Gut microbiota dysbiosis exaggerates ammonia-induced tracheal injury via TLR4 signaling pathway

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Abstract

Background: Ammonia is a toxic air pollutant that causes severe respiratory tract injury in animals and humans. Gut microbiota has been found to be involved in the development of respiratory tract injury induced by air pollutants, however, the specific mechanism requires investigation.

Results: Here, we found that, inhaled ammonia induced tracheal injury by reducing expression of claudin-1, increasing expression of muc5ac, TLR4, MyD88, NF-κB and cytokines (TNF-α, IL-1β, IL-6 and IL-10), and also altering tracheal microbiota composition. Spearman correlation analysis indicated that gut microbiota dysbiosis positively correlated with TLR4 level in the trachea. Antibiotic depletion intestinal microbiota treatment reduced the severity of ammonia-induced tracheal injury via TLR4 signaling pathway. Microbiota transplantation induced the tracheal injury via TLR4 signaling pathway even without the ammonia exposure.

Conclusions: These results indicate that gut microbiota dysbiosis exaggerates ammonia-induced tracheal injury via TLR4 signaling pathway. In addition, the [Ruminococcus]_torques_group, Faecalibacterium, unclassified_f_Lachnospiraceae may be the core gut microbiota contributing to the alterations of tracheal microbiota composition.

Background

Ammonia (NH₃) is a kind of contaminant in environmental that has been not effectively regulated in the world and is also a part of haze (PM₂.₅), which seriously affects the health of both humans and animals. Moreover, NH₃ is considered to be one of the most toxic pollutants in the broiler house (David et al., 2015). Ni (2012) reported that the typical range of average NH₃ level in broiler houses was about 35 ppm. As the first barrier of defense against inhaled air pollutants, respiratory tract is first stimulated by ammonia exposure (David et al., 2015; Xiong et al., 2016). Exposure to NH₃ causes the tracheal cilia to produce increased mucus or the cilia to be removed, and also leads to chronic respiratory diseases and significantly worsens symptoms of emphysema and asthma (Coltar et al., 2013; Warren, 1962; Dutton et al., 1959).

Recent studies in animals have been reported associations between gut microbiota and respiratory tract injury in many pathologies (Alexander et al., 2018; Marjolaine et al., 2019; Cui et al., 2020; Wang et al., 2021). With the development of science and technology, we have a deeper understanding of the gut microbiota, especially the concept of gut-airway axis. A research reported that intestinal microbiota can affect the pulmonary immune response in allergic airway diseases in mice through the gut-airway axis (Li et al., 2018). A previous study in Frontier in Immunology found the gut-airway axis or a potential target for the treatment of pulmonary tuberculosis (Negi et al., 2019). A paper also showed that the intestinal microbiota can affect the individual’s susceptibility to allergic airway diseases such as asthma through the gut-airway axis (Wypych et al., 2021). However, the mechanism of gut microbiota impacts on the respiratory tract injury induced by ammonia is still unknown. In addition, Lanaspa (2017) has also
reported that the microbial population of respiratory tract is closely related to respiratory diseases. It is found that high ammonia exposure will change the composition of microbiota community by changing the proportion of beneficial bacteria and harmful bacteria in the respiratory tract, and then cause respiratory diseases (Hamilton et al., 1998; Wang et al., 2019; Liu et al., 2020). Given the evidence that trachea in the airway not only has the function of respiratory ventilation, but also is located above the respiratory tract. Trachea is also one of the target organs stimulated by inhaled ammonia (Puchelle et al., 1995; Xiong et al., 2016; Wang et al., 2020). However, the contribution of gut microbiota to the tracheal injury induced by ammonia remains unknown.

