and was drawn by R packages (version 3.5.2). Each representative sequence was annotated with the SILVA database. LDA effect size (LEfSe) was performed to confirm the discriminating genera between the groups. The predicted functions of microorganism were uncovered with the PICRUSt2 program. Correlation network was conducted using the Spearman's rank correlation analysis. Solid lines mean positive relationship while dotted lines mean the negative relationship. The thickness of the line represents the correlation strength. The size of the nodes elucidates the relative abundance of species. In this study, we also drew phylogram tree, bubble plot and Venn diagrams with corresponding software.

2.5. Statistical analysis

All data were normalized and presented as mean ± standard deviation using IBM Statistical Product and Service Solutions software (version 25.0; SPSS Inc., Chicago, IL, USA). One –way ANOVA analysis with shortest significant ranges method (Duncan multiple comparison) was used to contrast differences, meanwhile, the significant difference between each two groups was examined by the Tukey–Kramer multiple comparison test. Different lowercase alphabets in bar charts represents there is a statistical significance ($P < 0.05$) between different groups.

3. Results

3.1. Changes of inflammatory markers in feces

The contents of inflammatory markers (LTF, MPO, MMP-9, and Calprotectin) in four groups were shown in Fig. 1. There was no significant difference in the levels of inflammatory markers between the Se and C groups. All inflammatory index in feces in the A group were significantly increased compared with those in the C group ($P < 0.05$), LTF, MPO, MMP-9, and Calprotectin increased by 178.67%, 199.44%, 596.95%, and 261.51%, respectively, which indicated that there was infiltration of inflammation-mediated cells in jejunum. LTF, MPO, MMP-9, and Calprotectin in the A + Se group increased by 115.76%, 114.95%, 235.63% and 145.76% ($P < 0.05$), respectively, compared with the C group. However, LTF, MPO, MMP-9, and Calprotectin in the A + Se group were significantly reduced ($P < 0.05$) compared to those in the A group. The results indicated that high NH_3 could induce intestinal inflammatory reaction in fattening pigs, and Se could antagonize intestinal injury caused by NH_3.

3.2. Screening of sequencing data for all samples

As shown in Fig. 2a, 133 OTUs were same in four groups, 309 OTUs

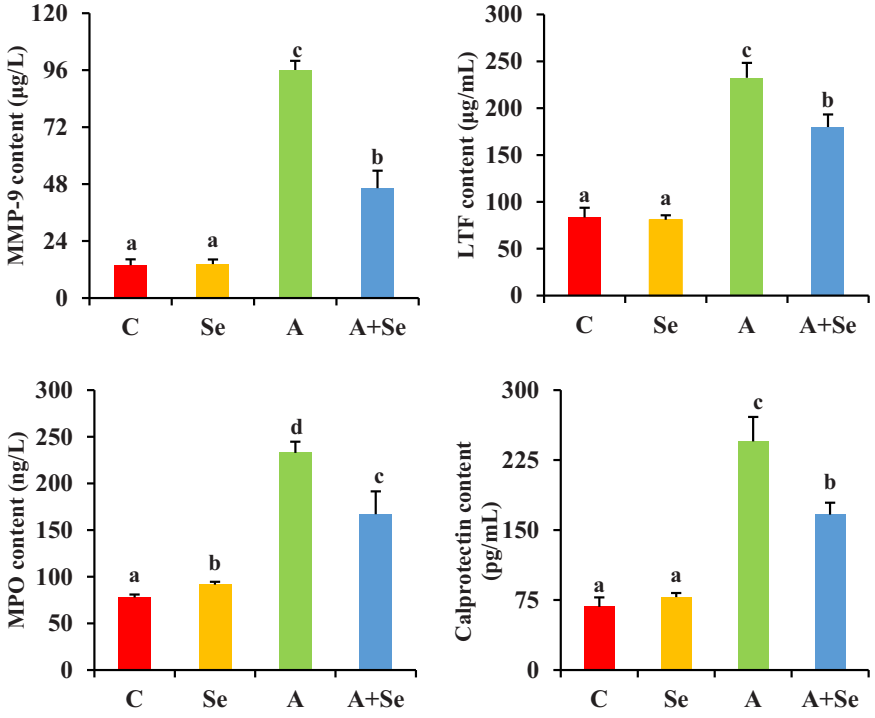

Fig. 1. Effect of NH_3 and Se treatments on inflammatory marker content in feces. Data in each group were described as mean ± SD (n = 6). Bars with different lowercase represent statistical significance ($P < 0.05$) between two groups using a Tukey–Kramer multiple comparison test.

