Neutralizing gut-derived lipopolysaccharide as a novel therapeutic strategy for severe leptospirosis

Xufeng Xie[a], Xi Chen[a], Shilei Zhang[a], Jiuxi Liu[a], Wenlong Zhang[a*], Yongguo Cao[a,b*]

[a]Department of Clinical Veterinary Medicine, College of Veterinary Medicine, Jilin University, Changchun, 130062, People's Republic of China; [b]State Key Laboratory for Diagnosis and Treatment of Severe Zoonotic Infectious Diseases, Key Laboratory for Zoonosis Research of the Ministry of Education, Institute of Zoonosis, and College of Veterinary Medicine, Jilin University, Changchun 130062, China

* Corresponding author. E-mail addresses: zwenlong123@126.com and ygcao82@jlu.edu.cn

Abstract

Leptospirosis is an emerging infectious disease caused by pathogenic *Leptospira* spp. Humans and some mammals can develop severe forms of leptospirosis accompanied by a dysregulated inflammatory response, which often results in death. The gut microbiota has been increasingly recognized as a vital element in systemic health. However, the precise role of the gut microbiota in severe leptospirosis is still unknown. Here, we aimed to explore the function and potential mechanisms of the gut microbiota in a hamster model of severe leptospirosis. Our study showed that leptospires were able to multiply in the intestine, cause pathological injury, and induce intestinal and systemic inflammatory responses. 16S rRNA gene sequencing analysis revealed that *Leptospira* infection changed the composition of the gut microbiota of hamsters with an expansion of Proteobacteria. In addition, gut barrier permeability was increased after infection, as reflected by a decrease in the expression of tight junctions. Translocated Proteobacteria were found in the intestinal epithelium of moribund hamsters, as determined by fluorescence in situ hybridization, with elevated LPS levels in the serum. Moreover, gut microbiota depletion reduced the survival time, increased the leptospiral load, and promoted the expression of proinflammatory

cytokines after *Leptospira* infection. Intriguingly, fecal filtration and serum from moribund hamsters both increased the transcription of *TNF-α*, *IL-1β*, *IL-10*, and *TLR4* in macrophages compared with those from uninfected hamsters. These stimulating activities were inhibited by LPS neutralization using polymyxin B. Based on our findings, we identified an LPS neutralization therapy that significantly improved the survival rates in severe leptospirosis when used in combination with antibiotic therapy or polyclonal antibody therapy. In conclusion, our study not only uncovers the role of the gut microbiota in severe leptospirosis but also provides a therapeutic strategy for severe leptospirosis.

Introduction

Leptospirosis is a worldwide emerging zoonotic disease caused by *Leptospira* spp. [1]. *Leptospira interrogans* impacts approximately one million people resulting 60000 deaths every year [1]. In human leptospirosis, the clinical manifestations can range from mild illness to multiorgan failure, characterized by jaundice, kidney, and liver failure, and even death [2, 3]. Currently, one important challenge is that leptospirosis is often misdiagnosed as other febrile diseases, and once the optimal treatment period is missed, leptospirosis quickly develops into the severe form, which often ends in death [4]. However, antibiotic treatment is often useless in severe leptospirosis and even aggravates it, called the Jarisch-Herxheimer Reaction [5]. Thus, it is imperative to explore precision medicine strategies for the treatment of severe leptospirosis.

90% of infected humans may resolve spontaneously while 10% of patients are designated as susceptible hosts [6]. Intestinal bleeding is a common but neglected symptom of severe leptospirosis in humans and hamster [7]. A healthy gut environment, shaped by the presence of a healthy functional microbial ecosystem, is fundamental to instruct the immune system towards homeostasis. The intestine harbours a densely populated resident microbial community, which consists of bacteria, viruses, and fungi, called microbiota [8]. Gut dysbiosis has been linked to increased susceptibility

to disseminated bacterial infections and sepsis in humans. In addition, studies of patients in intensive care units (ICUs) have found that dysbiosis increases the risk of nosocomial infections, sepsis, and mortality [9]. Our previous study proved that the gut microbiota contributed to the defence against *Leptospira* infection in mice, a resistant model of leptospirosis, without obvious intestinal lesions or increased intestinal permeability [10], while diarrhea was a common symptom in the late leptospirosis of hamster. As we known, the gut microbiota is closely related to the homeostasis of intestine [11]. Previous study demonstrated that the variation of the gut microbiota mediated the different susceptibility to *Cholera* infection [12]. Focusing on the role of the gut microbiota in leptospirosis may help explain the differences in susceptibility to *Leptospira* infection in humans and develop targeted therapy for acute leptospirosis. However, whether a relationship exists between the gut microbiota and severe leptospirosis is still unclear.

In this study, we used the hamster model of leptospirosis to explore the functional role of the gut microbiota in acute leptospirosis. We provided evidence that *Leptospira* infection disrupted intestinal homeostasis, and targeting gut-derived LPS improved the survival rates of hamsters with severe leptospirosis. Our study provides a basis for the development of novel therapeutic strategies to control severe leptospirosis.

Materials and methods

Ethics statement

A previous study reported that sex influenced host susceptibility to leptospirosis [13] and that sex may affect the composition of the gut microbiota [14]. To reduce variability in infection outcomes [15], most infection experiments were performed in female Syrian golden hamsters *(Mesocricetus auratus*). For experiments utilizing neutralizing LPS therapy combined with antibiotic or antibody therapy for severe leptospirosis, both sexes were used. Specific pathogen-free female Syrian hamsters

were maintained on standard rodent chow with water supplied ad libitum during the experimental period. All animal experiments were conducted according to the regulations of the Administration of Affairs Concerning Experimental Animals in China. The protocol was approved by the Institutional Animal Care and Use Committee of Jilin University (20170318).

Bacterial strains and animals

Pathogenic *L. interrogans* serovar Lai strain Lai (56601) and *L. interrogans* serovar Autumnalis (56606) was grown in liquid Ellinghausen-McCullough-Johnson-Harris (EMJH) medium at 29 °C without agitation. A Petroff-Hauser chamber was used to count leptospires. Six-week-old hamsters were provided by Liaoning Changsheng Biotechnology Co. Ltd.

Experimental infections

Part I hamster infection

To explore the effect of challenge route on leptospiral colonization in the intestine and the survival rate and whether it was specific to the routine of infection, six-week-old female hamsters were injected intraperitoneally or subcutaneously with 10^7 leptospires. Hamsters were euthanized at 0 h, 6 h, 48 h, 96 h post infection (p.i.), and articulo mortis stage (AM). The ileums and colons were collected aseptically. The intestinal contents were excluded from the intestine with PBS, and the tissues were stored at -80 °C for leptospiral load analysis. For the survival assay, hamsters were monitored daily for signs of illness including ruffled fur, gait difficulty, loss of appetite, or weight loss of ≥10% of the animal's maximum weight, which was considered moribund [4]. Hamsters were euthanized when they were moribund.

To explore the effect of *Leptospira* infection on the gut microbiota, six-week-old female hamsters were injected intraperitoneally with a lethal dose of 10^7 leptospires. Fresh fecal pellets were collected aseptically at 0 days, 2 days, and AM p.i.,

immediately frozen in liquid nitrogen, and stored at −80 °C. Colons were also collected aseptically and the intestinal contents were excluded from the intestine with PBS. Then, tissues were stored at -80 °C for gene expression measurement.

To examine the role of the gut microbiota on acute leptospirosis, the gut microbiota depletion and fecal microbiota transplantation (FMT: a therapeutic procedure aimed at restoring a normal intestinal microbiota by application of fecal microorganisms from a healthy subject into the gastrointestinal tract of a dysbiotic subject [16]) were performed as described previously with some modifications [17]. Briefly, six-week-old female hamsters were administered antibiotics (ampicillin, 100 mg/kg; metronidazole, 100 mg/kg; neomycin sulfate, 100 mg/kg (Sigma-Aldrich, USA) and vancomycin, 50 mg/kg (BiochemPartner, China) intragastrically once daily for ten consecutive days [18]. Fresh fecal pellets were collected from uninfected female hamsters and then resuspended in sterile normal PBS (1 fecal pellet in 1 mL of PBS). The pellets were immediately homogenized and the homogenate was centrifuged at 100 rpm, 4 °C for 5 min. 200 μL of the supernatant was given to the gut microbiota-depleted hamster by oral gavage for 5 consecutive days after antibiotic treatment was stopped. Then, hamsters were intraperitoneally infected with 10^6 *L. interrogans*. Hamsters were monitored daily for 21 days to record the survival rate. Hamsters were euthanized when they were moribund. Blood was collected to determine leptospiral load and the gene expression of inflammatory cytokines at 6 days p.i..

To explore the effect of LPS neutralization on severe leptospirosis, six-week-old male or female hamsters were inoculated intraperitoneally with 10^7 leptospires. Treatments were started immediately after detection of the first death regardless of the assigned group [19]. Group 1: saline control (400 μL/hamster, i.p.); Group 2: Polymyxin B (PMB) (1 mg/kg, i.p., Solarbio, China); Group 3: Antibody against *Leptospira* (Ab) (16 mg/kg, subcutaneous injection); Group 4: Doxycycline (Dox) (5 mg/kg, i.p., Solarbio, China); Group 5: PMB (1 mg/kg, i.p.) and Ab (16 mg/kg, subcutaneous injection); Group 6: PMB (1 mg/kg, i.p.) and Dox (5 mg/kg, i.p.).

Hamsters were treated twice a day for 3 consecutive days. Hamsters were monitored daily for 21 days. Hamsters were euthanized when they were moribund. Blood from female hamsters was collected to determine the leptospiral load and gene expression of inflammatory cytokines at 6 days p.i..

To explore the effect of LPS neutralization on another leptospiral serotype infection, six-week-old female hamsters were inoculated intraperitoneally with lethal 10^6 leptospires (56606), of which the infective doses was determined by previous study [20]. Treatments were started immediately after detection of the first death regardless of the assigned group. Group 1: saline control (400 μL/hamster, i.p.); Group 2: PMB (1 mg/kg, i.p., Solarbio, China); Group 3: Dox (5 mg/kg, i.p., Solarbio, China); Group 4: PMB (1 mg/kg, i.p.) and Dox (5 mg/kg, i.p.). Hamsters were treated twice a day for 3 consecutive days. Hamsters were monitored daily for 21 days. Hamsters were euthanized when they were moribund.