Lipopolysaccharides (LPS) may be an important way in which gut microbiota affects the tracheal injury induced by ammonia exposure. LPS, as a specific component of the cell wall of Gram-negative bacteria, could translocate into the blood and reach the respiratory tract via gut-airway axis and then activate TLR4 signaling pathway (Takeuchi et al., 1999) and then induced the inflammatory injury in the airway. Moreover, the activation of TLR4 signaling pathway is an important mechanism of intestinal microbiota dysbiosis affecting the development of lung in the condition of pulmonary hypertension caused by postnatal growth restriction inducing an increasing in intestinal Enterobacteriaceae (Wedgwood et al., 2020). Our previous research indicated that exposure to ammonia changed the composition of intestinal microbiota and particularly increased the relative abundance of Gram-negative bacteria (Zhou et al., 2021). Token together, we hypothesized that gut microbiota dysbiosis mediated tracheal injury induced by inhaled ammonia exposure possibly via activating tracheal TLR4 signaling pathway.

The aim of our experiment was conducted to investigate the contribution of intestinal microbiota to the tracheal injury induced by exposure to ammonia. In order to clarify the relationship between tracheal microbiota and intestinal microbiota, we used the 16 S rRNA gene sequence to analysis the changes of tracheal microbiota and intestinal microbiota. Tracheal inflammatory injury was detected by the histological observation and also expression of claudin-1, muc5ac, caspase3, TLR4, MyD88, NF-κB, IL-1β, TNF-α, IL-6, IFN-γ, IL-10 by RT-PCR. Then We determined the correlation between gut microbiota and tracheal injury through intestinal microbiota depleted by antibiotic treatment and microbiota transplantation. These data will provide some basic information for alleviating the ammonia toxic and therapy.

**Results**

*Inhaled ammonia exposure causes tracheal injury.*

As shown in Fig. 1, compared to the control group (Fig. 1A, B), in the ammonia exposure group (Fig. 1C, D), the tracheal inflammation was significant, and the tracheal mucosa was significantly thickened; mucosal epithelium was severely damaged (green arrow), a large number of epithelial cells fell off, and local squamous metaplasia was seen (red arrow). In addition, inhaled ammonia exposure decreased the tracheal claudin-1 expression \( (P<0.05; \) Fig. 1E) and increased the tracheal muc5ac and caspase3 expression \( (P<0.05; \) Fig. 1E). Furthermore, inhaled ammonia exposure significantly increased TLR4,
MyD88, NF-κB expression \( (P < 0.05) \) and also increased the cytokines such as IL-1β, TNF-α, IL-6, IL-10 expression \( (P < 0.05) \). Thus inhaled ammonia exposure caused the tracheal injury.

**Inhaled ammonia exposure disrupts the tracheal and ileal microbiota in broiler.**

To address how inhaled ammonia exposure may affect the tracheal microbiota, we studied the tracheal microbiota by PCoA analysis based on Bray Curtis distance, and found that inhaled ammonia exposure altered the structure of tracheal microbiota \( (R^2 = 0.2449, P = 0.004, \text{Fig. 2A}) \). Then we also analyzed the relative abundance of tracheal microbiota at the phylum level (Fig. 2B) and genus level (Fig. 2C), the results indicated that the composition of tracheal microbiota was significant different between control and 35 ppm group. To further analysis the tracheal microbiota composition between control and 35 ppm groups, we used the Student’s test to compare the microbiota difference at the phylum level (Fig. 2D) and genus level (Fig. 2E) respectively and found that exposure to inhaled ammonia significantly increased the abundance of [Ruminococcus]_torques_group, unclassified_f_Lachnospiraceae, Faecalibacterium, Ruminococcaceae_UCG-014, Alistipes.

Having established that inhaled ammonia exposure alters tracheal microbiota in broiler chickens, we next asked whether exposure to inhaled ammonia could alter the ileal microbiota community. To accomplish this, we characterized ileal microbiota community using 16S rRNA gene sequencing. Inhaled ammonia exposure had a significant effect on the ileal microbiota composition. The difference in community composition under inhaled ammonia exposure was driven by relative depletion of the Firmicutes phylum and relative enrichment of the Proteobacteria and Bacteroidetes phylum \( (P < 0.05 \text{ for both; Fig. 2F}) \). Ammonia-exposed broiler showed significant relative enrichment of Escherichia-Shigella, Faecalibacterium, Streptococcus, Ruminococcaceae_UCG-014, Rothia, unclassified_f_Lachnospiraceae, [Ruminococcus]_torques_group, unclassified_f_Ruminococcaceae \( (P < 0.05) \) compared to the control group (Fig. 2G).

