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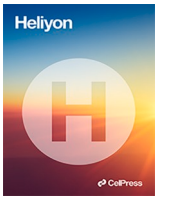

Research article

Furazolidone reduces the pathogenesis of *Trueperella pyogenes* and *Pseudomonas aeruginosa* co-infection in a mouse model

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ABSTRACT

The prevalence of abscess disease significantly limits the population expansion of captive forest musk deer, which is an endangered species protected by the legislation of China. Our prior work had demonstrated that *Trueperella pyogenes* and *Pseudomonas aeruginosa* are two important microorganisms in causing the abscess disease of forest musk deer, and furazolidone could inhibit the growth and virulence of the pathogens *in vitro*. In this study, the *in vivo* protection activity of furazolidone was evaluated by using mouse models chronically infected with *T. pyogenes* and *P. aeruginosa*. The results showed that furazolidone treatment significantly increased the survival rates of mice in the co-infection group, all the mice survived at 14 days post-infection. The damage degree of the lung tissues caused by bacterial infection was ameliorated by the treatment of furazolidone from 7 to 14 days post-infection, which also reduced the residual bacterial burden in the lungs. Compared to the untreated control group, the expression levels of genes activated by the quorum-sensing system of *P. aeruginosa* and the core virulence regulatory genes of *T. pyogenes* were significantly suppressed by furazolidone. In addition, the results of transcriptomic analyses showed that 270 DEGs were identified in the co-infection group. This finding further revealed that the immune responses of mice could be enhanced by the treatment of furazolidone, and this might also contribute to the clearance of bacteria from the lungs. Therefore, this study clearly reveals the protection activity of furazolidone against *P. aeruginosa* and *T. pyogenes* infection, and thus provides a promising candidate in the treatment of abscess disease.

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Abbreviations

T. pyogenes	Trueperella pyogenes
P. aeruginosa	Pseudomonas aeruginosa
Plo	pyolysin
M. berezovskii	Moschus berezovskii
QS	quorum-sensing
MDR	multi-drug resistant
PCR	Polymerase Chain Reaction
CFU	colony forming units
HE	hematoxylin-eosin
DEGs	differentially expressed genes
KEGG	Kyoto Encyclopedia of Genes and Genomes

1. Background

Trueperella pyogenes (*T. pyogenes*), a well-documented Gram-positive, facultative anaerobic bacterial pathogen, is typically isolated from the skin, gastrointestinal tract, and endometrium of domestic ruminants and wild animals [1,2]. This bacterium could cause cruel suppurative injuries in many host organs such as lungs [3,4]. The virulence of *T. pyogenes* is attributed to several known mechanisms and produces several virulence factors, including pyolysin (PLO), fimbriae, collagen-binding protein, and neuraminidases, contributing to bacterial adhesion, invasion, and colonization processes [5,6]. In addition, it is considered as the key pathogen in the formation of abscess disease in forest musk deer (*Moschus berezovskii*) [7]. *M. berezovskii* is an endangered wildlife, which has been farmed for more than 70 years in China. And the abscess disease is the crucial factor causing its population decrease [8,9]. Notably, *Pseudomonas aeruginosa* (*P. aeruginosa*) was also frequently detected with *T. pyogenes* in the abscess samples and was the predominant bacteria during the late-stage of respiratory abscess disease in *M. berezovskii* [7,10].

P. aeruginosa is a versatile Gram-negative bacterium with a complex quorum-sensing (QS) regulatory network and expresses a variety of virulence factors, such as extracellular proteases, exotoxin A, and pyocyanin [11]. The QS system-related cell–cell communication is a vital element in the pathogenesis of *P. aeruginosa*. In an interesting study, it was shown that the signalling molecules *N*-(3-oxo-dodecanoyl)-L-homoserine lactone, which are synthesized by the QS system of *P. aeruginosa*, could reduce the growth of *T. pyogenes* [12]. To date, the therapy of abscess disease largely depends on traditional antibacterial drugs. Moreover, because of the widespread use of antimicrobials, many *T. pyogenes* and *P. aeruginosa* strains are resistant to several commercial antibiotics by multifarious resistance mechanisms and pose serious obstacles for veterinary practice. Thus, developing a novel antibacterial strategy will be essential to treat abscess disease caused by *T. pyogenes* and *P. aeruginosa*.

Given the rising challenge of antimicrobial resistance worldwide [13], blocking the virulence factors of bacterial pathogens has emerged as a promising strategy to combat multi-drug resistant (MDR) bacteria-mediated infections [14–16]. The furanone compounds, which share a core structure similar to the HSL signals of *P. aeruginosa*, significantly decreased the production of virulence factors by *P. aeruginosa* [17]. Our previous study has demonstrated that furazolidone, a nitrofuran antibiotic used to treat gastrointestinal infections caused by bacteria and protozoa, inhibited the growth of *T. pyogenes* and remarkably reduced the expression of virulence factors of *P. aeruginosa* in *vitro* [18]. However, little information is available whether furazolidone could be applied in an animal model caused by *T. pyogenes* and *P. aeruginosa* infection.

In the present study, a mouse model of chronic pulmonary infection was established to assess the protective effectiveness of furazolidone. The pathological changes and bacterial burdens of the lungs were detected. In addition, quantitative PCR and transcriptome sequencing were performed to evaluate the anti-virulence effects and immunomodulation of furazolidone in the infected mice. Finally, we found that furazolidone treatment promoted the bacterial clearance, mitigated lung injuries and effectively improved survival of mice during co-infection. Intriguingly, furazolidone treatment significantly upregulated immune-related responses of host in a co-infected mouse model. This study will facilitate understanding of the biochemical mechanisms of the host feedback to bacterial co-infection and will provide an effective therapeutic strategy against abscess disease.