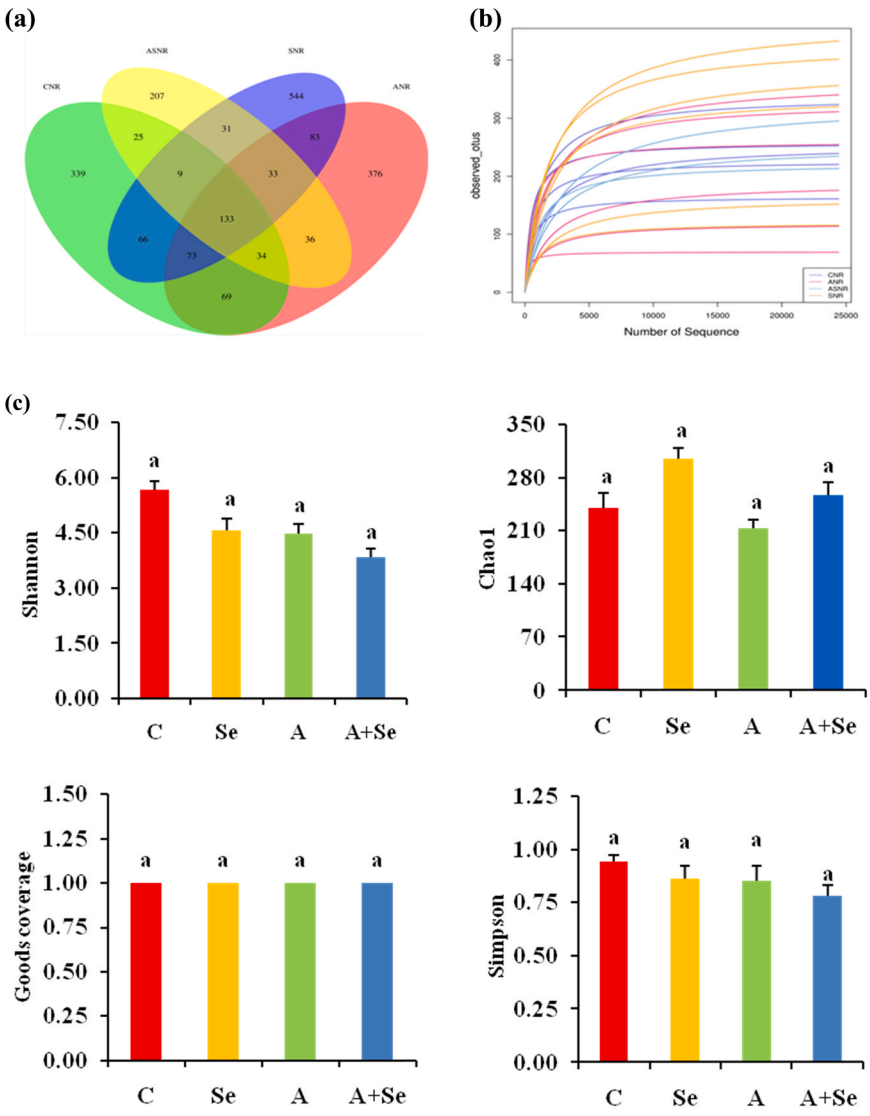

Fig. 2. Feature profiling for high-quality reads in four groups and effect of NH_3 and Se treatments on alpha diversity of gut microbiota in the jejunum of fattening pigs. (a) Venn diagram of C group, Se group, A + Se group and A group for operational taxonomic units (OTUs). Blue, yellow, Green and pink represented Se group, A–Se group, C group and A group, respectively. (b) Observed OTUs curve. CNR: intestinal contents in the C group; ANR: intestinal contents in the A group; SNR: intestinal contents in the Se group; ASNR: intestinal contents in A + Se group. (c) Comparison of Simpson, Shannon, Goods coverage and Chao1 index in the C, Se, A, and A + Se groups. Data in each group are described as mean ± SD (n = 6). Bars with the same lowercase represent no significant difference ($P < 0.05$) between two groups using a Tukey–Kramer multiple comparison test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

were same between C group and A group, 281 OTUs were same between C group and Se group and 236 OTUs were same between A + Se group and A group. However, there were 339, 207, 544, and 376 unique OTUs in the C group, A + Se group, Se group and A group, respectively. Sequencing data of all samples from the four groups were showed in Table 2. A total of 1,633,052 reads were obtained from 24 samples. After denoising, a total of 1471449 clean reads were obtained. Average percentage of Q20 was more than 94.88% when the average percentage of GC content was more than 52.37%, indicating the sequencing results had perfect credibility. A total of 1,080,523 high-quality reads (54,026.15 ± 11,398.9 per sample) were screened from the valid tags, including 230,973 reads in the C group, 364,014 reads in the Se group, 299,422 reads in the A group and 186,114 reads in the A + Se group. These reads above were clustered into 2058 OTUs and assigned to 27 phyla, 65 classes, 133 orders, 241 families and 543 genera.

Table 2
Summary statistics of 16 S rDNA high-throughput sequencing.

Sample	Raw tags	Valid tags	Valid %	Q20%	Q30%	GC %
Control	83,036	71,521	86.04%	93.56%	84.38%	52.18%
Se	84,433	76,019	89.96%	95.86%	88.98%	52.31%
Ammonia	77,019	70,751	91.82%	94.43%	86.20%	52.49%
A + Se	83,056	77,742	93.58%	96.00%	89.14%	52.55%

3.3. Alpha diversity analysis

The effects of high NH_3 and organic Se addition on alpha diversity in the jejunum of fattening pigs were shown in Fig. 2b–c. Alpha diversity parameters included Chao1, Observed-species, Goods coverage, Simpson, and Shannon. The rarefaction curves of observed species showed a flattening trend, indicating enough sequence depth to cover almost overall sequences, that is, almost all bacterial species are present in the jejunum contents of fattening pigs. There were no significant differences in Simpson, Shannon, Goods coverage and Chao1 among the four groups ($P > 0.05$).

3.4. Beta diversity analysis

The effects of high NH_3 and organic Se addition on beta diversity of jejunum in fattening pigs were in Fig. 3. In the results of the PCoA and PCA, different colored dots represent different groups. The closer dots suggest that the composition of the microbial structure between samples is more similar. In this study, the PCA and PCoA results based on Unweighted-UniFrac showed a relatively long distance between nodes in the C group, the A group and the A + Se group, indicating the structure of intestinal microflora in fattening pigs was strongly influenced by environmental factors (high NH_3 environment) and intake level of micronutrient (Se). NMDS is an important index of Beta diversity