Part rabbit infection

The purification of polyclonal antibody (Ab) was performed as previously described [20]. Briefly, six eight-month-old female New Zealand White rabbits were injected intravenously into the marginal ear vein with a dose of $2 \pm 4\times10^8$ live leptospires/mL in PBS according to the following schedule: day 1, 1 mL; day 6, 2 mL; day 11, 4 mL; and day 16 and 21, 6 mL each. One week after the last injection, the rabbits were anaesthetized and then exsanguinated through cardiocentesis. The rabbits were then euthanized. During the immunization period, the rabbits did not exhibit clinical signs of leptospirosis. Antisera were collected by centrifuging blood at 3,000 g. The polyclonal Ab IgG was purified from the antisera using the caprylic acid ammonium sulfate precipitation method from the antisera. The concentration of the IgG-polyclonal Ab was determined with the BCATM Protein Assay Kit (Thermo, USA) and adjusted to a final concentration of 20 mg/mL with phosphate buffered saline (PBS). The Ab was stored at -20 °C until used for treatment. The protocol was

approved by the Committee on the Ethics of Animal Experiments of the First Norman Bethune Hospital of Jilin University, China [(2013) clinical trial (2013-121)] [20].

Bacterial isolation and culture

For isolation of the translocated microbiota, six-week-old female hamsters were intraperitoneally infected with 10^7 leptospires. Hamsters were anaesthetized by intraperitoneal injection of a mix of ketamine/xylazine (200 and 10 mg/kg, respectively). Blood from the uninfected group and AM group by cardiac puncture were plated on LB or MacConkey agar plates aerobically or anaerobically at 37 °C for 36 h.

***In vivo* barrier permeability**

Six-week-old female hamsters were intraperitoneally infected with 10^7 leptospires. The permeability of the intestine was determined at 0 days, 2 days, and AM p.i.. Hamsters were starved for 6 h before gavage with FITC-dextran (4 kDa, 400 mg/kg body/hamster). After 2 h, hamsters were anaesthetized by intraperitoneal injection of a mix of ketamine/xylazine (200 and 10 mg/kg, respectively) and blood was collected and centrifuged at 3000 rpm and 4 °C for 10 min. The samples were analyzed with a fluorescence spectrophotometer (Synergy II plate reader with Gen5 software; BioTek Instruments, Winooski, VT) [21].

Minimum inhibitory concentration (MIC) determination

The MIC of PMB and Dox against *L. interrogans* serovar Lai strain Lai 56601 was determined according to a previous method [22]. Briefly, leptospires in the exponential-phase were deposited at a final concentration of 2×10^6 leptospires/mL in each well of 96 well plates with serial two-fold dilutions of PMB and Dox ranging from to 0.016 μg/L in EMJH. The final volume was 200 μL in each well. The plates were incubated for 3 days at 30 °C, and then 20 μL of Alamar Blue (Invitrogen,

Thermo Fisher Scientific) was added to each well. Then, the samples were incubated at 30 °C for 2 days. Each strain–drug combination was tested in duplicate, and positive (bacteria and no antibiotic added) and negative (no bacteria added) controls were included in each plate. The results were recorded by a microplate reader (MultiSkan FC, Thermo Fisher Scientific).

Limulus amebocyte lysate assay

Blood from uninfected hamsters and AM hamsters was collected and centrifuged at 3000 rpm for 10 min. Then, serum was diluted (1:10) and detected by the Limulus amebocyte lysate assay according to the manufacturer's instructions (LAL Chromogenic Endotoxin Quantitation Kit; Xiamen Biotechnology Co., Ltd., China). *E. coli* LPS at 1 EU/mL and *E. coli* LPS at 10 EU/mL (Sigma-Aldrich, USA) were used as positive controls.

Fluorescence in situ hybridization (FISH)

Six-week-old female hamsters were injected intraperitoneally with 10^7 leptospires. Hamsters were euthanized at 0 days, 2 days, and AM p.i.. Colons were collected aseptically. The intestinal contents were excluded from the intestine with PBS and a segment of colon was fixed in 4% paraformaldehyde solution overnight, washed and passed through 15% and 30% sucrose solutions. The sample was then embedded in optimal cutting temperature compound (O.C.T., Tissue-Tek) and cryo-sectioned into 5 μm longitudinal sections (Leica). Slides were equilibrated in hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl, 0.01% sodium dodecyl sulfate, 10% formamide, pH 7.5) and incubated in Cy3-lablled 10 ng/μl FISH probe (Comate Bioscience Co.,Ltd., China) for 14 h at 42 °C in a humidified chamber. The probe information is listed in Table S1. Slides were then incubated for 20 min in wash buffer (0.9 M NaCl, 20 mM Tris-HCl, pH 7.5) preheated to 42 °C and washed three times. Samples were then incubated in the dark with 10 μg/mL DAPI (Servicebio Technology Co., Ltd., China)

in PBS for 10 min at room temperature, washed three times with PBS and mounted with Vectashield mounting medium (Vector Labs). Images were acquired on a Nikon A1 confocal microscope.

FISH and immunofluorescence (IF) double labelling (FISH/IF)

After the preparation and digestion of paraffin-embedded sections and before hybridization, the probe hybridization solution was added at a concentration of 1 μM and the sections were incubated in a humidified chamber to hybridize with the Cy3-lablled 10 ng/μL FISH probe (Comate Bioscience Co.,Ltd., China) for 14 h at 42 °C. Then, the sections were washed, blocked, and incubated with rabbit anti-*L. interrogans* serovar Lai strain Lai 56601 (1:500) as the primary antibody overnight at 4 °C. The next day, the samples were washed with PBS three times for 5 min. After washing, the samples were blocked with 5% BSA and incubated at room temperature for 1 h with the secondary antibody. Samples were then incubated in the dark with 10 μg/mL DAPI (Servicebio Technology Co., Ltd., China) in PBS for 10 min at room temperature, washed three times with PBS and mounted with Vectashield mounting medium (Vector Labs) [23, 24]. Images were acquired on a Nikon A1 confocal microscope.

Isolation of primary macrophages

Primary macrophages were isolated as previously described [10]. Six-week-old female hamsters were injected intraperitoneally with 2 mL of 3% thioglycolate dissolved in distilled deionized water. Hamsters were euthanized after 4 days, and macrophages were lavage by sterile PBS and enriched by plating the lavage cells on tissue culture plates in RPMI 1640 supplemented with 10% FCS and 1% penicillin and streptomycin at 37 °C for 2 h. Then, the plate was washed 3 times with sterile PBS to remove nonadherent cells.

Culture of primary macrophages with fecal filtration or serum

1×10^6 cells/well were seeded in a 12-well plate. Fecal filtration or serum were collected from uninfected hamsters and AM hamsters. Fecal pellets were homogenized using PBS (1 mL per 0.1 g of fecal matter). The homogenate was centrifuged at 100 rpm and 4 °C for 5 min and filtered using 0.22 μm syringe filters. The quantity of the fecal filtration or serum were adjusted to 5% or 20% of the total volume to stimulate macrophages *in vitro*. After incubation at 37 °C for 4 h, RNA was extracted by TRIzol.

To determine the role of LPS in the induction of cytokine release, fecal filtration or serum were pre-incubated with LPS neutralizing reagent polymyxin B (PMB; Solarbio, China) for 2 h at 37 °C resulting in a final concentration of 50 μg/mL PMB after addition to the macrophages. After 4 h of incubation at 37 °C, RNA was extracted with TRIzol.

Real-time and reverse transcription-qPCR (RT-qPCR)

Total RNA of the colon samples was extracted by TRIzol (Invitrogen, USA). RNA was reverse transcribed into cDNA by using random primers from a TransScript One-Step gDNA Removal kit and cDNA Synthesis SuperMix (TransGen Biotech, China). The primers used in this study are listed in Table S1. The PCR conditions were as follows: 50 °C for 2 min, 95 °C for 10 min, followed by 45 cycles of amplification at 95 °C for 15 s and 60 °C for 60 s [25]. qPCR was conducted by an Applied Bioscience 7500 thermocycler and FastStart Universal SYBR Green Master Mix (Roche Applied Science, Germany). The expression of target genes was normalized to that of GAPDH using the $2^{-\Delta\Delta CT}$ method.

Bacterial load and qPCR assay

The leptospiral burdens in organs were determined by quantitative PCR using an Applied Bioscience 7500 thermocycler and FastStart Universal SYBR green Master

(Roche Applied Science, Germany). The specimens (0.09 to 0.15 g) of tissues were homogenized with PBS (w/v, 1/10). The homogenate was centrifuged at 2000 rpm and 4 °C for 5 min. The supernatant was transferred into a new tube. Then, it was centrifuged at 12000 rpm and 4 °C for 5 min. The pellets were extracted using the TIANamp Bacteria DNA kit (Tiangen, China) according to the manufacturer's instructions [26]. The concentration of DNA was measured by spectrometry. The genomic DNA of a counted number of *L. interrogans* was used as a calibrator. The primers specific for *LipL32* was used to detect leptospires (Forward primer, 5'-TCGCTGAAATRGGWGTTCGT-3'; Reverse primer, 5'-CGCCTGGYTCMCCGATT-3') . The leptospiral load was presented as the number of genome equivalents per μg of tissue DNA [27].

Histopathological examination

Colons from the uninfected group and AM group were collected and fixed in buffered 4% formaldehyde. Hematoxylin and eosin (H&E) staining was performed on 4–5 μm paraffin-embedded sections. Blinded samples were then scored by two pathologists. Each section was evaluated for inflammation, epithelial hyperplasia, erosion and ulceration, and the extent of lesion as listed in Table S2 [28].