2. Materials and methods

2.1. Chemicals and reagents

Furazolidone was obtained from the MedChem Express (Monmouth Junction, NJ, USA). Lysogeny broth (LB) was purchased from Sigma Aldrich (St. Louis, MO, USA). Brain heart infusion medium (BHI), fetal bovine serum (FBS) and TRIzol reagents was purchased from Thermofisher (Waltham, MA, USA). Ketamine was purchased from Putney, Inc. (Portland, MA, USA).

2.2. Bacterial strains and culture conditions

T. pyogenes TP13 was isolated from the lung pus of *M. berezovskii*, and the *P. aeruginosa* wild-type strain PAO1 were maintained in

our laboratory [7,19]. *T. pyogenes* and *P. aeruginosa* were cultured in LB or BHI supplemented with 5 % of FBS at 37 °C for 24 h.

2.3. Animals

C57BL/6 female mice (8 weeks, 18–20 g) were purchased from Beijing Huafukang Biological Experimental Animal Company (Beijing, China) and routinely housed in the specific-pathogen-free laboratory of Sichuan University. Animal experiments were approved by the Ethics Committee of the State Key Laboratory of Biotherapy (2021559A). They were carried out in compliance with the guideline of Institutional Animal Care and Use Committee of Sichuan University.

2.4. Mouse model of chronic pulmonary bacterial infection

To establish chronic pulmonary bacterial infection in mouse, agar-bead-embedded bacteria were prepared as described in a published report [20]. Briefly, the agar beads-embedded TP13 or PAO1 were diluted into the same cell concentration (1.0×10^6 CFU) in 50 μl of sterile saline solutions. Mice were anaesthetized with ketamine (50 μg ml^{-1}) in sterile saline, and the agar-bead-embedded bacteria were intranasally administrated into the lungs of mice (n = 29 mice/group). After 24 h, the mice were intranasally instilled with 50 μL of sterile saline or furazolidone (50 μM) once daily for 14 days. The survival of infected mice was recorded for the subsequent 14 days (n = 11 mice/group). The lungs of infected mice were isolated for histological analysis, bacterial burdens detection, quantitative PCR and RNA-sequencing as described below (n = 18 mice/group).

2.5. Histological analysis

The randomly selected mice were euthanized at indicated time points (day1, day3, day 7, and day 14). The whole lungs of mice were aseptically removed for gross appearance, and a slice of a pulmonary left lobe from each mouse was applied for histological examination by hematoxylin-eosin (HE) staining. Semiquantitative procedures were used to convert qualitative tissue changes into pathological scores as previously described [21].

2.6. Bacterial burdens in the lungs

The randomly selected mice were euthanized at indicated time points (day1, day3, day 7, and day 14). The right lower lobe lungs of mice were aseptically harvested and bacterial burdens of mice lungs were determined by serial dilution of samples on BHI-FBS agar plates or LBA plates. The composition of *T. pyogenes* and *P. aeruginosa* in the co-infection samples were determined by counting the CFU of them on BHI-FBS agar plates, because the phenotypes of *T. pyogenes* and *P. aeruginosa* are different and they are easily distinguished.

2.7. Quantitative PCR (qPCR)

Total RNAs from lung of infected mice at indicated time points (day1, day3, day 7, and day 14) were obtained using TRIzol reagents (ThermoFisher). The cDNA was synthesized by a PrimeScript™ II Reverse Transcriptase kit (Takara Bio Inc., Kusatsu, Japan). Real-time PCR reactions were performed using TB Green Premix Ex *Taq*II (Tli RNaseH Plus) Kit (Takara) to detect the relative expression of QS-related genes of PAO1, including *lasR*、*rhlR*、*pqsR*、*lasB*、*rhlA*、*pqsA*、*pqsD*、*pqsE* and *hcnA*. The expression of virulence genes of *T. pyogenes* was also examined at indicated time points. The primers were used as previously described [18]. Gene expression was analyzed by the $2^{-\Delta\Delta CT}$ method.

2.8. RNA-sequencing (RNA-seq)

Total RNA from lung of infected mice was isolated at day 3 using TRIzol reagent (ThermoFisher) following the manufacturer's instructions. The complementary DNA libraries were sequenced by an Illumina HiSeq PE150 platform in Novogene company (Beijing, China). High-quality reads were mapped to the reference genome (*Mus musculus*, GCA_000001635.9) using Bowtie2 v2.2.3 software. Differential gene expression between the drug-treated and control groups or the blank group, was calculated and normalized to fragments per kilobase million (FPKM) by HTSeq v0.6.1 and DESeq2 software. For the enrichment functional analysis, the differentially expressed genes (DEGs) of different groups were entered into the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database (http://www.genome.jp/kegg/) according to a previous report [22].