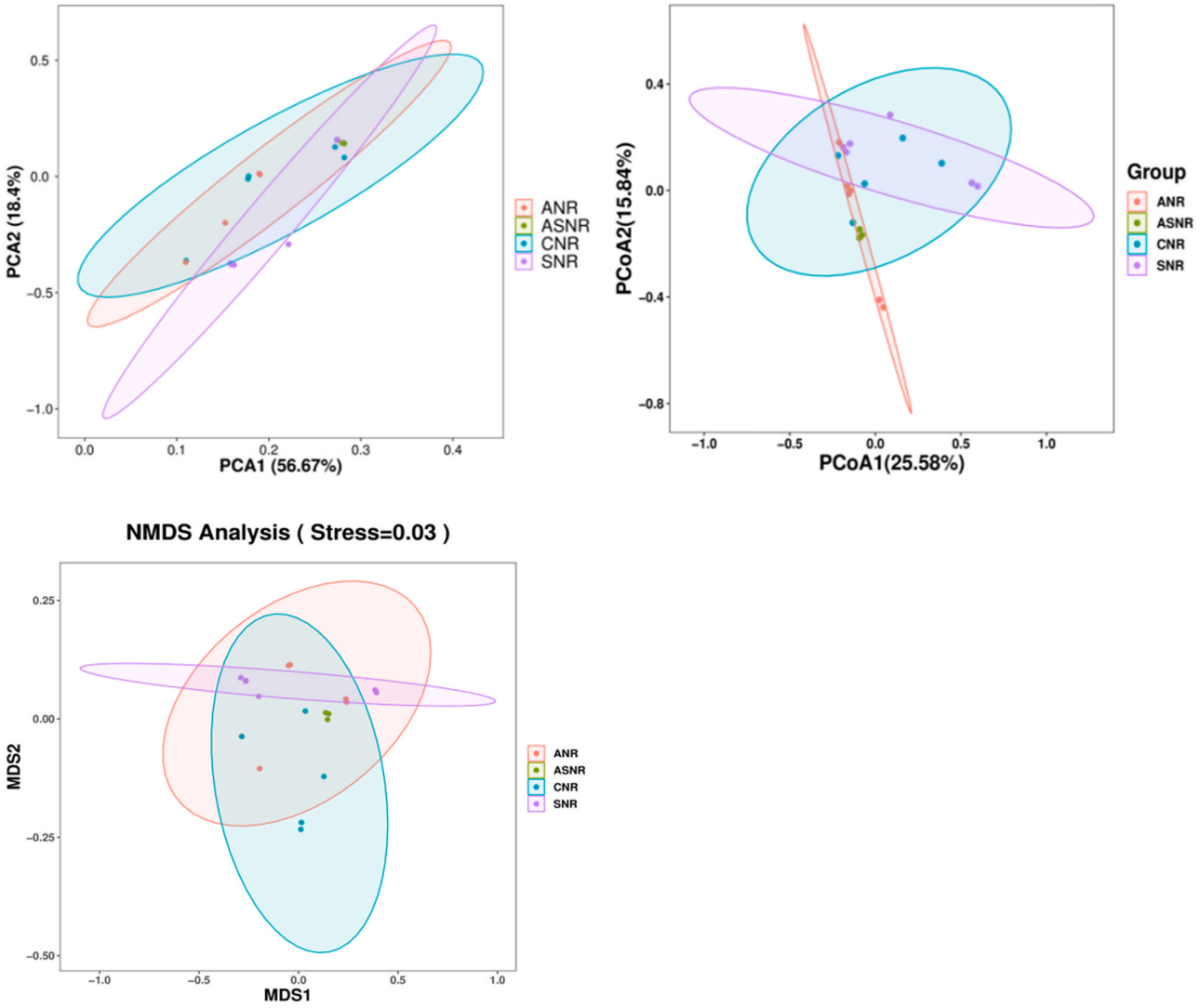

Fig. 3. Effect of NH_3 and Se treatments on beta diversity of gut microbiota in the jejunum of fattening pigs. Data in each group are described as mean ± SD (n = 6). The Kruskal–Wallis test was used to test for species differences between any two groups. (a) PCA: Principal component analysis; (b) PCoA: Principal coordinates analysis; (c) NMDS: Multidimensional scaling. CNR: intestinal contents in the C group; ANR: intestinal contents in the A group; SNR: intestinal contents in the Se group; ASNR: intestinal contents in the A + Se group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

analysis, and its analysis result is evaluated by stress coefficient. Our results showed that the stress coefficient was 0.03, and there were significant differences ($P < 0.05$) in species composition among the C group, A group and A + Se group.

3.5. Species analysis

A taxonomic analysis of the intestinal microflora in the jejunum of fattening pigs was shown in Fig. 4. At phylum level, Proteobacteria phylum and Firmicutes phylum acted as the predominance taxon in four groups. The Bacteroidetes phylum richness in the C group was the highest compared with that in the other three groups (Fig. 4a). Compared with the C group, levels of Clostridia and Alphaproteobacteria class significantly increased ($P < 0.05$) in the A group, levels of Gammaproteobacteria, Bacteroidia, Fusobacteriia and Actinobacteria significantly decreased ($P < 0.05$) (Fig. 4b). Interestingly, Se supplementation caused Saccharimonadia class to peak value and restored Actinobacteriato near-normal levels. In addition, microbial flora was also compared at the family level under the same conditions. The addition of Se caused the increase of Pseudomonadaceae and Caulobacteraceae, and decreased the proportion of Streptococcaceae, Peptostreptococcaceae and Pasteurellaceae (Fig. 4c). At genus level, there were significant differences in 10 microbial flora between the A group and the C group, among which the relative abundance of *Pseudomonas*, *Terrisporobacter*, *Brevundimonas*, *Clostridium_sensu_stricto_1* increased, and the relative abundance of *Actinobacillus*, *Moraxella*, *Porphyromonas*, *Bergeyella*, *Neisseria*, *Alloprevotella* decreased (Fig. 4d).

3.6. Advanced analysis

The results of phylogram tree, LDA effect size, Sparcc and PICRUSt2 of the intestinal microflora in the jejunum of fattening pigs were shown in Fig. 5. All genera of microorganism taxonomical classification were assigned to 27 phyla and their evolutionary relationships were evaluated by phylogram-tree. The genetic distance between two genera was closer, their kinship was more obvious (Fig. 5a). In Tenericutes phylum, the kinship between the *Terrimicrobium* and *Chthoniobacter* was closer than that between the *Terrimicrobium* and *Akkermansia*. In addition, although Treponema_2 and Rokubacteriales_unclassified belong to the same phylum, they have a distant evolutionary relationship in the