Moreover, we analyzed the dynamic changes of four key bacterial genera in the trachea and ileum with ammonia concentration, and the results indicated that [Ruminococcus]_ torques_group, Ruminococcaceae_UCG-014, Faecalibacterium, Unclassified_f_Lachnospiraceae showed similar changes both in the trachea and ileal under inhaled ammonia exposure (Fig. 2H). Taken together, the above results indicated inhaled ammonia exposure disrupted both the tracheal and ileal microbiota in broiler, and microbiota of both sites may be cross-talk.

**In inhaled ammonia-exposed broiler, variation in tracheal inflammation correlates with variation in ileal microbiota.**

We next asked whether variation in ileal microbiota could explain variation in tracheal inflammation in ammonia-exposed broiler. We compared the composition of ileal microbiota with tracheal inflammation using the spearman correlation analysis (Fig. 3A, B). Tracheal concentrations of IL-10, IL-6, TNF-α and IL-1β were significantly positively correlated with the community composition of ileal microbiota \( (P < 0.05) \). In addition, as shown in Fig. 3A, enrichment of ileal microbiota with the Streptococcus and Escherichia-
*Shigella* was correlated with increased tracheal TLR4 concentration ($P < 0.05$; Fig. 3B). The above results indicated ileal microbiota correlated with tracheal inflammation in inhaled ammonia exposed-broiler.

**Intestinal microbiota transplantation induces the tracheal injury.**

To further determine the contribution of ileal microbiota to the tracheal inflammation injury caused by inhaled ammonia exposure, we transplanted the ileal microbiota collected from the inhaled ammonia exposed-broilers to the healthy broiler. The results showed that there was diffuse infiltration of inflammatory cells in the mucosa; Submucosal edema, increased tissue space, accompanied by connective tissue hyperplasia in the transplantation group, however, the histological structure was normal and no obvious pathological changes were found in the PBS group (Fig. 4A, B). In addition, the concentrations of TLR4, IL-1β, TNF-α were significantly increased in the transplantation group as opposed to the PBS group (Fig. 4C). Taken together, the ileal microbiota played an important role in the tracheal inflammation induced by exposure to inhaled ammonia via TLR4 signaling pathway.

We also analyzed the tracheal microbiota between the transplantation group and PBS group, the results showed that microbiota transplantation also significantly increased the relative abundance of *Ruminococcus* _torques_ group, *Faecalibacterium*, *Unclassified_f_Lachnospiraceae*, etc in the trachea (Fig. 4D). This result indicated that these bacteria may be the microbiota-bridge between the trachea and ileal.

**Antibiotic treatment reduces ammonia-induced tracheal injury.**

Having determined that ileal microbiota was altered by inhaled ammonia exposure and correlated with tracheal inflammation, we next sought to determine whether the intestinal microbiota play a causal role in the pathogenesis of ammonia induced tracheal injury. To accomplish this, we compared the effects of inhaled ammonia exposure in broiler with conventional microbiota and experimentally manipulated microbiota, using broad antibiotics. As shown in Fig. 5, local necrosis and abscission of epithelial cells were observed; There was diffuse infiltration of inflammatory cells in lamina propria in the group of inhaled ammonia exposure treatment (Fig.5A), however, the histological structure was normal and no obvious pathological changes were found in the group of antibiotic treatment under inhaled ammonia exposure (Fig.5B). This result indicated antibiotic treatment reduces ammonia-induced tracheal injury. In addition, the concentrations of TLR4, TNF-α, IL-10 were reduced in the group of antibiotic treatment ($P < 0.05$; Fig.5C).