Microbiota assay

Fresh fecal pellets were collected under sterile conditions, immediately frozen in liquid nitrogen, and then stored at −80 °C. Total genomic DNA from the samples was extracted using the CTAB/SDS method. DNA concentration and purity were monitored on 1% agarose gels. Then, 16S rRNA genes of distinct regions were amplified using specific primers with barcodes (27F, AGAGTTTGATCMTGGCTCAG; 1492R, ACCTTGTTACGACTT). All PCRs were carried out with TransStart FastPfu DNA Polymerase (TransGen Biotech). PCR products were purified using the Qiagen Gel Extraction Kit (Qiagen, Germany).

Sequencing libraries were generated using the SMRTbellTM Template Prep Kit (PacBio) following the manufacturer's recommendations. The library quality was assessed on the Qubit 2.0 Fluorometer (Thermo Scientific) and FEMTO Pulse system. Finally, the library was sequenced on the PacBio Sequel platform. Raw sequences were initially processed through the PacBio SMRT portal. Sequences were filtered for a minimum of 3 passes, and a minimum predicted accuracy of 90% (minfullpass = 3, minPredictedAccuacy = 0.9). The predicted accuracy was 90%, which was defined as the threshold below which CCS was considered noise. The files generated by the PacBio platform were then used for amplicon size trimming to remove sequences outside the expected amplicon size (minLength 1340 bp, maxLength 1640 bp). The reads were assigned to samples based on their unique barcode and trimmed by removing the barcode and primer sequence. The reads were compared with the reference database using the UCHIME algorithm to detect chimeric sequences, and then the chimeric sequences were removed to obtain clean reads. Alpha diversity (Chao1 and Shannon) was calculated with QIIME (Version 1.9.1) and displayed with R software (Version 2.15.3). Principal coordinate analysis (PCoA) was performed to obtain principal coordinates and visualize the complex, multidimensional data. A distance matrix of weighted UniFrac among samples obtained before was transformed to a new set of orthogonal axes, by which the maximum variation factor was demonstrated by the first principal coordinate, and the second maximum variation factor was demonstrated by the second principal coordinate. PCoA analysis results were displayed by the WGCNA package, stat packages and ggplot2 package in R software (Version 2.15.3).

Statistical analysis

Survival differences between the study groups were compared by using the Kaplan-Meier log-rank test. All values are expressed as the mean ± SEM. Differences

between mean values of normally distributed data were analyzed using the Wilcoxon rank-sum test. The results were considered statistically significant at $p < 0.05$.

Results

L. interrogans proliferated in the intestine, impaired the intestinal structure, and induced proinflammatory response

Recently, Inamasu *et al*. reported that subcutaneous infection with leptospires led to intestinal dissemination [29]. We wondered whether different challenge routes affected the dissemination of *Leptospira* into the intestine. Thus, we compared the common intraperitoneal challenge with subcutaneous challenge in a hamster model of leptospirosis. Our study indicated that subcutaneous challenge and intraperitoneal challenge both led to leptospiral proliferation in the ileum and colon (Supplementary Fig. S1A and Fig. 1B). There were approximately 7 times more leptospires in the colon than in the ileums at AM p.i. after intraperitoneal challenge (Fig. 1B). Hamsters infected by intraperitoneal challenge had shortened survival time compared with hamsters infected by subcutaneous challenge (Supplementary Fig. S1B). *L. interrogans* infection disrupted the intestinal structure, caused bleeding, and led to the infiltration of inflammatory cells (Fig. 1C). In addition, *L. interrogans* infection promoted the inflammatory response in the colons as determined by the upregulated gene expression of *lipocalin 2* (*Lcn2*)/*neutrophil gelatinase-associated lipocalin* (*NGAL*), a marker of intestinal inflammation [30], *TNF-α*, *IL-1β*, *Nos2*, and *TLR4* (a well-known receptor of LPS that is vital against *Leptospira* infection [31]) and the downregulated gene expression of *IL-10* (Fig. 1D-I). We wondered whether proinflammatory gene expression was also related to other TLRs [32]. The results showed that the gene expression of *TLR2* and *TLR9* was significantly increased, while *TLR3*, *TLR5*, and *TLR7* showed no obvious difference in the AM group compared with the D0 group (Supplementary Fig. S2A-E). It is well-known that *Leptospira* infection can cause leptospiremia and cytokine storms at late stages [6, 33]. Our results

also proved that the gene expression of *TNF-α*, *IL-1β*, and *IL-10* were increased in the blood of the AM group compared with that of D0 group (Supplementary Fig. S2F-H). These results indicated that *L. interrogans* was able to proliferate in the intestine and impair the intestinal structure, along with inducing intestinal inflammation and systemic inflammation.

The disruption of the gut microbiota after *L. interrogans* infection

To obtain insight into the composition of the gut microbiota during leptospirosis, we analyzed the feces from *Leptospira*-infected hamsters by 16S rRNA gene sequencing at the indicated time points (Fig. 2A). The results showed that the bacterial richness (Observed species and Ace) and the diversity (Simpson index and Shannon index) of the gut microbiota were decreased at 2 days p.i., while they were increased at AM p.i. (Fig. 2B-E). Then, we pooled all groups together to perform principal coordinate analysis (PCoA). The results showed that the composition of the gut microbiota in the AM group was significantly different from that in the D0 group (Fig. 2G). There was a large degree of dispersion among different individuals 2 days p.i. (Fig. 2F), and the composition of the gut microbiota in the D2 group was also different from that of the other two groups (Supplementary Fig. S3A-B). To identify bacterial taxa that were common in the D0 group or the AM group, we performed linear discriminant analysis effect size (LEfSe) analysis. The results showed that the abundance of Proteobacteria was increased in the AM group (Fig. 2I and 2K), while the abundances of *Lactobacillus* and *Allobaculum* were decreased compared with those in the D0 group (Fig. 2I, 2L, and 2M). The composition of the bacterial taxa in the D2 group was also various compared with that of other two groups, especially the abundance of *Lactobacillus* was also decreased in the AM group compared with that of the D2 group (Supplementary Fig. S3C-D). Although the relative abundance of Firmicutes seemed to gradually decrease after *Leptospira* infection (Fig. 2H), the ratio of Firmicutes to Bacteroidetes showed no difference in the D0 and AM groups (Fig.

2J). These results demonstrated that *L. interrogans* infection changed the composition of the gut microbiota of hamsters.

***L. interrogans* infection increased intestinal permeability by disrupting tight junctions**

The intestinal barrier is essential for maintaining intestinal homeostasis [34]. Epithelial tight junctions define the paracellular permeability of the intestinal barrier, which is related to some tight junction proteins, such as Claudins, Occludin, and Zona Occludens 1 (Zo-1) [35]. The epithelium separates the organism from the external environment and defines individual compartments within tissues. At some sites, the epithelial cells form a nearly complete barrier, the disruption of which is catastrophic [35]. Most studies have relied on a probe, FITC-4 kDa dextran, which has a hydrodynamic diameter of 28 Å, to probe intestinal permeability [36-38]. As shown in Fig. 1B, we detected leptospiral proliferation in the gut. We wondered whether *Leptospira* infection could influence intestinal permeability. The results showed that the intestinal permeability of hamsters was increased significantly after infection (Fig. 3A). Then, we further investigated the gene expression of tight junction proteins and *Mucin-2* in hamsters. The results showed that the gene expression of *Claudin-2* was increased, while the gene expression of *Claudin-3*, *JAMA*, *ZO-1* and *Mucin-2* was decreased in the AM group compared with the D0 group (Fig. 3B-3F).

***L. interrogans* infection caused the translocation of Proteobacteria**

Damaged intestinal structure may increase the translocation of pathobionts [39, 40] and our 16S rRNA sequencing showed an increase in Proteobacteria in the AM group. We hypothesized that the pathogenic Proteobacteria could translocate into the intestinal epithelium or bloodstream after infection. First, we found that the intestinal epithelium of the AM group had more bacteria than that of the D0 and D2 groups, as determined by the EUB 338 probe, which was supposed to hybridize with all bacteria

(Fig. 4). Then, we examined the intestines of the D0 group and the AM group by FISH assays with the GAM42a probe, which was supposed to hybridize γ-Proteobacteria, and IF assays with the anti-*L. interrogans* serovar Lai strain Lai 56601 antiserum. The results showed that the intestinal epithelium of the AM group indeed had γ-Proteobacteria that was not colocalized with leptospires (Supplementary Fig. S4A). Then, we tested whether the gut microbiota could translocate into the blood. However, no bacteria were recovered from the blood by aerobic or anaerobic culture on LB and MacConkey agar plates (Supplementary Fig. S4B).

Gut microbiota depletion exacerbated leptospirosis

To determine the role of the gut microbiota in hamsters during *L. interrogans* infection, we treated hamsters with broad-spectrum antibiotics to deplete the gut microbiota, as shown in Fig. 5A. 16S rRNA sequencing analysis indicated that the richness of the gut microbiota decreased after antibiotic treatment (Fig. 5B-C). In addition, the diversity of the gut microbiota in the Abx-treated hamsters was lower than that of the control group, and this change in diversity was reversed by FMT (Fig. 5D-E). Antibiotic treatment changed the composition of the gut microbiota in hamsters (Fig. 5F, Supplementary Fig. S5A-D), leading to the expansion of Proteobacteria and a decrease in Bacteroidota (Fig. 5F-G). The survival time of Abx-treated hamsters was significantly reduced after infection with 10^6 leptospires (Fig. 5I). In addition, we also measured leptospiral load and the gene expression of inflammatory cytokines in the blood. The results showed that the leptospiral load of the Abx group was significantly increased compared with that of the control group, and this increase was partially reversed by FMT (Fig. 5J). Abx treatment slightly increased the gene expression of *TNF-α* and *IL-1β* compared with those of the untreated group. Consistent with this result, the Abx-treated infection group exhibited higher expression of *TNF-α* and *IL-1β* than the untreated infection group, which was also partially reversed by FMT (Fig. 5K-L). Although *Leptospira* infection

significantly increased the gene expression of *IL-10* compared with that of the untreated group, there was no significant difference among the control infection group, the Abx-treated infection group, and the FMT infection group (Fig. 5M). These results indicated that gut microbiota homeostasis helped fight *Leptospira* infection in hamsters.