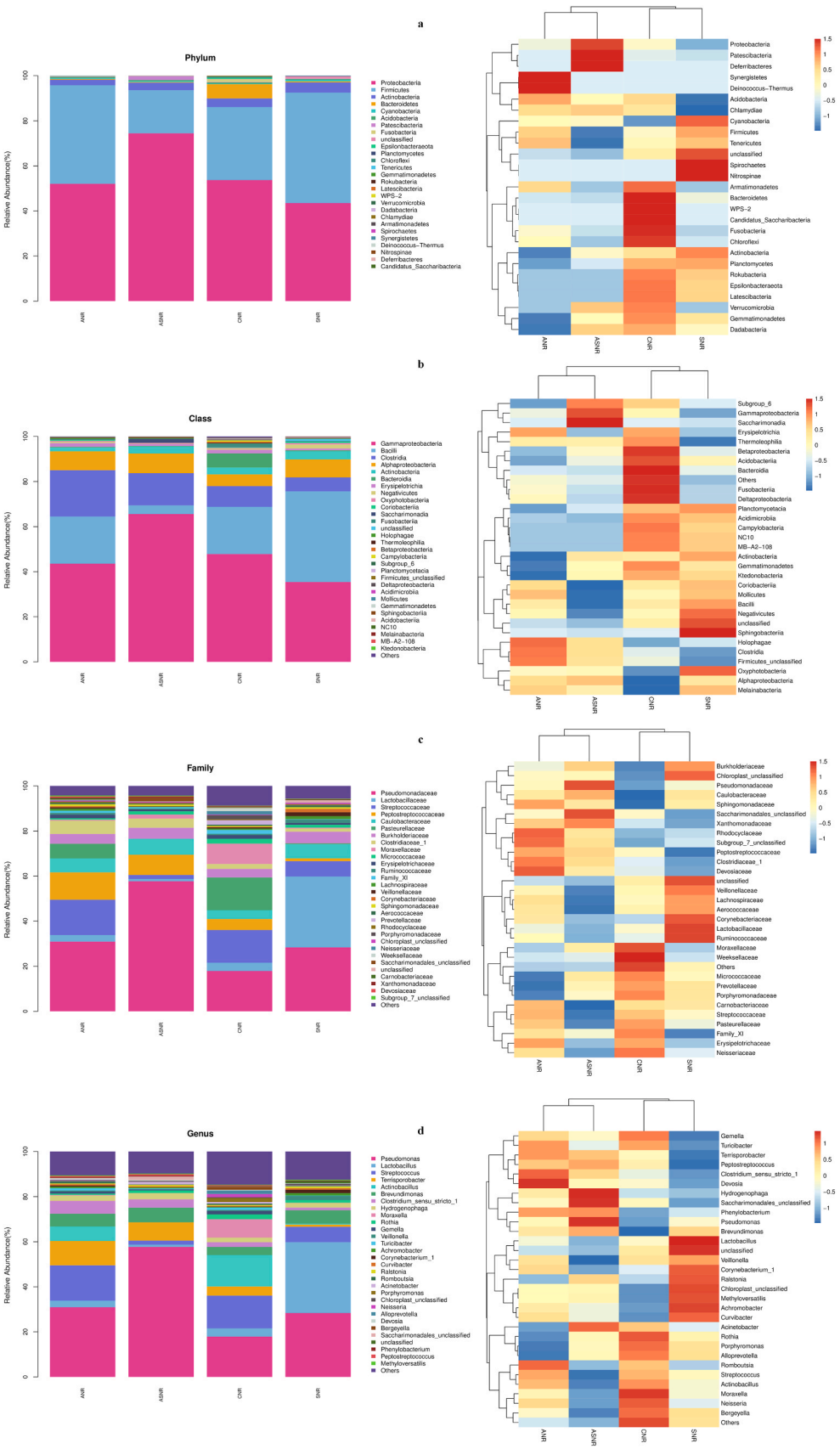

Fig. 4. Effect of ammonia and Selenium treatment on microbial diversity structure of jejunum in fattening pigs. Data in each group are described as mean ± SD (n = 6). The Kruskal–Wallis test was used to test the difference between any two groups. CNR: intestinal contents in the C group; ANR: intestinal contents in the A group; SNR: intestinal contents in the Se group; ASNR: intestinal contents in the A + Se group.