2.9. Statistical analysis

Graphpad Prism version 9.0 was employed for the analysis of data. Mean values were compared using one-tailed unpaired Student *t*-tests or a Mann-Whitney *U* test. Mouse survival curves were analyzed by Log-rank (Mantel-Cox) test. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Furazolidone enhances the survival of mice co-infected with P. aeruginosa and T. pyogenes

Our previous research demonstrated that furazolidone provided anti-virulence effects on *T. pyogenes* and *P. aeruginosa in vitro* [18]. However, the biological processes of the modulation of furazolidone in an animal model is not generally known. To address this issue, the mice were chronic infected with *T. pyogenes* TP13, *P. aeruginosa* PAO1, or co-infected with TP13 and PAO1 by intranasally instillation. As shown in Fig. 1, the survival analysis did not show significant differences between the control group and furazolidone group in TP13-infected (Fig. 1A) or PAO1-infected (Fig. 1B) mice. Interestingly, the treatment of furazolidone effectively protected mice from the co-infection of TP13 and PAO1 (Fig. 1C), suggesting furazolidone had a protective activity against TP13 co-infection with PAO1.

3.2. Furazolidone mitigates lung injury in mice

To explore the protection effect of furazolidone on the pathological alterations of infected mice, the lungs of infected mice were harvested from 1 to 14 days post-infection. The infection efficiency was confirmed by the gross appearance (Fig. 2A) and hematoxylin and eosin staining (Fig. 2B) of mice lungs compared to the blank group. As shown in Fig. 2C–J and 2AA, the results of histopathological analysis demonstrated furazolidone treatment mitigated lung injury in TP13-infected mice lungs compared to the control group from 7 to 14 days post-infection. Likewise, furazolidone treatment reduced pathological changes in PAO1-infected mice lungs compared to the control group from 3 to 7 days post-infection (Fig. 2K–R and 2AB). Importantly, in the context of mice co-infected with TP13 and PAO1, furazolidone treatment remarkably improved the recovery of lung damage, which displayed a milder degree of inflammatory cell infiltration compared to the control group at 3 days post-infection (Fig. 2S–Z and 2AC).

3.3. Furazolidone facilitates the clearance of bacteria from mice lungs

To investigate whether furazolidone treatment affect the bacteria burdens of mice lungs during monoinfection or coinfection, we next examined its effects on the clearance of PAO1 or TP13 by conloy-forming unit (CFU) assay. Our results showed that furazolidone treatment significantly increased clearance of PAO1 or TP13 in the mice lungs from 7 to 14 days post-infection (Fig. 3A) or day 14 post-infection (Fig. 3B). Similarly, the bacterial burdens of PAO1 or TP13 were also declined in the furazolidone-treated mice co-infected with TP13 and PAO1 from 7 to 14 days post-infection (Fig. 3C and D). Intriguingly, the number of TP13 colonies in the mice lung was undetectable in the coinfection group from 7 to 14 days post-infection (Fig. 3D). However, PAO1 colonies were always detectable from 1 to 14 days post-infection, suggesting PAO1 tend to be the dominant species during chronic coinfections. Taken together, these results demonstrated that furazolidone played a pivotal role in the clearance of both *T. pyogenes* and *P. aeruginosa* from mouse lungs, especially during chronic coinfections.

3.4. Furazolidone reduces the expression of bacterial virulence genes

Based on our previous finding that furazolidone could inhibit the expression of the virulence genes of *T. pyogenes* and *P. aeruginosa in vitro* [18], we further determine the effect of furazolidone on the expression of virulence genes in *T. pyogenes* or *P. aeruginosa in vivo* during the chronic infection. We found that the expression of *plo* was dramatically declined in the mice lungs treated with furazolidone compared to control group at 1 day post-infection (Fig. 4A). Likewise, the expression levels of *plo*, *ploS*, and *ploR* were significantly lower in the furazolidone-treated group than the untreated group from 3 to 14 days post-infection (Fig. 4B–D). For the monoinfection group by PAO1, the main QS-related genes of PAO1 were significantly decreased in the mice lungs treated with furazolidone compared to control group from 1 to 14 days post-infection (Fig. 4E–H). Due to the small proportion of TP13, the virulence genes of TP13 were

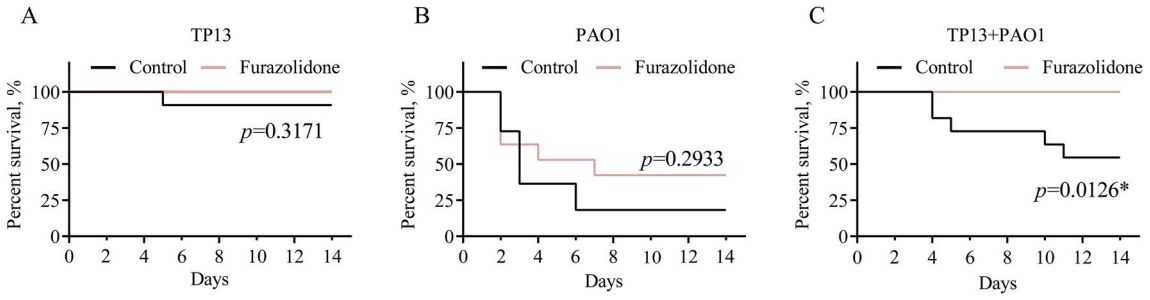

Fig. 1. The survival rate of mice infected intranasally with *T. pyogenes* or *P. aeruginosa*. Mice were intranasally challenged with 1.0×10^6 CFU TP13 (A), PAO1 (B), or PAO1 plus TP13 (CFU at a ratio of 1:1, C) for 24 h, and were intranasally treated with 50 μL of NaCl (0.9 %, control group) or furazolidone (50 μM) once-daily for 14 days. Survival rate of mice was monitored for the subsequent 14 days (n = 11 mice/group). $^*p < 0.05$ (determined by Mantel-Cox test).