Gut-derived LPS in the intestine and serum induced inflammation

Gut dysbiosis is a major determinant of endotoxaemia via dysfunction of the intestinal barrier scaffold, which is a prerequisite for LPS translocation into systemic circulation [41]. A previous study demonstrated that gut-derived LPS was a central mediator in alcoholic steatohepatitis [42], white adipose tissue inflammation [43], and peripheral inflammation [44]. Cytokine storms are characteristic of severe leptospirosis [6]. Our results showed that the proportion of Proteobacteria and intestinal permeability were increased in the AM group; thus, we hypothesized that gut-derived LPS promoted the inflammatory response in severe leptospirosis. We found that the LPS level was higher in the serum of the AM group than in that of the D0 group (Supplementary Fig. S6B). The LPS level of in the Abx-treated AM group was lower than that in the PBS-treated AM group (Supplementary Fig. S6B). To further gain insight into the role of gut-derived LPS in inflammation, we cocultured macrophages with different concentrations of fecal filtration or serum and measured the changes in gene expression (Fig. 6A). The results showed that fecal filtration from uninfected hamsters and AM hamsters both upregulated the gene expression of *IL-1β*, *IL-10*, and *TLR4*, while only fecal filtration from AM hamsters upregulated the gene expression of TNF-α (Fig. 6B-E). The serum of uninfected hamsters significantly increased the transcription of *TNF-α* (Fig. 6F), while the serum of AM hamsters only slightly upregulated the gene expression of *TNF-α* (Fig. 6F). Serum from uninfected hamsters and AM hamsters promoted the transcription of IL-1β (Fig. 6G). However, only the serum of AM hamsters significantly upregulated the gene expression of *IL-10* and

TLR4 (Fig. 6H-I). The identification of TLR4, a well-known receptor of LPS, prompted us to examine whether the proinflammatory activity of fecal filtration and serum of AM hamsters was mediated by LPS. A previous study demonstrated that cytokine responses to heat-killed leptospires were not inhibited by PMB [31]. Our results showed that LPS neutralization with PMB inhibited gene expression of *TNF-α*, *IL-1β*, *IL-10*, and *TLR4* in macrophages after stimulation by fecal filtration or serum from AM hamsters (Fig. J-Q). These results indicated that gut-derived LPS might be an essential factor that causes inflammatory storm in hamsters with severe leptospirosis.

LPS neutralization synergized with antibiotic therapy or polyclonal antibody therapy improved the survival rate in severe leptospirosis

Based on the above results, we proposed that in addition to leptospires, gut-derived LPS might also play a pathogenic role in severe leptospirosis and induce an uncontrolled inflammatory response, in which case neutralizing LPS could be effective in treating severe leptospirosis. Therefore, we explored the effect of LPS neutralization therapy compared with antibiotic therapy and our published rabbit polyclonal antibody therapy in severe leptospirosis [20]. As shown in Fig. 7A, moribund hamsters were treated twice a day for 3 consecutive days. LPS neutralization therapy, antibiotic therapy, and rabbit polyclonal antibody therapy did not significantly improve the conditions of female hamsters with severe leptospirosis. However, LPS neutralization combined with antibiotic therapy or rabbit polyclonal antibody therapy significantly increased the survival rate up to 50% or 66.7% (Fig. 7B). To further explore the factors leading to differences in survival, we determined the leptospiral load and gene expression of inflammatory cytokines in the blood at 6 days p.i.. The results showed that antibiotic therapy and rabbit polyclonal antibody therapy decreased the leptospiral load in the blood, while LPS neutralization therapy did not reduce the leptospiral load in the blood (Fig. 7C). Although antibiotic therapy

reduced the leptospiral load, it significantly increased the gene expression of proinflammatory cytokines (*TNF-α* and *IL-1β*) compared with that of the control group (Fig. 7D-E). LPS neutralization therapy combined with *Leptospira*-killing therapy decreased the gene expression of inflammatory cytokines (*TNF-α*, *IL-1β* and *IL-10*) compared with that of the control group (Fig. 7D-F). In addition, we examined the effectiveness of LPS neutralization therapy in male hamsters with severe leptospirosis and in female hamsters with different serotype. The results showed that LPS neutralization synergized with antibiotic therapy or polyclonal antibody therapy improved the conditions of male hamsters with severe leptospirosis and female hamsters infected by another pathogenic leptospiral serotype (Supplementary Fig. S8B and Supplementary Fig. S9B). Our results indicated that LPS neutralization could be a novel therapy for severe leptospirosis.

Discussion

Recently, Inamasu *et al*. investigated the pathological changes and distribution of leptospires in the gut of hamsters after subcutaneous infection [29]. Their study highlighted that not only the urine of infected animals but also the feces could be a source of infection. However, the exact role of the gut microenvironment in the pathogenesis of leptospirosis is still unknown. In our study, we found that leptospires could proliferate in the ileum and colon following intraperitoneal challenge or subcutaneous challenge, which was consistent with the results of Inamasu's study [29]. Hamsters infected intraperitoneally exhibited more rapid death than hamsters infected subcutaneously. A previous study reported that the route of infection affected the kinetics of hematogenous dissemination, kidney colonization, and inflammation [45]. In addition, the subcutaneous route induced a slower-progressing infection than the intraperitoneal route in a leptospirosis rat model [46]. However, there was a higher leptospiral load in the colons than in the ileums of hamsters infected by the intraperitoneal route. The capacity of *L. interrogans* to breach the immune defence at

each port of entry determined when *Leptospira* gained access to the blood. Although intraperitoneal challenge may be not a natural route of infection, both intraperitoneal challenge and subcutaneous challenge led to the colonization of leptospires in the intestine, indicating that leptospiral colonization in the intestine was independent on the routine of infection. *Leptospira* infection destroyed the intestinal structure and induced intestinal inflammation in the colon. However, Inamasu *et al*. reported that *Leptospira* was not involved in the pathogenesis of the colon, suggesting that the jejunum and ileum are the main sites of lesions. Differences in bacterial strains and the route of infection might lead to variations in results. Infection via the intraperitoneal and subcutaneous routes bypass the epidermal defence system. Early damage to the intestinal structure and environment may be one of the causes of rapid death following infection by intraperitoneal injection. The proliferation of leptospires in the intestine makes feces a potential excretion route. Our previous study demonstrated that leptospires were rapidly cleared in the intestine and that *Leptospira* infection did not increase the intestinal permeability of mice [10]. A recent study reported that pathogenic *Leptospira* spp. were highly present in human and gorilla stool samples in the Congo but were absent in other African nonhuman primates, therefore suggesting a possible gorilla-humans transfer [47]. Our 16S rRNA sequencing results also showed that the relative abundance of Spirochetes was increased in the AM group compared with that of the D0 group, which might be related to the excretion of leptospires in the feces. However, no leptospires were detected in the feces from *Leptospira*-infected rats [46]. Serovar specificity and species differences may determine the excretion route of leptospires and the disruption of intestinal homeostasis may influence the progression of leptospirosis.

Our 16S rRNA sequencing results showed that the species richness and diversity of the gut microbiota in the AM group were even higher than those in the D2 group. The species richness and diversity of the gut microbiota decreased first and then increased with disease progression. This phenomenon was also reported in melioidosis and

HIV-1 infection [48, 49]. Lankelma *et al.* explained that several "big players" in the gut microbiota were eliminated by the host immune system during severe systemic bacterial infection, giving way to the proliferation of other bacteria [48]. Specific microbiota community composition, rather than species richness, drives microbiota-mediated resistance against *Leptospira* infection. Becattini *et al.* demonstrated that microbiota composition and the type of immune stimulus impacted bacterial responses and that the intestinal metabolome was rapidly reshaped by host immune activation [50]. We hypothesized that the early colonization and proliferation of leptospires in the intestine rapidly activated intestinal immunity leading to the expansion of specific microbes in the AM group. The composition of the gut microbiota in the D2 group was discrete, as shown by PCoA. This difference might be related to the disease progression. In addition, we found that the levels of *Lactobacillus* and *Allobaculum* were higher in the D0 group than in the AM group at the genus level. Meanwhile, the level of Proteobacteria increased in the AM group. A previous study demonstrated that pre-treatment with *Lactobacillus plantarum* prevented severe pathogenesis in mice [51], which highlighted the *Lactobacillus* as candidate for leptospirosis prevention. Our study proved that *Leptospira* infection increased the gene expression of *TLR2*, *TLR4*, *TLR9*, and some proinflammatory cytokines, which created a proinflammatory environment in the intestine. A previous study demonstrated that intestinal inflammation increased the bioavailability of respiratory electron acceptors for Enterobacteriaceae, which promoted the expansion of pathogenic Enterobacteriaceae [52]. Therefore, it is plausible that *Leptospira* infection triggered intestinal inflammation, which promoted the expansion of Proteobacteria by increasing the bioavailability of respiratory electron acceptors for pathogenic Proteobacteria.

The intestinal barrier is critical for maintaining homeostasis in the host. The apical junctional complex (AJC) encircles pairs of neighbouring epithelial and endothelial cells to create an adhesive network and maintain barrier integrity [53]. Our results

showed that *Leptospira* infection significantly disrupted the intestinal permeability of hamsters at the late stage of infection. Previous studies have demonstrated that *Leptospira* infection reduced the signalling between cell-cell junction proteins and caused the mislocalisation of AJC proteins, such as occludin and Zo-1 [54]. A recent study reported that *L. interrogans* could disassemble AJCs in renal proximal tubule epithelial cells and transmigrate through the paracellular route for dissemination in the host [54]. Thus, we hypothesize that *L. interrogans* was also able to transmigrate through the intestinal epithelial barrier, and feces excretion route of leptospires was possible in susceptible hosts during leptospirosis. Our results showed that the gene expression of *Claudin-2*, *Claudin-3*, *Claudin-4*, *Zo-1* and *Mucin-2* were compensatively upregulated in the D2 group compared with those in the D0 group. However, the gene expression of *Claudin-3*, *JAMA*, *Zo-1* and *Mucin-2* was downregulated in the AM group compared with that in the D0 group. In contrast, the gene expression of *Claudin-2* was higher in the AM group that in the D0 group, which might explain why moribund hamsters had diarrhoea [36].