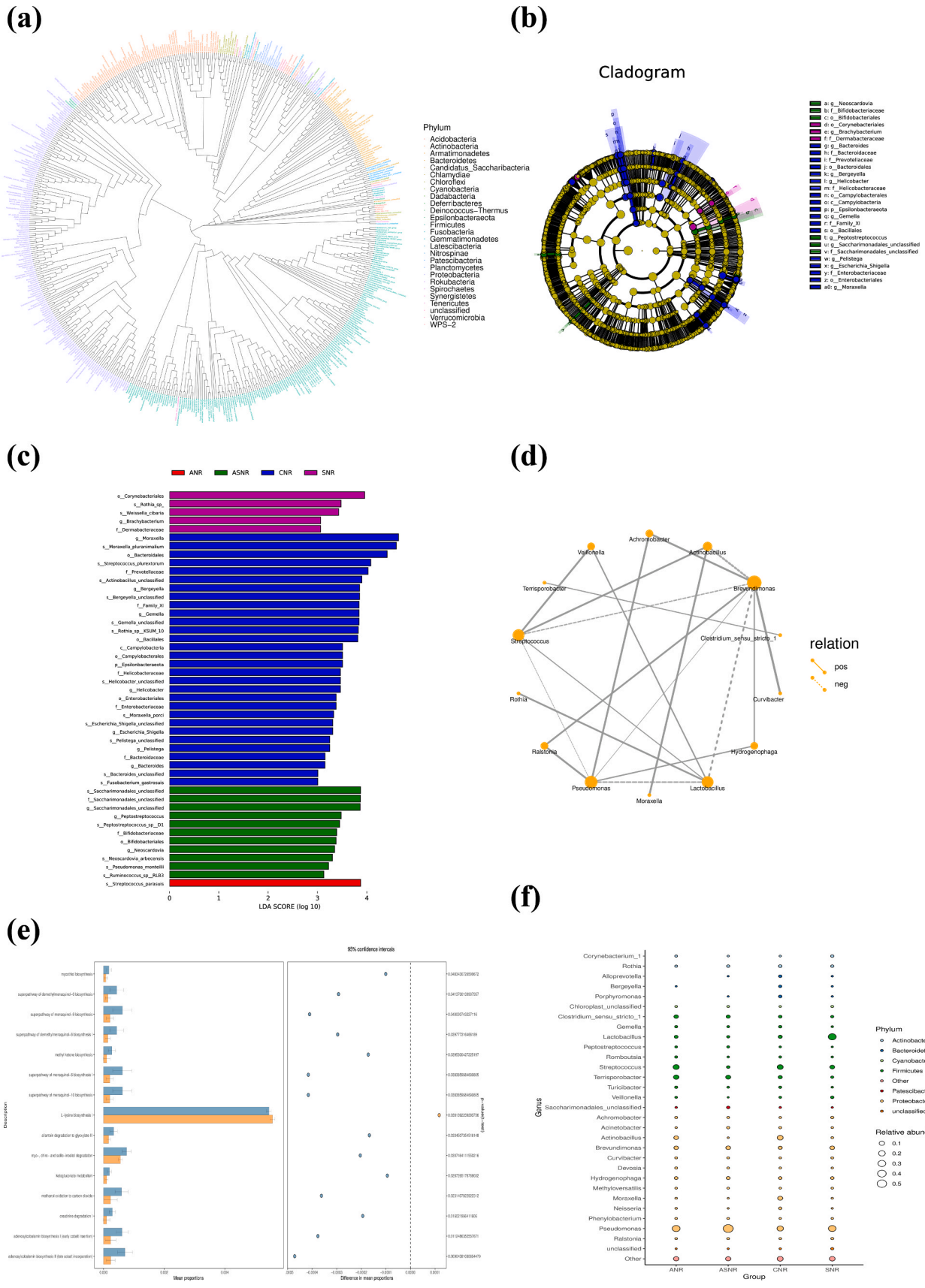

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Fig. 5. Advanced analysis of 16S rDNA sequencing technology. (a) Phylogram tree in the genus level. (b) Cladogram of LDA effect size (LEfSe) analysis based on taxonomical classification. Blue, green, pink and red represented C group, A + Se group, Se group and A group, respectively. LDA: Linear Discriminant Analysis. (c) Bar chart of LEfSe analysis. Blue, green, pink and red represented C group, A + Se group, Se group and A group, respectively. (d) Interaction network for genus level. Solid lines mean positive correlation and dotted lines mean negative correlation. The thickness of the line represented the association strength. Each dot represented the relative abundance of species. (e) Prediction of pathway functions using PICRUSt2 based on the KEGG pathway database. PICRUSt2: Phylogenetic Investigation of Communities by Reconstruction of Unobserved States- picrust2. (f) Bubble plot for top 31 genera. The color of each dot represented different phyla. The size of dots represented the relative abundance of each genera. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

phylogram tree.

In order to find the species with significant difference in abundance among different groups, LDA Effect Size (LEfSe) was used to analyze the difference of species abundance between the C and A groups. Our results showed that *Moraxella*, *Bergeyella*, *Gemella*, *Helicobacter, Escherichia_-Shigella, Pelistega and Bacteroides* were the top 7 abundant genera in the C group. In parallel, *Saccharimonadales_unclassified*, *Peptostreptococcus* and *Neoscardivia* were discriminating genera in the A + Se group. In the Se group, Corynebacteriales, Dermabacteraceas and *Brachybacterium* were biomarkers. Streptococcus_parasuis was the symbolic species in the A group that can be regarded as the referent of identification for NH_3 poisoning (Fig. 5b–c).

SparCC networks were established to evaluate the interaction correlation for different genera. The results displayed a positive relationship between *Achromobacter* and *Pseudomonas* and mutual exclusive relationship between *Brevundimonas* and *Lactobacillus* (Fig. 5d).

The shift in the presumptive biological functions of jejunal microbiota between the C group and A group were validated using PICRUSt2 and presented in Fig. 5e. For amino acid metabolism, the pathway associated with L-lysine biosynthesis in the A group was increased ($P < 0.05$). In the biological oxidation process, the pathway associated with the methanol oxidation to carbon dioxide was decreased ($P < 0.05$) in the A group.

In the bubble plot, top 31 genera in each group were classified to 8 phyla. Our results proved that the abundance of different genera showed significant changes in four groups. Proteobacteria phylum was the dominant phylum in the four treatment groups, and the relative abundance of *Lactobacillus* in the Se group was the highest (Fig. 5f).

3.7. Correlation of inflammatory markers and microbiota community

An analysis of the association between inflammatory markers (MMP-9, LTF, MPO, and Calprotectin) and predominant flora (*Pseudomonas*, *Lactobacillus*, and *Streptococcus*) was shown in Table 3. The results showed there was a positive correlation between MPO/*Pseudomonas,* MMP-9/*Pseudomonas,* Calprotectin/*Pseudomonas* ($P < 0.05$), but there was a negative correlation relationship between *Streptococcus* and MPO/LTF/Calprotectin ($P < 0.01$). Meanwhile, there was a strong positive correlation among four inflammatory markers ($P < 0.01$). In addition, there was a negative correlation between *Pseudomonas* and the other two genera (*Lactobacillus/Streptococcus*).

4. Discussion

More and more toxicological evidences have also shown that NH_3 is toxic to mammals, amphibians, aquatic organisms (Kim et al., 2019) and farm animals. Prolonged exposure to excessive NH_3 in livestock house can reduce the productivity and resistance of livestock and poultry, burn the nasal cavity, mouth and throat, and cause respiratory problems (Kearney et al., 2014), as well as liver, kidney, spleen and heart disorders (Wang et al., 2020). It has been demonstrated that various environment pollutants may aggravate inflammatory responses (Wang et al., 2018b), including NH_3 (An et al., 2019). MMP-9, LTF, MPO and Calprotectin in feces are important biomarkers for the evaluation of intestinal inflammation (Doron et al., 2018). MMP-9 belongs to one member of the family of zinc- and calcium- dependent endopeptidases (Dominika and Anna, 2020), which plays an important role on the decomposition of tissue remodeling-related extracellular matrix, such as gelatin. MMP-9 can eliminate chemokines, cytokine (Chopra et al., 2019) and release signaling proteins, which can mediate pathological pathways through cascaded reaction induced by cell signal molecule (Huang, 2018). Previous investigation has indicated that the knockdown of ORMDL3 gene can attenuate airway inflammation in asthmatic mice via the decrease of MMP-9 (Wang et al., 2019b). Moreover, a recent study showed that *Staphylococcus aureus* relied on MMP-9 to mediate aorta inflammation through a deprivation of ROS (Tsai et al., 2018). Therefore, we speculated that NH_3 in jejunum can promote the combination of related protein messenger to specific receptor through the secretion of MMP-9, thus leading to inflammatory damage of jejunum. LTF, a milk-derived glycoprotein which involved in the defense against inflammatory infection (Drago-Serrano et al., 2017) through sequestration of iron and interaction with infectious factors. LTF also dramatically inhibited the secretion of neutrophil extracellular traps that could hydrolyze pathogenic microbiota and enhanced inflammatory susceptibility by reducing IL-1 (Koshu et al., 2016). Calprotectin, a novel marker for intestinal inflammation, reflects abnormal neutrophil migration into the enteric lumen. The results showed that high concentration of NH_3 exposure could mediate the migration of excessive neutrophils into the jejunum and disturbs the immune response system, thereby causing jejunal inflammation through the synergism of LTF and Calprotectin. MPO, an enzyme from neutrophil grain, is released into the extracellular fluid in the inflammatory condition and plays an important role on the organic natural defense and immune response. In the inflammatory periodontitis model, the addition of cucurbit B reduced inflammation by reducing MPO content (Zhong et al., 2020). A large number of investigations, including the present experiment, also obtained similar conclusion with this study. In summary, in the present study, excessive NH_3 exposure resulted in increased levels of these four inflammatory markers, indicating that NH_3 caused inflammatory damage in jejunum. However, the inflammatory damage of jejunum could be alleviated in a certain extent after L-Selenomethionine supplementation. Therefore, we hypothesized that L-Selenomethionine supplementation could modulate these

Table 3
An analysis of the association between inflammatory markers and predominant flora.