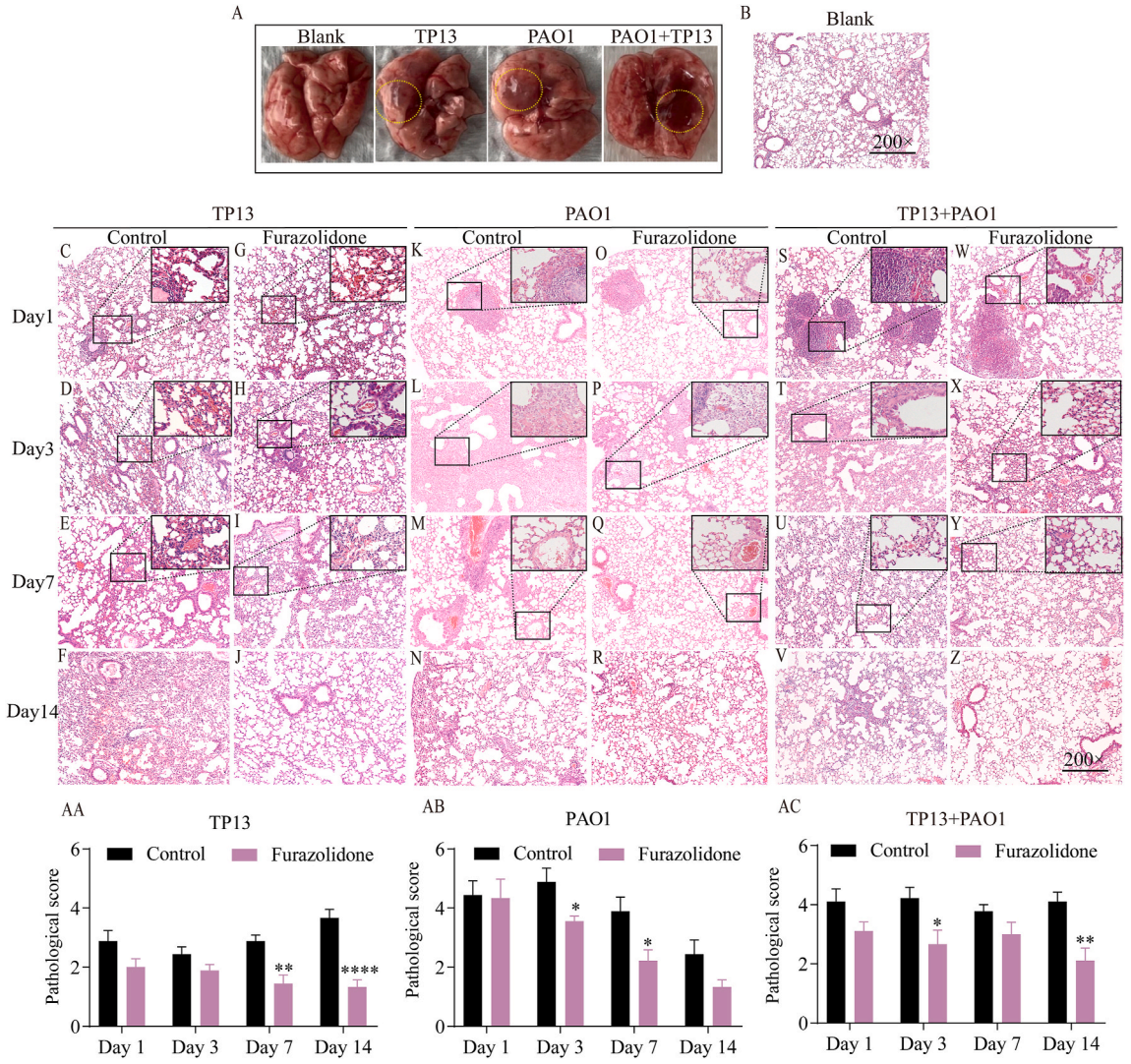

Fig. 2. Gross appearance and histology of lungs of mice infected intranasally with *T. pyogenes* or *P. aeruginosa*. The mice were intranasally challenged with 1.0×10^6 CFU TP13, PAO1, or PAO1 plus TP13 (CFU at a ratio of 1:1) for 24 h, and were intranasally treated with 50 μL of NaCl (0.9 %, control group) or furazolidone (50 μM) once-daily for 14 days (n = 18 mice/group). (A) Gross appearance of lungs was detected. The yellow circle indicates the location of the lesion. Blank group indicates the normal mice without any treatment. (B) Hematoxylin and eosin (HE) staining of lung tissue sections of normal mouse. 200 × magnification. (C–Z) HE staining of lung tissue of mice infected with TP13 (C–J), PAO1 (K–R), or TP13 plus PAO1 (S–Z). 200 × magnification. (AA-AC) Pathological scores of lung tissue of mice infected with TP13 (AA), PAO1 (AB), or TP13 plus PAO1 (AC). Data are shown as mean ± SEM of 9 mice per group. $^*p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$ (determined by Mann-Whitney *U* test, two-tailed).

not detected in the samples of chronic coinfection models. As shown in Fig. 4J–L, most QS-related genes of PAO1 were significantly decreased in the mice lungs treated with furazolidone from 3 to 14 days post-infection, except for *lasR* and *lasB*. However, only the *rhlR* and *pqsA* genes of PAO1 were significantly inhibited upon furazolidone treatment in the coinfection group at 1 day post-infection (Fig. 4I). Therefore, these results collectively emphasize the ability of furazolidone to inhibit the QS system of *P. aeruginosa* and the main virulence genes of *T. pyogenes* during coinfection.