In addition, we also investigated the tracheal microbiota between the antibiotic treatment and ammonia exposure group, and found that antibiotic treatment significantly decreased the relative abundance of *Ruminococcus* _torques_ group, *Faecalibacterium*, *Unclassified_f_Lachnospiraceae*, etc in the trachea (Fig. 5D). This result further indicated tracheal microbiota and ileal microbiota were cross-talk under inhaled ammonia exposure, which might be communicated by changing the relative abundance of some bacteria such as *Ruminococcus* _torques_ group, *Faecalibacterium*, *Unclassified_f_Lachnospiraceae* in the two parts.
Discussion

The mechanism of inhaled ammonia exposure toxicity is of great ecological significance and health implications due to its contribution to form haze and difficult to regulated effectively worldwide. It has been reported that gut microbiota dysbiosis played an important role in the respiratory tract injury. However, the understanding of this mechanism is not comprehensive. Here, our study first demonstrated that the contribution of gut microbiota in exaggerating ammonia-induced tracheal injury via TLR4 signaling pathway.

In our present study, we found that ileal microbiota dysbiosis was correlated with tracheal inflammation injury induced by inhaled ammonia exposure via TLR4 signaling pathway, which was determined by spearman analysis, intestinal antibiotic treatment and also microbiota transplantation. Increasing evidences indicated that intestinal microbiota played an important role in the respiratory tract injury caused by some respiratory disease or virus infection through gut-lung axis, such as lung inflammation injury caused by *mycoplasma gallisepticum* infection (Wang et al., 2021), pulmonary inflammation response caused by Klebsiella pneumoniae (Marjolaine et al., 2019), lung injury induced by influenza virus (Alexander et al., 2018), asthma (Cui et al., 2020). To our knowledge, Lipopolysaccharides (LPS) derived by Gram-negative bacteria could translocate into the blood and reach the respiratory tract via gut-lung axis and then activate TLR4 signaling pathway (Takeuchi et al., 1999), which was consist with the increase of the relative abundance of Gram-negative bacteria in our present study. Moreover, the relationship between TLR4 signaling pathway and intestinal microecology was also be demonstrated in the condition of acute lung injury (Tang et al., 2021). Taken together, ileal microbiota dysbiosis exaggerated tracheal inflammation induced by inhaled ammonia exposure via TLR4 signaling pathway in broiler.

In addition, we also found that exposure to inhaled ammonia altered the tracheal and ileal microbiota, and also significantly increased the relative abundance of *Ruminococcus* _torques_group, Faecalibacterium, unclassified_f_Lachnospiraceae both in the two parts. Combined with the results of intestinal antibiotic treatment and microbiota transplantation treatment, we also found that when the ileal microbiota was eliminated or added, the relative abundance of *Ruminococcus* _torques_group, Faecalibacterium, unclassified_f_Lachnospiraceae in the trachea showed similar trends. This result indicated that these bacteria might be the microbiota-bridge in the ileum and trachea under inhaled ammonia exposure, which suggested the two parts were cross-talk. To our knowledge, *Ruminococcus_torques group* commonly exists in the intestine and was a “bad bacteria”, and its increase could disrupt the intestinal barrier and also be associated with Crohn (Martinez-Medina et al., 2006; Png et al., 2010), IBD (Lyra et al., 2009), gastrointestinal related diseases (Malinen et al., 2010; De Cesare et al., 2017). *Faecalibacterium* was relatively high in the healthy condition (Miquel et al., 2013) and was an anti-inflammatory bacteria. We found that in the present study, *Faecalibacterium* increased under inhaled ammonia exposure, which may be due to the concentration and duration of ammonia exposure that made the broiler be low grade inflammation. Taken together, under inhaled ammonia
exposure, *Faecalibacterium, [Ruminococcus]_torques_group, unclassified_f_Lachnospiraceae* may be the core gut microbiota contributing to the alterations of tracheal microbiota composition.

Limitedly, the crosstalk between intestinal microbiota and tracheal injury needs more time points or more ammonia exposure concentration to further determine it. In the present study, we should also measure the LPS concentrations in the blood, trachea, or ileum. In addition, intestinal microbiota may also be through other pathways to affect the tracheal inflammation injury under inhaled ammonia exposure, such as NOD-like receptors or short chain fatty acids.