	Pseudomonas	*Lactobacillus*	*Streptococcus*	MPO	LTF	MMP-9
Lactobacillus	− 0.542*					
Streptococcus	− 0.812**	0.708**				
MPO	0.607*	− 0.474	− 0.686**			
LTF	0.683**	− 0.570*	− 0.751**	0.954**		
MMP-9	0.545*	− 0.507	− 0.632*	0.967**	0.957**	
Calprotectin	0.625*	− 0.493	− 0.704**	0.959**	0.977**	0.974**

Note: *($P < 0.05$), **($P < 0.01$).

inflammation-related mediums and activate inflammation pathways.

The composition of microorganisms in the gut plays an important regulatory role in maintaining homeostasis (Parker et al., 2020). Our results showed that a variety of microbiota communities existed in the jejunum of fattening pigs, and there was remarkable significance among different treatment groups, indicating that NH_3 and Se could induce changes in the symbiotic relationship of microorganism. The dynamic intestinal microbiome composition system can promote the host to resist the invasion of pathogens, activate the immune response (Levy et al., 2017) and improve the resistance, which is related to the occurrence and development of many diseases. As shown in our results, the shift in typical gut microorganism abundance accompany with the abnormal secretion of inflammatory factors which means the appearance of chronic inflammation in the jejunum. High throughput 16S rDNA sequencing technology provides the possibility for further study on the structure and interaction relationship of commensal microorganisms (Kumara et al., 2019). In the current experiment, Proteobacteria and Firmicutes were dominant in the jejunum of finishing pigs in the four treatment groups. Interestingly, Bacteroidetes has a significant relative superiority only in the C group, while there is almost no Bacteroidetes phylum exists in another three groups, indicating the abundance of Bacteroidetes can be influenced by NH_3 concentration and diet component. It was reported that the richness of Bacteroidetes in the gut of crucian carp (*Carassius auratus)* exposed to 50 mg/L NH_3 concentration for 30 days was limited (Qi et al., 2017). Previous studies have shown that the Bacteroidetes in the intestine can decompose any form of dietary carbohydrates into its own energy. There was an increase in the Firmicutes/Bacteroidetes during weaning treatment of piglets (Meng et al., 2020). Our results showed that the ratio of Firmicutes to Bacteroidetes increased abnormally in the A group, while the ratio of Firmicutes to Bacteroidetes decreased in the A + Se group. These studies demonstrated that changes in environmental factors could significantly affect the ratio of Firmicutes to Bacteroidetes in gut microbes. In addition, *Terrisporobacter* and *Clostridium_sensu_stricto* genera in the A group were significantly higher than those in the C group, suggesting that they might mediate a series of inflammatory changes, while the addition of organic Se can alleviate the negative effect of NH_3 on the intestine by regulating *Terrisporobacter* and *Clostridium_sensu_stricto*.

Enteric microbiota composition widely involved in metabolism (Sonnenburg and Bäckhed, 2016), drug conversion and immune response (Tai et al., 2016). Inflammatory bowel disease (IBD), which could be influenced by many exogenous or endogenous factors, is a chronic inflammation in intestine elicited by the anomalous interaction between microorganism taxa in enteric canal and intestinal immune system (Yan et al., 2020). Gut microbiota flora could mediate cascaded inflammation response in organism through the changes of own structure composition and metabolite. A recent study (Pedret et al., 2018) demonstrated that *Bifidobacterium* could promote the growth of *Akkermansia* (Akk), and both of which could secrete abundant short-chain fatty acids (SCFAs), which were negative correlation with inflammatory lesion. A recent microbiological report also found that many bacteria in intestine interacted through secondary metabolic product except for the interaction between inflammation-related factors and microbiome. The above research theory was consistent with our study, which indicated that *Pseudomonas* could inhibit the growth of *Lactobacillus* and *Streptococcus*, and there was a positive interaction relationship between *Lactobacillus* and *Streptococcus.* Qi et al. (2017) reported that the relative abundance of *Flavobacterium* in the gut of crucian carp exposed to a high concentration of NH_3 increased, while the abundance of *Bacteroides* decreased. Our results showed that prolonged exposure to excessive NH_3 caused changes in inflammatory markers and intestinal flora, and relative content of inflammatory markers changed with the change of jejunal microbial niche, which were the first to demonstrate a correlation between inflammatory markers and intestinal flora in fattening pigs. However, there were little researches on the correlation between NH_3-mediated inflammation and gut microflora changes. In summary, we speculated that excess NH_3 inhalation could cause the shift in gut microbiota composition, and affect the function of tight junctions and intestinal barrier in jejunal epithelial cells (Zheng et al., 2019), which in turn disturb the balance in intestinal tract and lead to the chronic inflammation reaction in the jejunum.

In conclusion, our results indicated that prolonged exposure to high-level NH_3 could cause changes in inflammatory markers and beta diversity of intestinal microflora in fattening pigs. In the high NH_3 environment, there was a positive correlation between MMP-9 and *Pseudomonas*. In addition, the addition of L-selenomethionine could improve the imbalance of enteric microbial flora and the inflammatory injury of jejunum caused by NH_3. Changes in intestinal microflora and inflammatory markers can be used as important indicators to evaluate NH_3 toxicity, and studying changes in intestinal microflora is also an important mechanism to reveal NH_3 toxicity.

CRediT authorship contribution statement

Jun Bao and Yutao Li: Conceived and designed the current experiments. **Runxiang Zhang, Xiang Li and Jianhong Li**: Performed the experiments. **Wenbo Ji and Xiangyin Zeng**: analyzed these data. **Yutao Li**: Wrote the manuscript. **Jun Bao and Yutao Li**: Revised the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

An, Y., Xing, H., Zhang, Y., Jia, P., Gu, X., Teng, X., 2019. The evaluation of potential immunotoxicity induced by environmental pollutant ammonia in broilers. Poult. Sci. 98, 3165–3175.