3.5. *Furazolidone may accelerate host immune response*

To better investigate the biological processes of the host response to furazolidone treatment during infection, the DEGs were determined between untreated and treated samples at 3 days post-infection. RNA-seq was performed to define the transcriptional changes in mouse lungs infected by PAO1 or coinfected by TP13 and PAO1 with and without furazolidone treatment. Due to the small proportion of TP13 and hard to detect the colony numbers in the late chronic infection, the TP13 monoinfection group was not included in the comparative transcriptome analysis. Raw data obtained from the RNA-seq were deposited in the NCBI BioProject database (accession number PRJNA1015350). A total of 3557 DEGs were identified in the PAO1 infected mice compared to the blank

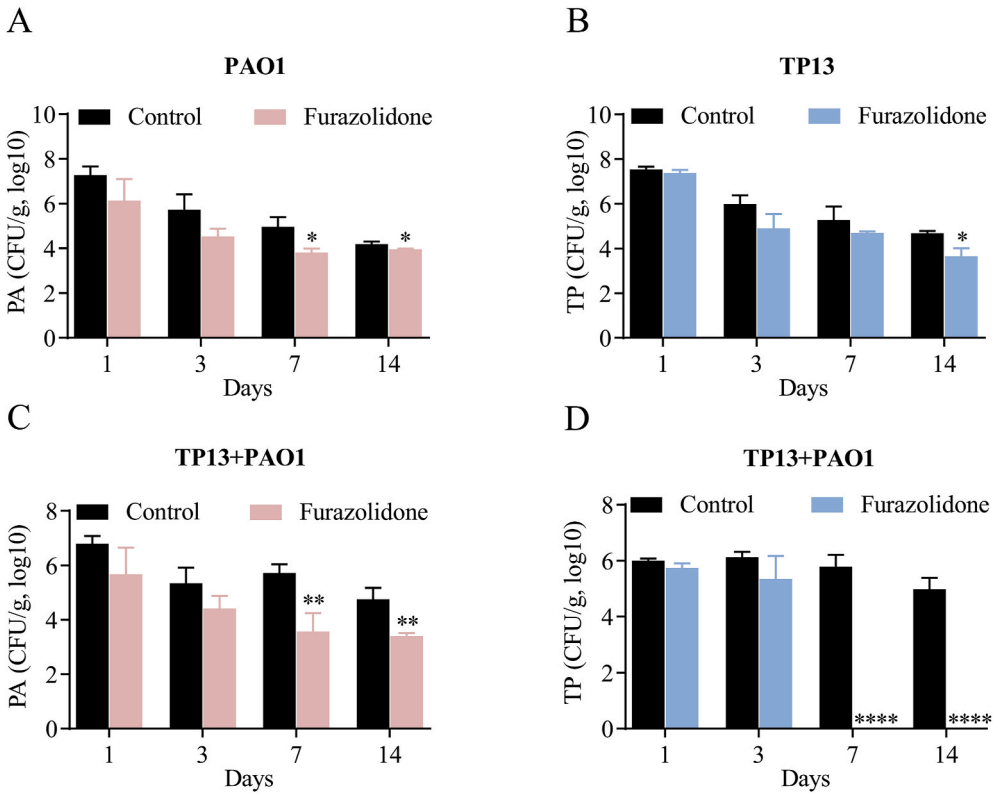

Fig. 3. Residual bacteria of mice during chronic lung infection. (A–D) Mice were challenged as described in Fig. 2. The bacterial burdens of the lungs were determined by plate counting at the indicated time points. (A and C) *P. aeruginosa* burden in the lung day 1 to day 14 post-intranasal inoculation. (B and D) *T. pyogenes* burden in the lung day 1 to day 14 post-intranasal inoculation. Data are mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$ (determined by Student's *t*-test).

group (Fig. 5A). And 242 DEGs were identified in the PAO1 infected mice with furazolidone treatment compared to the control group, including 137 down-regulated genes and 105 up-regulated genes (Fig. 5A), which significantly enriched in several inflammatory response-related KEGG terms such as "Cytokine-cytokine receptor interaction" and "Th17 cell differentiation" (Fig. 5B). For the TP13 and PAO1 co-infection group, the treatment of furazolidone induced 126 down-regulated genes and 144 up-regulated genes (Fig. 5A), which significantly enriched in several infection-related or inflammatory response-related KEGG terms compared to the untreated group (Fig. 5C). We then checked the expression levels of inflammation-related genes in the up-regulated KEGG terms of each infection group. Compared to the uninfected blank group, up-regulation of pro-inflammatory genes such as *Ccl20*, *Il17f*, and *Il22* were detected in all groups (Fig. 5D). In the PAO1-infection group, furazolidone treatment mainly up-regulated 10 genes, such as the *H2-T22*, *H2-T24*, and *Il22* genes, and down-regulated *Ifng*, *Ccl21a* and *Bst2* genes. Moreover, 32 out of 33 genes associated with inflammatory responses were up-regulated in the co-infection group with furazolidone treatment, and only *Ccl20* showed down-regulation (Fig. 5D). These distinct gene expression patterns indicated furazolidone might have a key role in the modulation of host immune activation and immune responses during bacterial infection.