Gut microbiota-depleted hamsters exhibited reduced survival time, increased leptospiral load, and upregulated gene expression of proinflammatory cytokines compared with untreated hamsters after *Leptospira* infection, while FMT partially reversed these effects. Our previous study demonstrated that the leptospiral load was increased in the organs of gut microbiota-depleted mice and that gut microbiota depletion diminished the bactericidal activity of macrophages during *Leptospira* infection [10]. These results indicated that the homeostasis of the gut microbiota is essential for both susceptible and resistant hosts to combat *Leptospira* infection. 16S rRNA sequencing analysis revealed that antibiotic treatment decreased the diversity of the gut microbiota, and FMT partially reversed these changes. Antibiotic treatment increased the relative abundance of proinflammatory bacteria, such as γ-Proteobacteria, while it reduced the relative abundance of anti-inflammatory bacteria, such as *Allobaculum*. The alteration of the gut microbiota might skew the

host to an inflammatory condition and reduce antileptospiral capacity. FMT aims to replace an unfavourable resident gut microbiota with a favourable microbiota from a healthy donor. FMT has demonstrated clear efficacy in the treatment of recurrent *Clostridioides difficile* infection (rCDI), inflammatory bowel disease, and neurological disorders [16]. The efficiency of FMT determines the phenotypes of subjects in the infection model. Prolonging the duration of FMT might help recover the gut microbiota homeostasis and the capacity to combat anti-*Leptospira* infection.

Our study showed the translocation of Proteobacteria to the intestinal epithelium. Although no bacteria were found in the blood of the AM group, the level of LPS was increased in the PBS-treated AM group compared with that of the uninfected group, and it was decreased in the Abx-treated AM group compared with that of the PBS-treated AM group. This result indicated that gut-derived LPS was involved in severe leptospirosis. Gut dysbiosis is a primary determinant of low-grade endotoxaemia mediated by dysfunction of the intestinal barrier scaffold, which is a prerequisite for LPS translocation into the systemic circulation [41]. Importantly, mice (resistant to leptospirosis) can tolerate levels of LPS endotoxin 250 higher than the level that humans can tolerate [55]. In addition, classical gram-negative LPS had a 1000-fold higher potency than leptospiral LPS [56]. Fecal filtration from the AM group and the uninfected group both increased the gene expression of *TLR4*, while only serum from the AM group increased the gene expression of *TLR4*. TLR4 expression was inhibited by PMB treatment. In severe leptospirosis, excessive immune activation is a hallmark of disease [6]. A previous study demonstrated that the cytokine response to heat-killed whole leptospires was not inhibited by PMB in macrophages [31]. Leptospiral LPS has been described as atypical in terms of structure, and silver-stained electrophoretic profiles of LPS from pathogenic leptospiral strains were shown to be different from those of *E. coli* LPS [57]. Although it is still difficult to distinguish the specific roles of gut-derived LPS and leptospiral LPS in promoting inflammation, we hypothesize that gut-derived LPS may also be an essential driver of

inflammatory reactions in severe leptospirosis. In a rat cirrhosis model, gut decontamination with a 2-week course of antibiotics led to a redistributed microbiota, reduced proinflammatory activation of mucosal immune cells and diminished gut bacterial translocation [39]. Another study showed that endogenous endotoxins caused a pan enteric inflammatory ileus after colonic surgery and that gut decontamination alleviated small intestinal muscularis inflammation [58]. Our results showed that doxycycline treatment significantly reduced the leptospiral load in the blood in severe leptospirosis; however, the gene expression of proinflammatory cytokines was upregulated, which was consistent with the Jarisch-Herxheimer Reaction [5]. Recently, Li *et al*. proposed an all-in-one drug delivery strategy equipped with bactericidal, LPS neutralization, and detoxification activities to treat sepsis-associated infections and hyperinflammation [59]. Our study also highlighted the important role of endogenous gut-derived LPS induced by *Leptospira* infection. Thus, the neutralization of gut-derived LPS and antileptospiral therapy are both essential in the treatment of severe leptospirosis; thus, these strategies provide a novel avenue for the treatment of human acute leptospirosis in the future.

Our study has potentially important clinical implications for the care of critically ill patients with severe leptospirosis. In human infections, gastrointestinal symptoms such as abdominal pain, vomiting, and diarrhea have been frequently observed, which has been linked to an increased risk of organ dysfunction, and even death [2, 60]. Our findings implicate impairment of the intestinal barrier by *Leptospira* infection as a mechanism underlying the association between gut dysbiosis and disseminated gut-derived LPS in the ICU. More importantly, from a translational perspective, our findings suggest that the increased risk of death in severe leptospirosis can be reduced by LPS neutralization combined with anti-*Leptospira* therapy. In fact, gut microbiota dysbiosis in COVID-19 patients or HIV infection is also associated with microbial translocation, LPS biosynthesis, and an expansion of Proteobacteria [61, 62]. The application of LPS antagonist therapies, whether microbial or pharmaceutical, may be

a viable precision-medicine strategy to increase the survival rate of patients with severe leptospirosis or other gut-damaging infections by protecting against the spread of LPS in the bloodstream.

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Author contributions

WZ and YC conceived ideas, and XX and WZ designed experiments. XX, JL, ZN and XC performed the mouse experiments. JL and SZ conducted the bacteriology experiments. XX and JL analyzed data. WZ and YC performed bioinformatics analysis. XX and WZ wrote the original draft. YC supervised the study and reviewed the final version of the text.

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Competing interests

The authors declare no competing interests.

Data availability statement

Data are available in a public, open access repository. The bacterial 16S rDNA sequences were deposited to the sequence read archive. The accession number for the 16S sequencing data reported in this paper are PRJNA772361 and PRJNA1036417.

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Figure legends

Fig. 1. *L. interrogans* proliferated in the intestine, destroyed the intestinal structure, and increased intestinal inflammation. (A) Flow diagram of the experiment. Six-week-old female hamsters were injected intraperitoneally with 10^7 leptospires. Hamsters were euthanized at 0 h, 6 h, 48 h, 96 h, and AM p.i.. The ileums and colons were collected aseptically for further analysis. (B) Leptospiral burdens in the ileums and colons of *Leptospira*-infected hamsters were determined by qPCR (n = 6). (C) Left panel, histopathology of colons of D0 hamsters (scale bar, 50 μm; n = 4) and AM hamsters (scale bar, 50 μm; n = 4). Representative photographs are presented. Right panel, histopathology scores of colons. D0, uninfected hamster. AM, articulo mortis. (D-I) The gene expression of *Lcn2* (D), *TNF-α* (E), *IL-1β* (F), *IL-10* (G), *TLR4* (H), and *Nos2* (I) was analyzed by RT-qPCR. The mRNA levels of genes in the colons of the D0 group (n = 3) and the AM group (n = 3) were normalized to the expression of the housekeeping gene *GAPDH*. Each infection experiment was repeated three times. Data are shown as the mean ± SEM and analyzed by the Wilcoxon rank-sum test. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Fig. 2. *L. interrogans* infection changed the composition of the gut microbiota. (A) Flow diagram of the experiment. Six-week-old female hamsters were injected intraperitoneally with 10^7 leptospires. Fecal pellets were collected aseptically for 16S rRNA gene sequencing at 0 days, 2 days, and AM p.i.. D0, uninfected hamster. AM, articulo mortis. (B-C) Observed species (B) and Ace (C) in the feces of hamsters. Observed species and Ace indicate species richness. (D-E) Simpson index (D) and Shannon index (E) in the feces of hamsters. Simpson and Shannon index indicate species diversity. (F) Principal coordinates analysis (PCoA) of fecal samples based on 16S rRNA gene sequencing using weighted UniFrac. (G) PCoA of fecal samples based on 16S rRNA gene sequencing using weighted UniFrac. (H) Relative abundance of the top 10 phyla in the feces of hamster. (I) Linear discriminant analysis (LDA) of effect size (LEfSe) between D0 and AM hamsters (LDA score > 3). Cambridge blue bars indicate taxa enrichment in D0 hamsters, and pink bars indicate taxa enrichment in AM hamsters. (J) The ratio of Firmicutes to Bacteroidetes in the D0 and AM. (K-M) The relative abundance of Proteobacteria (K), *Lactobacillus* (L), and *Allobaculum* (M) in the D0 and AM groups. (D0, n = 10; D2, n = 10; AM, n = 8). Data are shown as the mean ± SEM and analyzed by the Wilcoxon rank-sum test. $^*p < 0.05$, $^{**}p < 0.01$.

Fig. 3. *L. interrogans* infection disrupted intestinal tight junctions. Six-week-old female hamsters were injected intraperitoneally with 10^7 leptospires. Blood was collected to determine intestinal permeability at 0 days, 2 days, and AM p.i.. Colons were collected aseptically to measure gene expression at 0, 2, and AM p.i.. (A) The intestinal permeability of *Leptospira*-infected hamsters was analyzed with a fluorescence spectrophotometer at indicated time (D0, n = 4; D2, n = 6; AM, n = 6). AM, articulo mortis. (B-G) The relative gene expression of *ZO-1* (B), *Claudin-3* (C), *JAMA* (D), *Claudin-2* (E), and *Mucin-2* (F) in *Leptospira*-infected hamsters (n = 6-10

per group) was determined by qPCR. D0, uninfected hamster. AM, articulo mortis. Each infection experiment was repeated three times. Data are shown as the mean ± SEM and analyzed by the Wilcoxon rank-sum test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Fig. 4. Translocated microbiota in the intestinal epithelium following *L. interrogans* infection. Six-week-old female hamsters were injected intraperitoneally with 10^7 leptospires. Hamsters were euthanized at 0 days, 2 days, and AM p.i.. AM, articulo mortis. Colons were collected aseptically. The intestinal contents were excluded from the intestine with PBS and a segment of colon was fixed in 4% paraformaldehyde solution overnight and analyzed by FISH with an EUB 338 probe (red). (A) Th results are representative photographs of three groups. (B) Number of positive bacteria per field in the three groups. n = 6 per group. D0, uninfected hamster. AM, articulo mortis. Each infection experiment was repeated three times. Data are shown as the mean ± SEM and analyzed by the Wilcoxon rank-sum test. ***$p < 0.001$.