Artíñano, B., Pujadas, M., Alonso-Blanco, E., Becerril-Valle, M., Coz, E., Gómez-Moreno, F.J., Salvador, P., Nuñez, L., Palacios, M., Diaz, E., 2018. Real-time monitoring of atmospheric ammonia during a pollution episode in Madrid (Spain). Atmos. Environ. 189, 80–88.

Bicer, Y., Dincer, I., 2018. Life cycle assessment of ammonia utilization in city transportation and power generation. J. Clean. Prod. 170, 1594–1601.

Chopra, S., Overall, C.M., Dufour, A., 2019. Matrix metalloproteinases in the CNS: interferons get nervous. Cell. Mol. Life Sci. 76, 3083–3095.

Dominika, W., Anna, H., 2020. Tamoxifen-induced alterations in the expression of selected matrix metalloproteinases (MMP-2, -9, -10, and -13) and their tissue inhibitors (TIMP-2 and -3) in the chicken ovary. Theriogenology 148, 208–215.

Doron, Y., Uri, K., Adi, L., Michal, M.A., Eyal, K., Dana, B.A.S., Sandra, N., Nina, L., Ella, F., Benjamin, A., Limor, S., Noam, O., Orit, P., Miri, Y., Abraham, R.E., Shomron, B.H., 2018. Su1178-Serum MMP-9- A novel biomarker for prediction of clinical relapse in patients with quiescent small bowel Crohn's disease. Gastroenterology 154 (6), s–495.

Drago-Serrano, M.E., Campos-Rodríguez, R., Carrero, J.C., De, lG.M., 2017. Lactoferrin: balancing ups and downs of inflammation due to microbial infections. Int. J. Mol. Sci. 18 (3), 501. https://doi.org/10.3390/ijms18030501.

Dąbrowska, K., Albrecht, J., Zielińska, M., 2018. Protein kinase C-mediated impairment of glutamine outward transport and SN1 transporter distribution by ammonia in mouse cortical astrocytes. Neurochem. Int. 118, 225–232.

Di Lorenzo, T., Melita, M., Cifoni, M., Galassi, D.M.P., Iannucci, A., Biricilti, S., Gori, M., Baratti, M., 2017. Effect of ammonia on the gene expression levels of the freshwater cyclopoid Eucyclopsserrulatus. Environ. Toxicol. Pharm. 51, 138–141.

Gili, E.N., Sílvia, F.H., Carlos, R., 2020. The diet-microbiome tango: how nutrients lead the gut brain axis. Curr. Opin. Neurobiol. 62, 122–132.

Helal, S.H.A.E.Z., Reham, Z.H., Metwally, M.M., Magda, M.E.M., Wed, A.A.H., 2019. Antioxidant, antiapoptotic, antigenotoxic, and hepatic ameliorative effects of L-carnitine and selenium on cadmium-induced hepatotoxicity and alterations in liver cell structure in male mice. Ecotoxicol. Environ. Saf. 173, 419–428.

Huang, H., 2018. Matrix metalloproteinase-9 (MMP-9) as a cancer biomarker and MMP-9 biosensors: recent advances. Sensors 18 (10), 3249. https://doi.org/10.3390/s18103249.

Jin, X., Xu, Z., Zhao, X., Chen, M., Xu, S., 2017. The antagonistic effect of selenium on lead induced apoptosis via mitochondrial dynamics pathway in the chicken kidney. Chemosphere 180, 259–266.

Jing, H., Wang, S., Wang, Y., Shen, N., Gao, X.J., 2020. Environmental contaminant ammonia triggers epithelial-to-mesenchymal transition-mediated jejunal fibrosis with the disassembly of epithelial cell-cell contacts in chicken. Sci. Total Environ. 726. https://doi.org/10.1016/j.scitotenv.2020.138686.

Kim, J.H., Kang, Y.J., Kim, K. Il, Kim, S.K., Kim, J.H., 2019. Toxic effects of nitrogenous compounds (ammonia, nitrite, and nitrate) on acute toxicity and antioxidant responses of juvenile olive flounder, *Paralichthys olivaceus*. Environ. Toxicol. Pharmacol. 67, 73–78.

Koshu, O., Mako, K., Yasuteru, U., Hiroshi, N., Jan, M.H., Tanya, M., Daigoro, H., Kazuo, S., Hiroshi, K., Mototsugu, T., Miho, K., Shinji, K., Keiichi, H., Masaomi, N., Toshiro, F., Matsuhiko, H., Junichi, H., 2016. Lactoferrin suppresses neutrophil extracellular traps release in inflammation. EBioMedicine 10, 204–215.

Kearney, G.D., Shaw, R., Prentice, M., Tutor-Marcom, R., 2014. Evaluation of respiratory symptoms and respiratory protection behavior among poultry workers in small farming operations. J. Agromed. 19, 162–170.

Kumara, D., Sun, Z., Cao, G., Xue, R., Hu, X., Gong, C., 2019. Study of gut bacterial diversity of *Bombyx mandarina* and *Bombyx mori* through 16S rRNA gene sequencing. J. Asia Pac. Entomol. 22, 522–530.

Levy, M., Blacher, E., Elinav, E., 2017. Microbiome, metabolites and host immunity. Curr. Opin. Microbiol. 35, 8–15.

Li, S., Tang, T., Guo, P., Zou, Q., Ao, X., Hu, L., Tan, L., 2019. A meta-analysis of randomized controlled trials: efficacy of selenium treatment for sepsis. Med. (Baltim.) 98 (9), e14733. https://doi.org/10.1097/MD.0000000000014733.

Meng, Q., Luo, Z., Cao, C., Sun, S., Ma, Q., Li, Z., Shi, B., Shan, A., 2020. Weaning alters intestinal gene expression involved in nutrient metabolism by shaping gut microbiota in pigs. Front. Microbiol. 11, 694. https://doi.org/10.3389/fmicb.2020.00694.

Plautz, J., 2018. Piercing the haze. Science 361, 1060–1063.

Parker, A., Fonseca, S., Carding, S.R., 2020. Gut microbes and metabolites as modulators of blood-brain barrier integrity and brain health. Gut Microbes 11 (2), 135–157.