4. Discussion

As the well-documented opportunistic pathogens, *T. pyogenes* and *P. aeruginosa* have been known for decades. Our previous study demonstrated that furazolidone significantly increased anti-virulence activity against *P. aeruginosa* and inhibited the growth of *T. pyogenes in vitro*. In addition, *P. aeruginosa* had an innate growth advantage in the competition with *T. pyogenes* [18]. Nonetheless, it is not fully understood whether the furazolidone provides protective immune responses against *T. pyogenes* and *P. aeruginosa* infection in a mouse model. In this study, we found that furazolidone treatment suppressed the growth and virulence of *T. pyogenes* or *P. aeruginosa* and increased the survival rate of co-infected mice. In addition, the *in vivo* transcriptome analysis revealed that the furazolidone-treated group upregulated immune-related responses of host in a co-infected mouse model. These findings suggested that furazolidone could regulate the immune responses of mice to kill the invasive bacteria and provide a promising strategy to control abscess diseases caused by *T. pyogenes* and *P. aeruginosa* co-infections.

First of all, to investigate whether furazolidone provide protection during chronic lung co-infection by *T. pyogenes* and *P. aeruginosa*, the mice were chronic infected with TP13, PAO1, or co-infected with TP13 and PAO1 by intranasally instillation. We found that furazolidone significantly protected mice against TP13 and PAO1 co-infection and increased survival rate of infected mice. However,

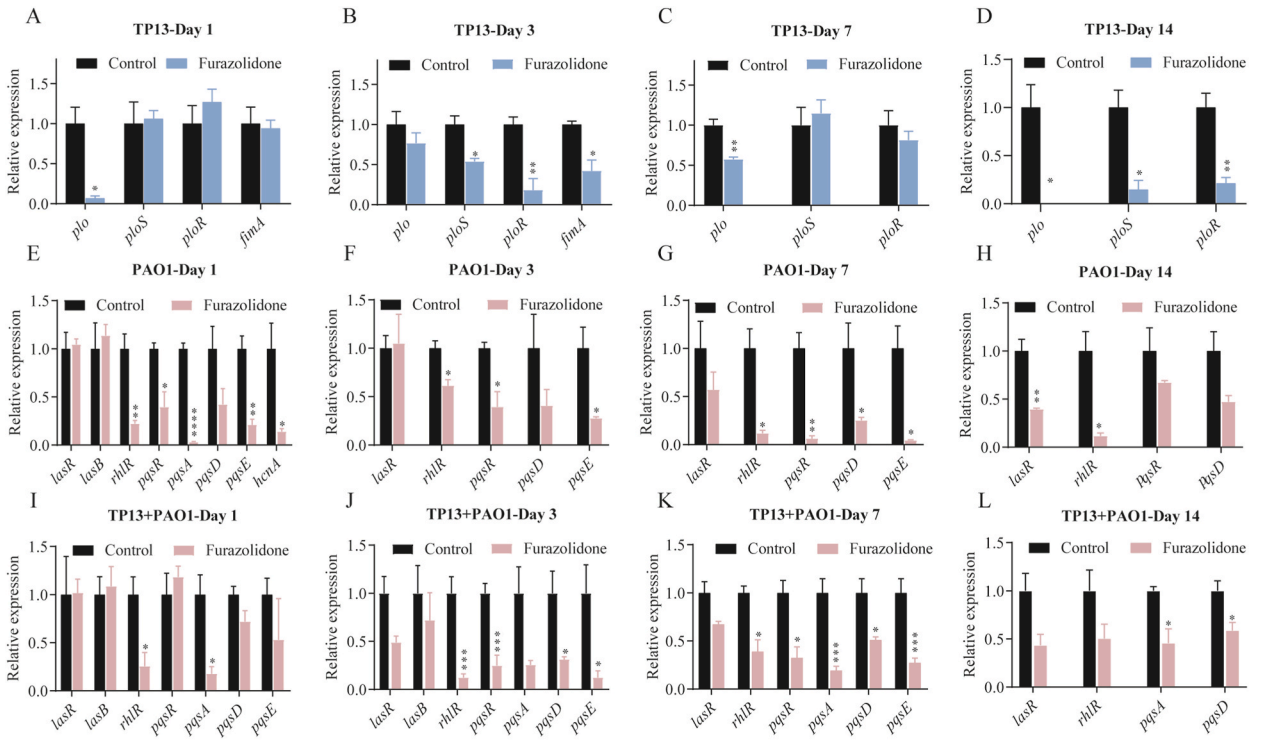

Fig. 4. Relative expression of virulence genes from *T. pyogenes* or *P. aeruginosa* during chronic lung infection. (A–L) Mice were challenged as described in Fig. 2. The relative expression of virulence genes from TP13 (A–D) or quorum sensing related genes from PAO1 (E–H) and TP13+PAO1 (I–L) during chronic lung infection were determined by qPCR at the indicated time points. Data shown are the mean ± SEM of three independent experiments. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$, (determined by an unpaired one-tailed *t*-test).