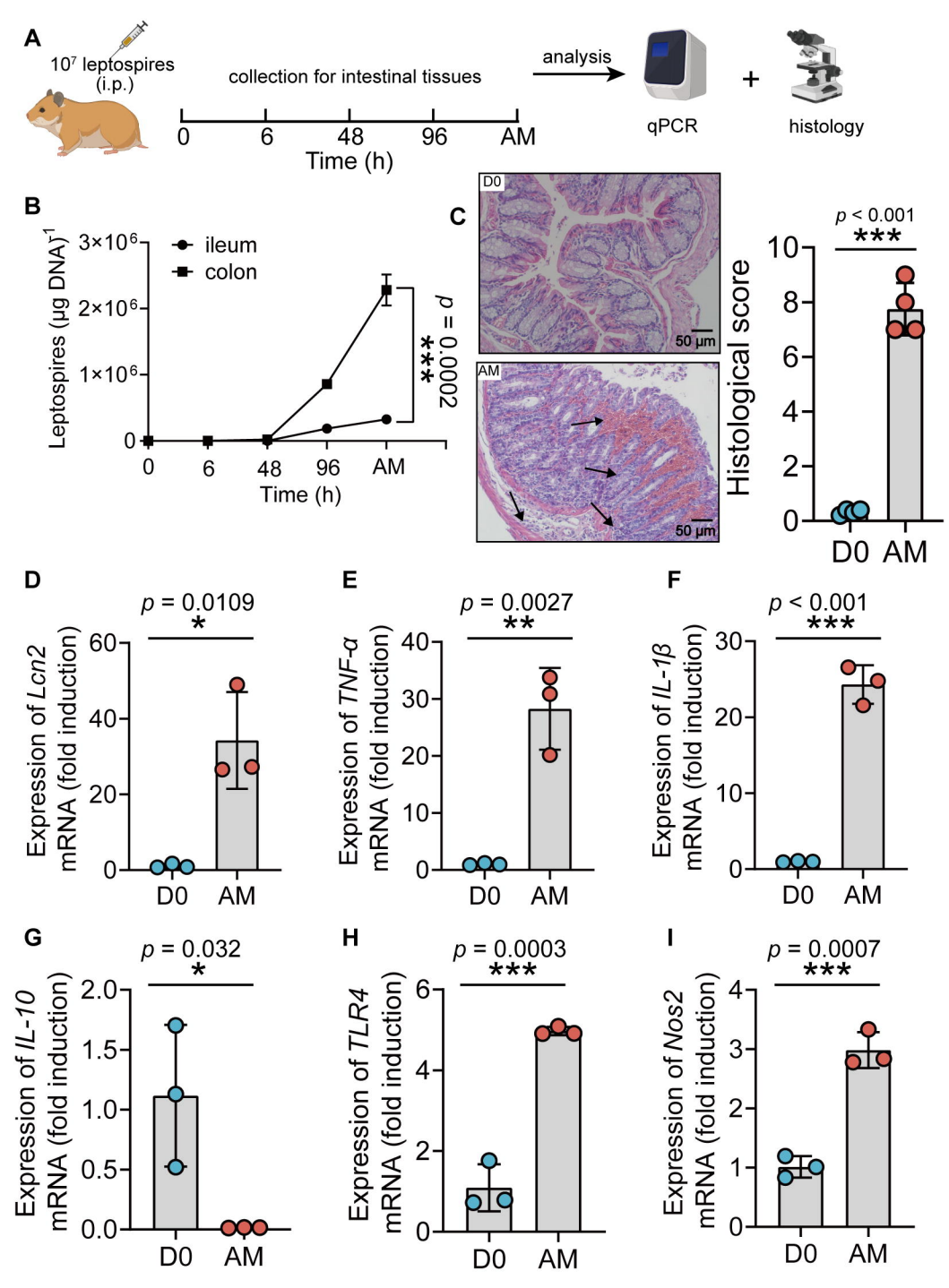
Fig. 5. Microbiota depletion exacerbated leptospirosis. (A) Flow diagram of the experiment. Hamsters were administered with Abx or water intragastrically once daily for ten consecutive days. Then, fecal pellets were collected aseptically for 16S rRNA gene sequencing. (B-C) Observed species (B) and Chao1 (C) in the feces of hamsters. Observed species and Chao1 indicate species richness. (D-E) Simpson index (D) and Shannon index (E) in the feces of hamsters. Simpson and Shannon index indicate species diversity. (F) Relative abundance of the top 10 phyla in the feces of hamsters. (G) The relative abundance of Bacteroidetes, Firmicutes, and Proteobacteria in the Con, Abx, and FMT groups. n = 4 per group. (H) Hamsters were treated with Abx as described above. After a 2-day washout period, hamsters were intraperitoneally infected with 10^6 *L. interrogans*. Then, the survival rate of the hamsters was recorded.

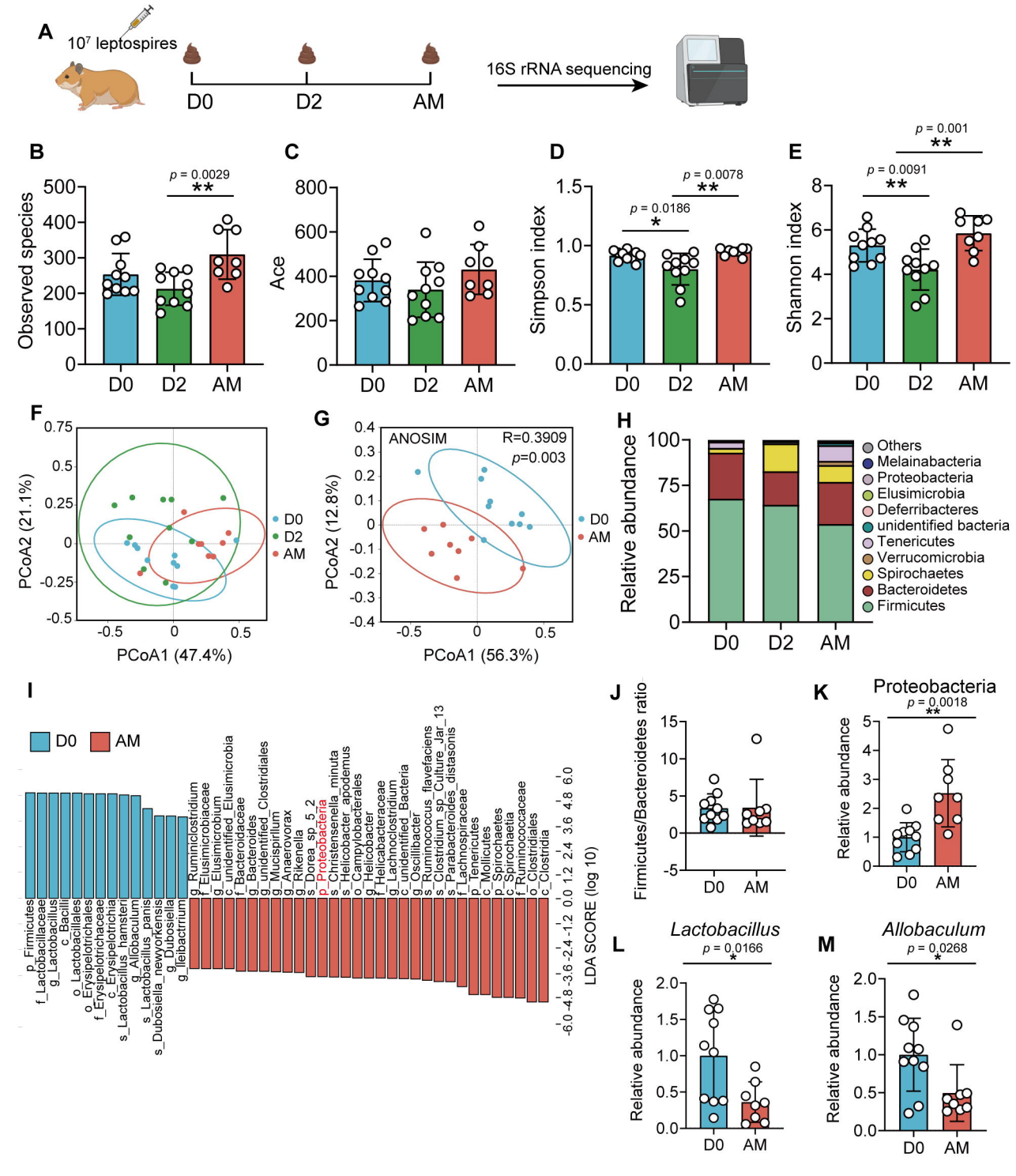
n = 6 per group. (J) Leptospiral load in the blood of the Con, Abx, and FMT groups. n = 6 per group. (K-M) Gene expression of *TNF-α* (K), *IL-1β* (L), and *IL-10* (M) in the blood of the three groups. n = 5 per group. Each infection experiment was repeated three times. Data are shown as the mean ± SEM and analyzed by the Wilcoxon rank-sum test. Survival differences between the study groups were compared by using the Kaplan-Meier log-rank test. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. ns, not significant.