**Conclusions**

In conclusion, these results suggest that gut microbiota dysbiosis could contribute to tracheal injury induced by exposure to inhaled ammonia via TLR4 signaling pathway. Moreover, the *[Ruminococcus]_torques_group, Faecalibacterium, unclassified_f_Lachnospiraceae* may be the core gut microbiota contributing to the alterations of tracheal microbiota composition.

**Methods**

**Animals, groups design and sample collection**

The experiment procedures were approved by the Animal Welfare Committee of Institute of Animal Sciences, Chinese Academy of Agricultural Sciences. 14-day-old 200 male Arbor Acres (AA) broilers were collected to adapt to the environment chamber for 1 w before the ammonia exposure experiment. Then 144 broilers were randomly selected and divided into two groups when they were 21 days old, and each group consisted of 72 healthy birds. In the control group, the concentration of ammonia exposure was < 3 ppm within the chamber, while broilers were exposed to ammonia exposure at a concentration of 35 ppm. The concentrations of NH$_3$ in the two chambers were monitored with a LumaSense Photoacoustic Field Gas-Monitor INNOVA 1412 (Santa Clara, CA, USA) during the entire experiment. The trial lasted 21 days.

At the last day of the experiment, all the broilers were weighted and then one broiler of average weight was randomly selected to euthanize. Tracheal tissues were collected to analyze the histological changes using the histological examination and also measure the expression of claudin-1, muc5ac, TNF-α, caspase3, IL-1β, MyD88, IL-6, TLR4, IL-10, NF-κB, IFN-γ. We also collected the ileal contents to analyze the changes of microbiota community using the 16S rRNA sequence.

**Antibiotic treatment**

To determine whether the intestinal microbiota played an important role in the respiratory tract inflammation induced by exposure to ammonia, we use the combinatorial antibiotics to feed the broilers to deplete the intestinal microbiota. Briefly, enteric broad spectrum antibiotics (100 mg/g streptomycin, 100 mg/g vancomycin, 100 mg/g metronidazole, 100 mg/g amoxicillin) were feed to the broilers
beginning 3 wk before the ammonia exposure treatment, and until the end of the experiment, as also described by the Zhou et al., 2021 and Wang et al., 2014.

**Microbiota transplantation**

To further clarify whether the intestinal microbiota played an important role in the respiratory tract injury induced by exposure to ammonia, we also transplanted the microbiota from the ammonia exposure broilers to the healthy broiler. Briefly, we collected the contents of ileal from ammonia exposure broiler and suspended in PBS, and centrifuged, and then took the supernatant to administer to the healthy broiler.

**Histopathological examination (HE)**

To analyze the tracheal histochemical changes, histopathological examination was used, and the specific method was according to the standard procedure.

**16S rRNA Miseq sequencing and bioinformatics analysis**

We used the 16S rRNA Miseq sequencing to detect the intestinal microbiota community. Briefly, the microbial DNA from contents of ileal were extracted by the E.Z.N.A.® Stool DNA Kit (Omega Bio-tek, Norcross, GA, U.S.). The amplified primers were the V3-V4 regions of 338F (5’-ACTCCTACGGGAGGCAGCAG-3’) and 806R (5’-GGACTACHVGGGTWTCTAAT-3’). All the bioinformatics analysis was on the cloud. majorbio.com.

**Real-time quantitative PCR**

According to the manufacturer’s instruction, we used the TRIzol reagent (Invitrogen, Carlsbad, CA) to extract the total RNA from the tracheal samples. The procedure of RT-PCR was performed by the light cycler 96 system (Roche, Basel, Switzerland). As shown in the table 2, we listed the reference gene, target gene and also their primer sequences.

**Enzyme-linked immunosorbent assay (ELISA)**

We use the ELISA kits to analyze the levels of IL-10, TLR4, IL-1β, TNF-α, IL-6 in the trachea (Jianglai biotechnology CO., Ltd, Shanghai, China). For specific methods, refer to the manual.