such effects were undetected in the TP13 mono-infected or PAO1 mono-infected mice. It is probably because *T. pyogenes* has a limited lethal effect on mice with an intact immune system during monoinfection. In addition, *P. aeruginosa* was more lethal to mice during monoinfection, and *P. aeruginosa* CFUs was twice than the co-infection condition (co-infection containing 50 % *T. pyogenes*), which resulted in the insignificant protective effect of furazolidone in mice during *P. aeruginosa* infection alone. Accumulating evidence has shown that *T. pyogenes* and *P. aeruginosa* are able to disseminate to lung via respiratory infection and contribute to their pathogenesis [23–25]. Therefore, the pathological alterations and the clearance of bacteria from the lungs are critical lines of host defense against *T. pyogenes* and *P. aeruginosa* co-infection. To explore whether furazolidone has an effect on the pathological alterations of infected mice, the lungs of infected mice were collected at the indicated time points. We found that furazolidone treatment mitigated lung injury in TP13-infected or PAO1-infected mice lung from 7 to 14 days post-infection or from 3 to 7 days post-infection. Interestingly, furazolidone provided more robust protection and remarkably improved the recovery of lung damage from 3 to 14 days post-infection. Moreover, furazolidone treatment significantly increased bacterial clearance of the lungs from 7 to 14 days post-infection in the co-infected mouse, which correlated with increased mouse survivability. However, the interactions between *P. aeruginosa* and *T. pyogenes* should be considered in the co-infection model. We need to focus on the fact that *P. aeruginosa* had an innate growth advantage in the competition with *T. pyogenes* mentioned above. Due to the dual effects of *P. aeruginosa* and furazolidone, we found that the number of *T. pyogenes* colonies in the mice lung was undetectable in the coinfection group after 7 days post-infection. Taken together, these findings suggested that furazolidone might be a novel target to control the co-infection caused by *T. pyogenes* and *P. aeruginosa*.

The QS system is a crucial cell–cell communication strategy of bacterial pathogens in infectious disease control. The QS of *P. aeruginosa* regulates many important biological activities such as virulence factor production [26,27]. Previous published studies have demonstrated that *T. pyogenes* expresses several virulence factors including hemolytic exotoxin pyolysin (Plo), PloS, PloR and fimbriae that play essential roles during infection [2,28]. Although furazolidone exhibited anti-virulence activity against *P. aeruginosa* and inhibited the growth of *T. pyogenes in vitro* at low concentrations [18], it is not clear whether the furazolidone has anti-virulence effects on *P. aeruginosa* or *T. pyogenes* infection in the mouse model. Thus, we further focused on the anti-virulence effects of furazolidone in mice with *T. pyogenes* or *P. aeruginosa* chronic infections. We found that furazolidone has significant impacts on the QS system of *P. aeruginosa*, by reducing the expression of QS regulatory genes such as *rhlR* and *pqsA*. In addition, such anti-virulence effects were also detected in the *T. pyogenes*-infected mice treated with furazolidone. Altogether, furazolidone exhibited the significant anti-virulence effects on *T. pyogenes* or *P. aeruginosa* infection and consequently alleviating symptoms of infection described above.

Numerous studies have determined the interaction dynamics between bacterial pathogens and hosts using RNA-seq, which is an