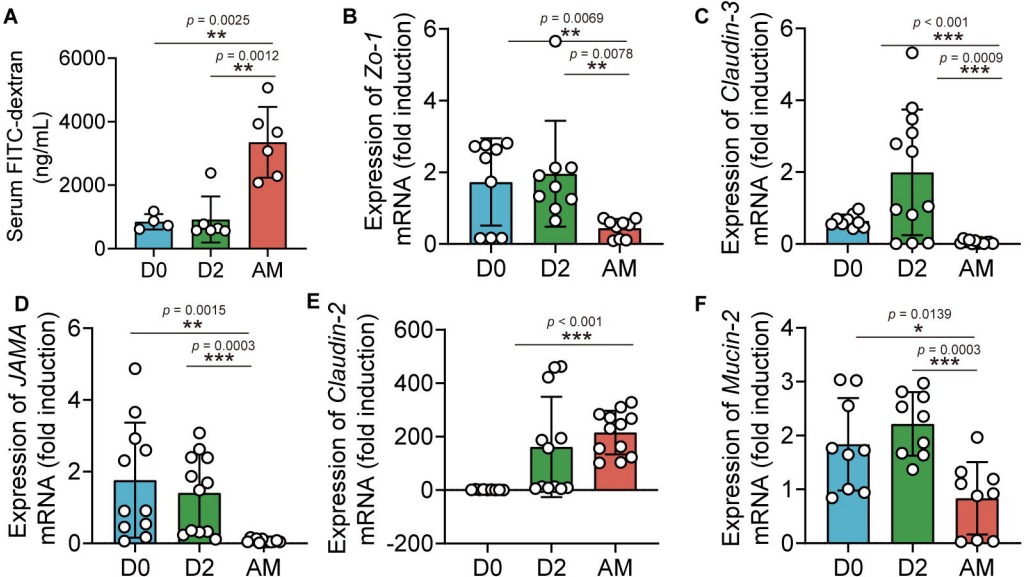
Fig. 6. *Leptopsira* infection induced intestinal and systemic inflammation that was partially inhibited by LPS neutralization. (A) Flow diagram of the experiment. Fecal filtration or serum of uninfected hamsters and AM hamsters was directly added into dishes or pretreated with PMB at 37 °C for 2 h, and then cocultured with macrophages for 4 h. RNA was extracted for gene expression analysis. (B-E) The gene expression of *TNF-α* (B), *IL-1β* (C), *IL-10* (D), and *TLR4* (E) of fecal filtration of uninfected hamsters (n = 3) and AM hamsters (n = 3) were analyzed by RT-qPCR. (F-I) The gene expression of *TNF-α* (F), *IL-1β* (G), *IL-10* (H) and *TLR4* (I) of serum of uninfected hamsters (n = 3) and AM hamsters (n = 3) were analyzed by RT-qPCR. (J-M) The effect of LPS neutralization on the gene expression of *TNF-α* (J), *IL-1β* (K), *IL-10* (L) and *TLR4* (M) in the fecal filtration of AM hamsters (n = 3) were analyzed by RT-qPCR. (N-Q) The effect of LPS neutralization on the gene expression of *TNF-α* (N), *IL-1β* (O), *IL-10* (P) and *TLR4* (Q) of in the serum of AM hamsters (n = 3) was analyzed by RT-qPCR. The mRNA levels in untreated controls were set as 1.0. Each infection experiment was repeated three times. Data are shown as the mean ± SEM and analyzed by the Wilcoxon rank-sum test. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

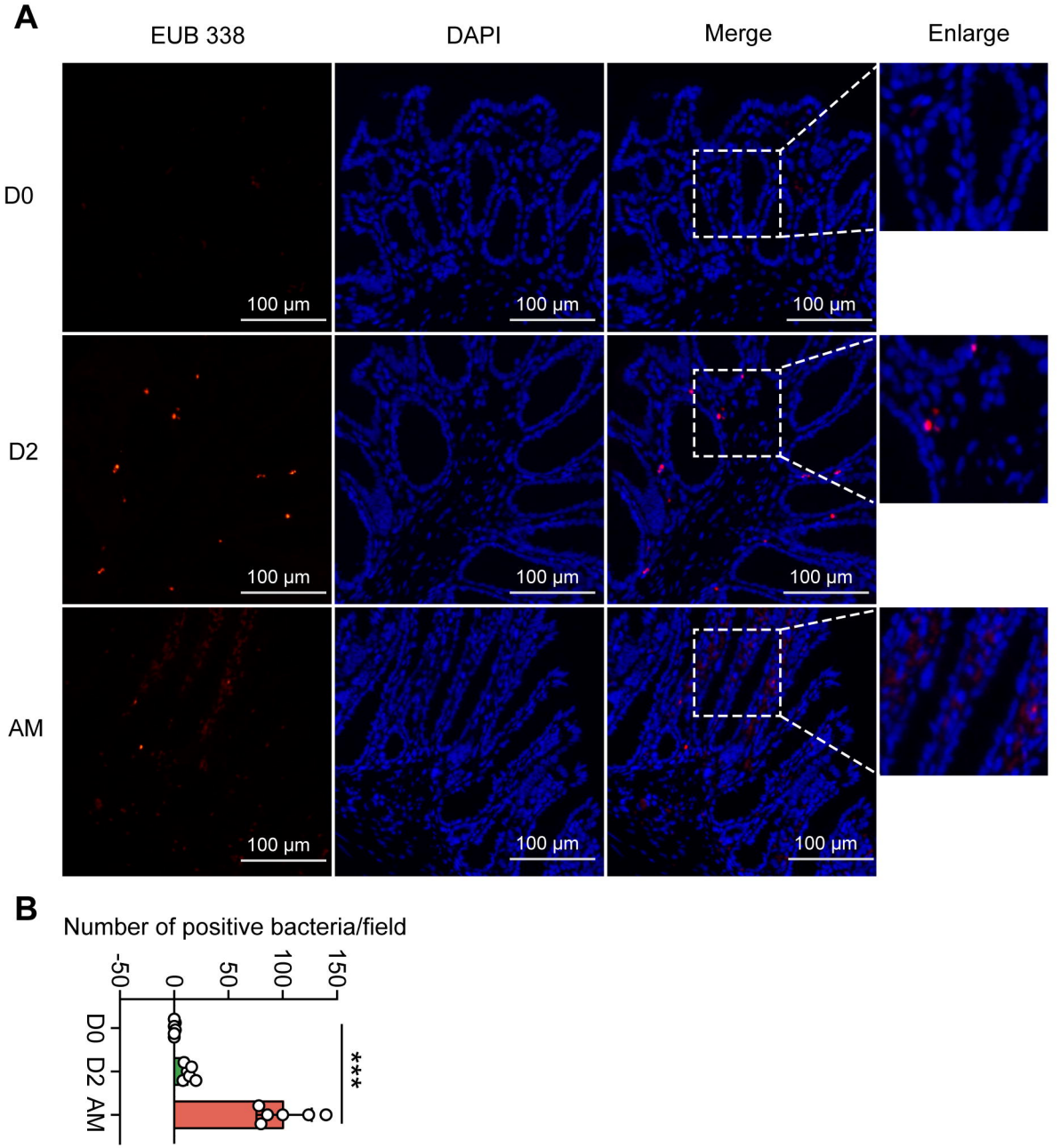
Fig. 7. LPS neutralization combined with antibody therapy or antibiotic therapy improved the survival rate of female hamsters with severe leptospirosis. (A) Flow diagram of the experiment. Six-week-old female hamsters were injected

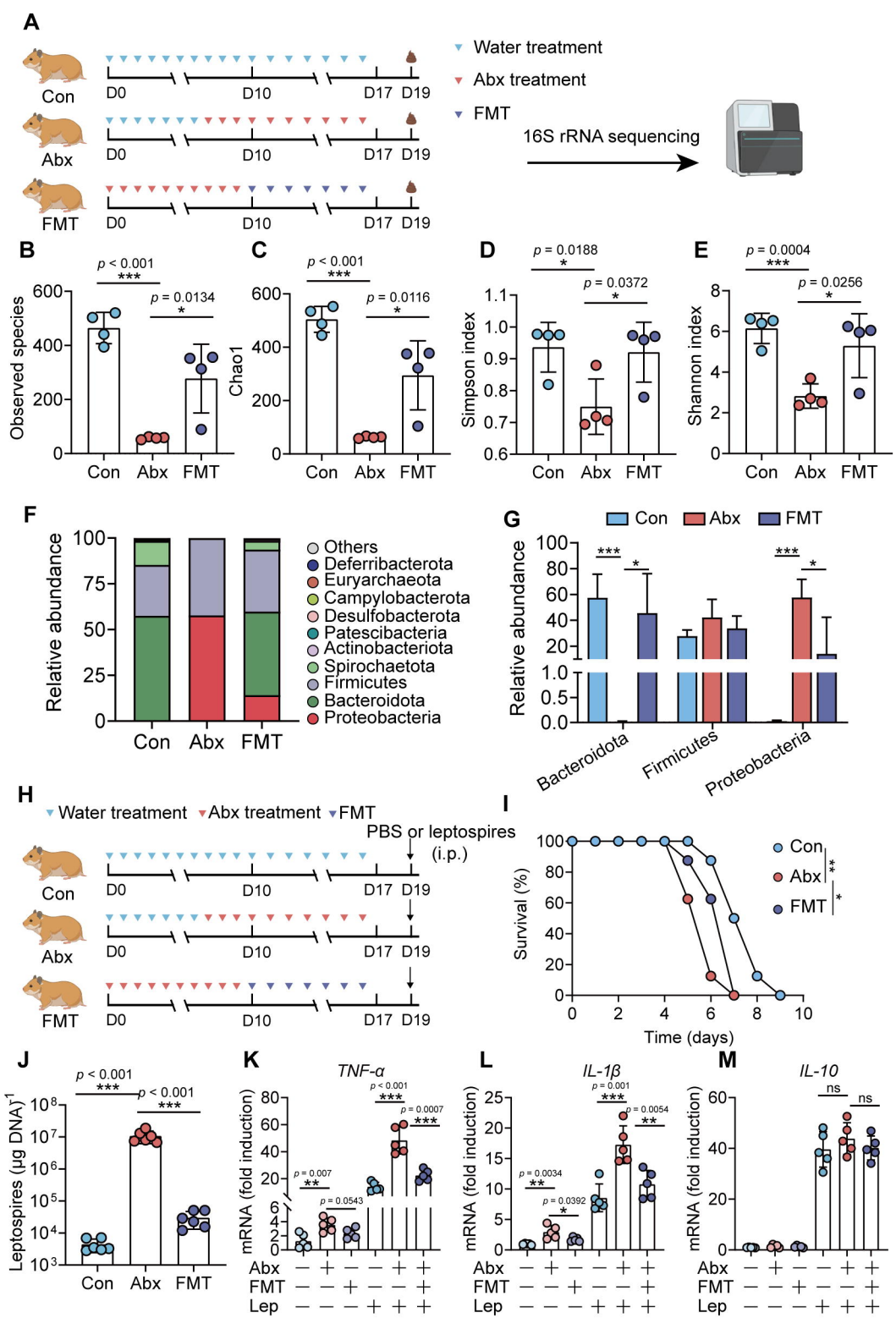
intraperitoneally with 10^7 leptospires. Group 1: saline control; Group 2: Polymyxin B (PMB) (1 mg/kg, i.p.); Group 3: Antibody against *Leptospira* (Ab) (16 mg/kg, subcutaneous injection); Group 4: Doxycycline (Dox) (5 mg/kg, i.p.); Group 5: PMB (1 mg/kg, i.p.) and Ab (16 mg/kg, subcutaneous injection); Group 6: PMB (1 mg/kg, i.p.) and Dox (5 mg/kg, i.p.); Hamsters were treated twice a day for 3 consecutive days. Hamsters were monitored daily for 21 days. (B) Survival rate of female hamsters of severe leptospirosis after different treatments. n = 6 per group. (C) Leptospiral load in the blood of different groups. n = 5 per group. (D-F) Gene expression of *TNF-α* (D, *IL-1β* (E), and *IL-10* (F) in the blood of different groups. The mRNA levels of cytokines in uninfected controls were set as 1.0. n = 5 per group. Each infection experiment was repeated three times. Survival differences between the study groups were compared by using the Kaplan-Meier log-rank test. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. ns, not significant.