**Statistical analysis**

Student-t test of SPASS 26.0 software (SPASS Inc., Chicago, IL, USA) were used to analyze the levels of claudin-1, muc5ac, caspase3, TNF-α, TLR4, IFN-γ, MyD88, IL-10, NF-κB, IL-1β, IL-6. Data were present as mean ± SE. $P < 0.05$ was set as“significance”.

**Declarations**
Acknowledgements

Not applicable.

Authors’ contributions

ZMH conceived, designed, and supervised the study. ZY, ZX did the animal experiment. ZY, ZX and FJH conducted bioinformatics analyses. ZY wrote the drafts of the manuscript. ZY, ZMH, ZX and FJH commented on and revised the drafts of the manuscript. All authors read and approved the final draft of the manuscript.

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Availability of data and materials

The datasets generated in the current study were deposited to the NCBISRA database under the BioProject accession no.PRJNA838096 and no.PRJNA838120.

Ethics approval and consent to participate

The experiment procedures were approved by the Animal Welfare Committee of Institute of Animal Sciences, Chinese Academy of Agricultural Sciences.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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References


23. Wang, T., He, Q., Yao, W., Shao, Y., Li, J., Huang, F., 2019. The variation of nasal microbiota caused by low levels of gaseous ammonia exposure in growing pigs. Front. in microbio. 10, 1083.


**Tables**

There are not any tables provided with this version

**Figures**

**Figure 1**

**Inhaled ammonia exposure causes tracheal injury.** A: Representative histological images of the tracheal tissues analyzed by H.E. staining in the control group (A, 100 × magnification; B, 200 × magnification) and ammonia exposure group (C, 100 × magnification; D, 200 × magnification); E: expression of markers of tracheal epithelial barrier and apoptosis mRNA; F: expression of tracheal TLR4, MyD88, NF-κB; G: expression of cytokines in the tracheal. (n=6)
Figure 2

**Inhaled ammonia exposure alters both tracheal and ileal microbiota in broilers.** A: PCoA analysis based on the Bray-Curtis distance in tracheal microbiota; B: Relative abundance of tracheal microbiota composition at the phylum level; C: Relative abundance of tracheal microbiota composition at the genus level; D: Student’s test on the phylum level in the trachea; E: Student’s test on the genus level in the trachea. F: Student’s test on ileal microbiology phylum composition. G: Student’s test on ileal microbiology genus composition. H: Changes in key bacterial relative normalized abundance over ammonia concentration in the trachea and ileum. y axis is normalized log10 abundance plotted versus ammonia concentration (x axis) for the 4 bacterial genera indicated.

Figure 3

**Ileal microbiota is correlated with tracheal inflammation.** A: The Spearman correlation analysis between the ileal microbiota and tracheal cytokines. B: The Spearman correlation analysis between the ileal microbiota and tracheal TLR4, MyD88 and NF-κB.
Intestinal microbiota transplantation collected from ammonia-exposed broiler to healthy broiler induced tracheal inflammation and altered tracheal microbiota. A: Changes in tracheal histomorphological in the ileal microbiota transplantation group. B: Changes in tracheal histomorphological in PBS group. C: Effect of microbiota transplantation on changes of TLR4, TNF-α, IL-1β, IL-6, IL-10 in the trachea. D: Difference
between tracheal microbiology genus composition in the NY group and P group. NY: transfer of intestinal microbiota from ammonia exposure broiler into healthy broiler, P: PBS-treated into the healthy broiler.

**Figure 5**

**Antibiotic treatment reduces ammonia-induced tracheal injury.** Changes in tracheal histomorphological: A: broilers were treated with ammonia exposure; B: broilers were treated with combinatorial antibiotics in...
diet under ammonia exposure. C: Effect of antibiotic depletion of intestinal microbiota under ammonia exposure on changes of TLR4, TNF-α, IL-1β, IL-6, IL-10 in the trachea. D: Difference between tracheal microbiology genus composition in the NK group and N group. N: 35 ppm ammonia exposure group, NK: combinatorial antibiotics in diet under ammonia exposure.