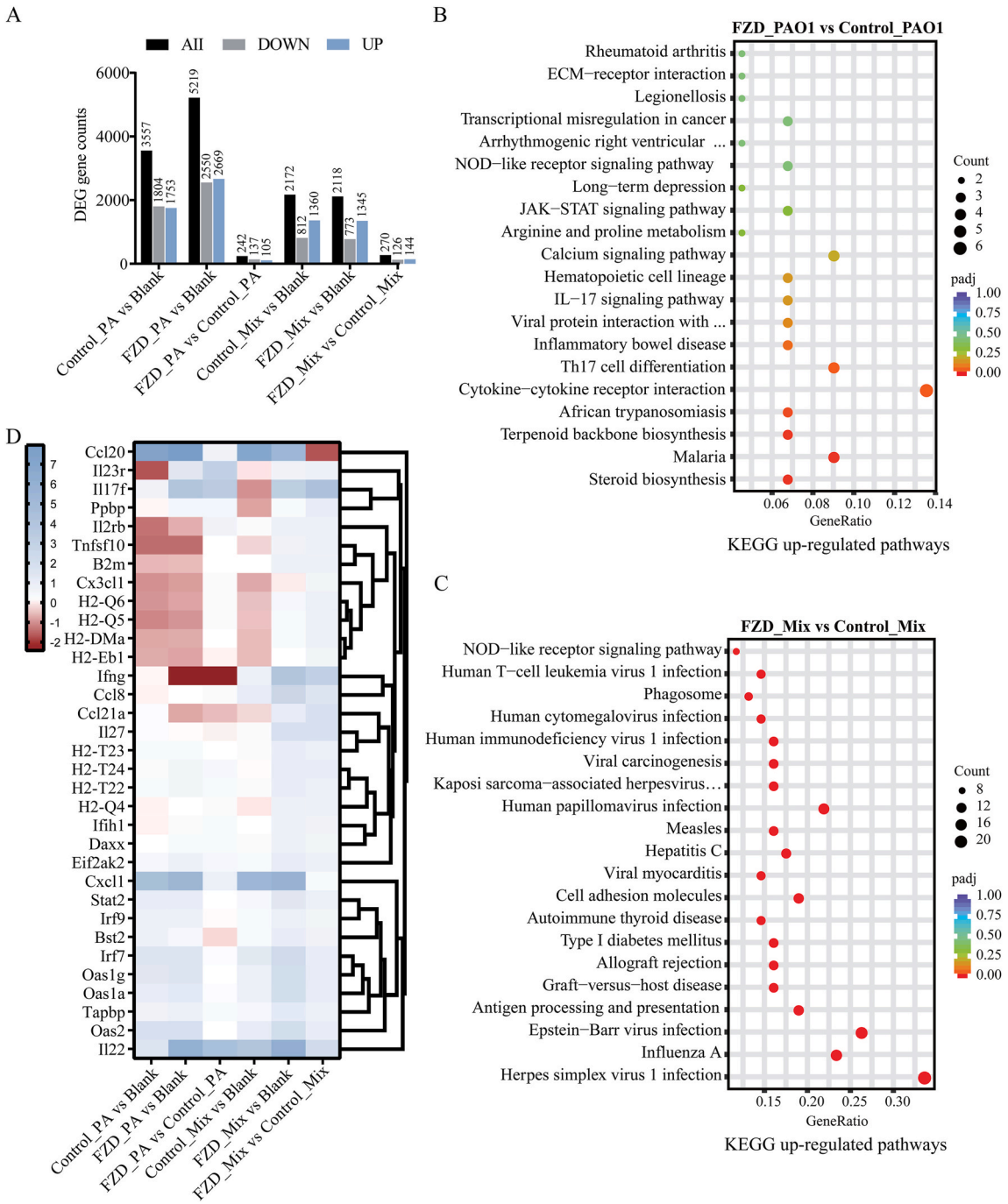

Fig. 5. Transcriptomic analysis for mice lung treated by furazolidone during chronic infection. (A) Number of downregulated and upregulated DEGs from the mice lung. Blank group indicates the normal mice without any treatment. (B) Enrichment of upregulated top-level KEGG terms in the PAO1 infection group. (C) Enrichment of upregulated top-level KEGG terms in the co-infection group (TP13+PAO1). (D) Hierarchical clustering analysis for the expression of the inflammatory response-associated genes. Abbreviations: FZD, Furazolidone; PA, PAO1 infection; Mix, PAO1 and TP13 coinfection.

effective approach to analyze transcriptomes [28–31]. These investigations provide systematic analyses of the transcriptional alterations and biochemical processes between bacteria and host immune response. To further investigate the DEGs and signal pathways that involved in host responses to *T. pyogenes* and *P. aeruginosa* co-infection, the transcriptome analysis were carried out to determine the DEGs functions and the dominant signal pathways. A total of 242 DEGs were identified in the PAO1 and furazolidone treated mice compared to control samples, providing the wide-ranging landscape of the host transcriptome. The 242 DEGs were assigned to 20

up-regulated KEGG pathways, "Cytokine-cytokine receptor interaction" represented the largest category, followed by "Th17 cell differentiation" and "Malaria". Moreover, 270 DEGs were obtained in the TP13 and PAO1 co-infection group treated by furazolidone, which significantly enriched in several infection-related KEGG terms compared to the untreated group, such as "Herpes simplex virus 1 infection", "Influenza A" and "Antigen processing and presentation". Notably, most upregulated DEGs expressed in the TP13 and PAO1 co-infection group involve host immune activation and inflammatory responses, such as *Il17f*, *Il22*, and *Il27*. In a similar study conducted by Anuradha et al., it was also indicated that type 17 (IL-17F) cytokines were involved in host immune responses during pathogens coinfection upon anthelmintic therapy [32]. Taken together, our data provide a global overview of the important DEGs and pathways involved in the immune-related response induced by TP13 and PAO1 co-infection, and furazolidone exhibited a potential role in immunoregulation and therapeutics during bacterial co-infection.

In conclusion, the current study first pinpoints the key role of furazolidone that protected mice from *T. pyogenes* and *P. aeruginosa* co-infection, and the mice survived at 14 days post-infection by decreasing the bacterial burdens in lungs and alleviated lung injury of infected mice. Additionally, furazolidone treatment inhibited the expression of virulence-related genes in *T. pyogenes* and *P. aeruginosa* in the infected mice from 1 to 14 days post-infection. Importantly, we identified 270 DEGs in the TP13 and PAO1 co-infection group, and found that furazolidone plays a key role in the modulation of host immune activation and immune responses by transcriptome analyses. This study provides a novel strategy to combat the *T. pyogenes* and *P. aeruginosa*-related diseases, which highlights the antibacterial and anti-virulence potency of furazolidone in the therapy of co-infection. Moreover, we acknowledge that only one drug examined or lack of furazolidone investigation in large ruminant treatment are the limitations of this study. More comprehensive studies by using furazolidone or another drug testing in large ruminants may contribute to controlling abscess diseases.

CRediT authorship contribution statement

Nan Yang: Methodology, Investigation, Funding acquisition. **Heyue Li:** Project administration, Methodology, Investigation. **Xiting Yang:** Investigation, Formal analysis. **Yi Wu:** Validation, Investigation. **Zheng Lv:** Software, Methodology. **Ziheng Zhang:** Methodology. **Xiaoling Ma:** Investigation. **Xikun Zhou:** Resources, Conceptualization. **Xiuyue Zhang:** Resources, Methodology. **Kelei Zhao:** Funding acquisition, Conceptualization. **Lianming Du:** Writing – review & editing, Supervision, Software. **Ting Huang:** Writing – review & editing, Writing – original draft, Supervision.

Ethics statement

Animal experiments were approved by the Ethics Committee of the State Key Laboratory of Biotherapy (2021559A).

Data availability statement

Data is available upon request to the corresponding author.

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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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