Pedret, A., Valls, R.M., Calderón, P.L., Llauradó, E., Companys, J., Pla, P.L., Moragas, A., Martín, L.F., Ortega, Y., Giralt, M., Caimari, A., Chenoll, E., Genovés, S., Martorell, P., Codoñer, F.M., Ramón, D., Arola, L., Solà, R., 2018. Effects of daily consumption of the probiotic *Bifidobacterium animalis* subsp. *lactis* CECT 8145 on anthropometric adiposity biomarkers in abdominally obese subjects: a randomized controlled trial. Int. J. Obes. https://doi.org/10.1038/s41366-018-0220-0.

Qi, X., Xue, M., Yang, S., Zha, J., Wang, G., Ling, F., 2017. Ammonia exposure alters the expression of immune-related and antioxidant enzymes-related genes and the gut microbial community of crucian carp (*Carassius auratus*). Fish Shellfish Immunol. 70, 485–492.

Ramanantenasoa, M.M.J., Gilliot, J.M., Mignolet, C., Bedos, C., Mathias, E., Eglin, T., Makowski, D., Genermont, S., 2018. A new framework to estimate spatio-tem- poral ammonia emissions due to nitrogen fertilization in France. Sci. Total Environ. 645, 205–219.

Sonnenburg, J.L., Bäckhed, F., 2016. Diet-microbiota interactions as moderators of human metabolism. Nature 535, 56–64.

Tsai, M., Wu, C., Lin, W., Cheng, C., Chuang, C., Chang, K., Jiang, R., Hsu, J., Lee, I., 2018. Infection with *Staphylococcus aureus* elicits COX-2/PGE_2/IL-6/MMP-9-dependent aorta inflammation via the inhibition of intracellular ROS production. Biomed. Pharmacother. 107, 889–900.

Tai, N., Peng, J., Liu, F., Gulden, E., Hu, Y., Zhang, X., Chen, L., Wong, F.S., Wen, L., 2016. Microbial antigen mimics activate diabetogenic CD8 T cells in NOD mice. J. Exp. Med. 213, 2129–2146.

Williams, T.A., Bonham, L.A., Bernier, N.J., 2017. High environmental ammonia exposure has developmental-stage specific and long-term consequences on the cortisol stress response in zebrafish. Gen. Comp. Endocrinol. 254, 97–106.

Wang, J., Ji, H., Wang, S., Liu, H., Zhang, W., Zhang, D., Wang, Y., 2018a. Probiotic lactobacillus plantarum promotes intestinal barrier function by strengthening the epithelium and modulating gut microbiota. Front. Microbiol. 9, 1953. https://doi.org/10.3389/fmicb.2018.01953.

Wang, W., Chen, M., Jin, X., Li, X., Yang, Z., Lin, H., Xu, S., 2018b. H_2S induces Thi/Th2 imbalance with triggered NF–κB pathway to exacerbate LPS-induce chicken pneumonia response. Chemosphere 208, 241–246.

Wang, H., Zhang, Y., Han, Q., Xu, Y., Hu, G., Xing, H., 2020. The inflammatory injury of heart caused by ammonia is realized by oxidative stress and abnormal energy metabolism activating inflammatory pathway. Sci. Total Environ. 742, 140532 https://doi.org/10.1016/j.scitotenv.2020.140532.

Wang, S., Li, X., Wang, W., Zhang, H., Xu, S., 2019a. Application of transcriptome analysis: oxidative stress, inflammation and microtubule activity disorder caused by ammonia exposure may be the primary factors of intestinal microvilli deficiency in chicken. Sci. Total Environ. 696, 134035 https://doi.org/10.1016/j.scitotenv.2019.134035.

Wang, H., Liu, Y., Shi, J., Cheng, Z., 2019b. ORMDL3 knockdown in the lungs alleviates airway inflammation and airway remodeling in asthmatic mice via JNK1/2-MMP-9 pathway. Biochem. Biophys. Res. Commun. 516, 739–746.

Wu, W., Kong, Q., Tian, P., Zhai, Q., Wang, G., Liu, X., Zhao, J., Zhang, H., Lee, Y., Chen, W., 2020. Targeting gut microbiota dysbiosis: potential intervention strategies for neurological disorders. Engineering 6, 415–426.

Xing, H., Peng, M., Li, Z., Chen, J., Zhang, H., Teng, X., 2019. Ammonia inhalation-mediated miR-202-5p leads to cardiac autophagy through PTEN/AKT/mTOR pathway. Chemosphere 235, 858–866.

Xu, Y., Li, Z., Zhang, S., Zhang, H., Teng, X., 2020. miR-187-5p/apaf-1 axis was involved in oxidative stress-mediated apoptosis caused by ammonia via mitochondrial pathway in chicken livers. Toxicol. Appl. Pharmacol. 388, 114869 https://doi.org/10.1016/j.taap.2019.114869.

Yan, S., Yang, B., Ross, R.P., Stanton, C., Zhang, H., Zhao, J., Chen, W., 2020. *Bifidobacterium longum* subsp. *longum* YS108R fermented milk alleviates DSS induced colitis via anti-inflammation, mucosal barrier maintenance and gut microbiota modulation. J. Funct. Foods 73, 104153. https://doi.org/10.1016/j.jff.2020.104153.

Yu, Z., Wu, X., Zheng, L., Dai, Z., Wu, L., 2020. Effect of acute exposure to ammonia and BFT alterations on Rhynchocypris lagowski: digestive enzyme, inflammation response, oxidative stress and immunological parameters. Environ. Toxicol. Pharmacol. 78, 103380 https://doi.org/10.1016/j.etap.2020.103380.

Zhong, H., Huang, Y., Deng, X., Liu, M., Luo, W., 2020. Cucurbitacin B supplementation reduces inflammatory responses and alveolar bone loss via regulating MPO, COX-2 and RANK/RANKL/OPG signals in a rodent model of ligature-induced periodontitis. J. King Saud Univ. Sci. 32, 1889–1895.

Zheng, S., Jin, X., Chen, M., Shi, Q., Zhang, H., Xu, S., 2019. Hydrogen sulfifide exposure induces jejunum injury via CYP450s/ROS pathway in broilers. Chemosphere 214, 25–34.

Zhang, K., Zhu, Y., Wang, X., Zhao, X., Li, S., Teng, X., 2017. Excess manganese-induced apoptosis in chicken cerebrums and embryonic neurocytes. Biol. Trace Elem. Res. 180, 297–305.

Zarrinpar, A., Chaix, A., Yooseph, S., Panda, S., 2014. Diet and feeding pattern affect the diurnal dynamicsof the gut microbiome. Cell Metab. 20, 1006–1017.