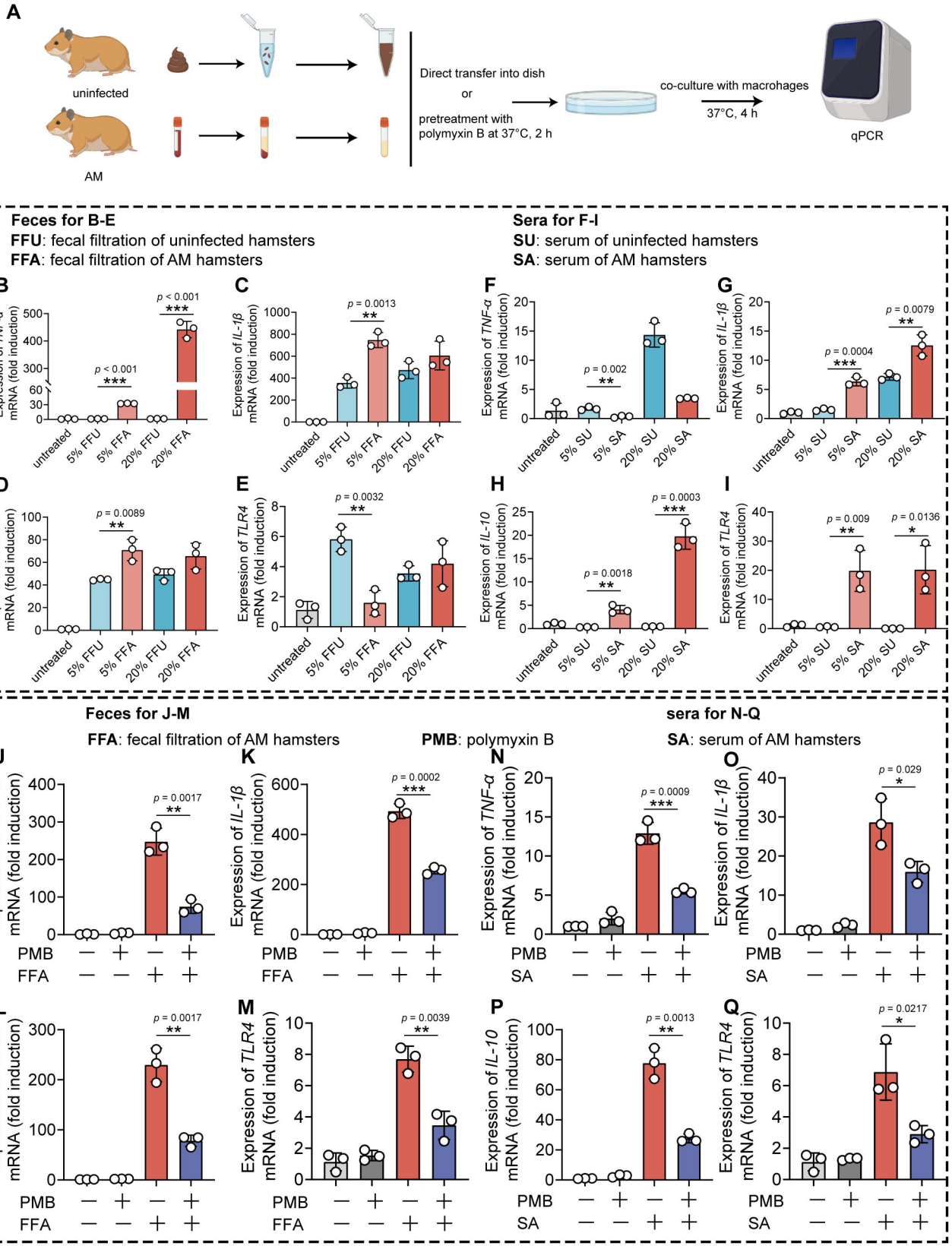

A
10⁷ leptospires (i.p.)
collection for intestinal tissues
0
6
48
96
AM
Time (h)
analysis
qPCR
+
histology
B
ileum
colon
Leptospires (µg DNA)⁻¹
3×10⁶
2×10⁶
1×10⁶
0
0
6
48
96
AM
Time (h)
p = 0.0002
***
C
D0
AM
50 µm
50 µm
p < 0.001
***
Histological score
10
8
6
4
2
0
D0
AM
D
p = 0.0109
*
Expression of Lcn2 mRNA (fold induction)
60
40
20
0
D0
AM
E
p = 0.0027
**
Expression of TNF-α mRNA (fold induction)
40
30
20
10
0
D0
AM
F
p < 0.001
***
Expression of IL-1β mRNA (fold induction)
30
20
10
0
D0
AM
G
p = 0.032
*
Expression of IL-10 mRNA (fold induction)
2.0
1.5
1.0
0.5
0.0
D0
AM
H
p = 0.0003
***
Expression of TLR4 mRNA (fold induction)
6
4
2
0
D0
AM
I
p = 0.0007
***
Expression of Nos2 mRNA (fold induction)
4
3
2
1
0
D0
AM

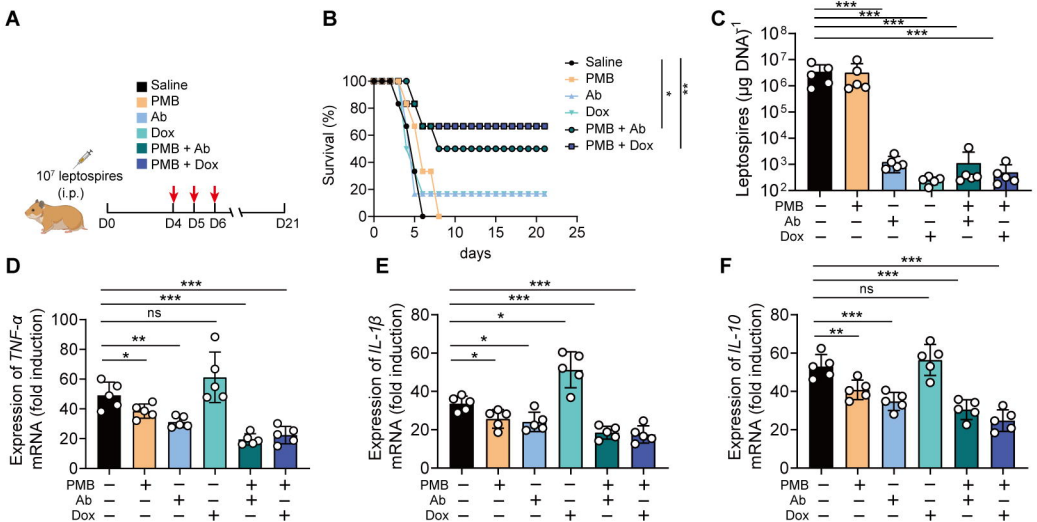

A
10⁷ leptospires
D0
D2
AM
16S rRNA sequencing
B
Observed species
p = 0.0029
**
C
Ace
D
Simpson index
p = 0.0186
*
p = 0.0078
**
E
Shannon index
p = 0.0091
**
p = 0.001
**
F
PCoA2 (21.1%)
PCoA1 (47.4%)
D0
D2
AM
G
ANOSIM
R=0.3909
p=0.003
PCoA2 (12.8%)
PCoA1 (56.3%)
D0
AM
H
Relative abundance
Others
Melainabacteria
Proteobacteria
Elusimicrobia
Deferribacteres
unidentified bacteria
Tenericutes
Verrucomicrobia
Spirochaetes
Bacteroidetes
Firmicutes
I
D0
AM
p_Firmicutes
f_Lactobacillaceae
g_Lactobacillus
c_Bacilli
o_Lactobacillales
o_Erysipelotrichales
f_Erysipelotrichaceae
c_Erysipelotrichia
s_Lactobacillus_hamsteri
g_Allobaculum
s_Lactobacillus_panis
s_Dubosiella_newyorkensis
g_Dubosiella
g_Ileibacterium
g_Ruminiclostridium
f_Elusimicrobiaceae
g_Elusimicrobium
c_unidentified_Elusimicrobia
f_Bacteroidaceae
g_Bacteroides
g_unidentified_Clostridiales
g_Mucispirillum
g_Anaerovorax
g_Rikenella
s_Dorea_sp_5_2
p_Proteobacteria
s_Christensenella_minuta
s_Helicobacter_apodemus
o_Campylobacterales
g_Helicobacter
f_Helicabacteraceae
g_Lachnoclostridium
c_unidentified_Bacteria
g_Oscillibacter
s_Ruminococcus_flavefaciens
s_Clostridium_sp_Culture_Jar_13
s_Parabacteroides_distasonis
f_Lachnospiraceae
f_Tenericutes
p_Mollicutes
c_Spirochaetes
p_Spirochaetes
f_Ruminococcaceae
o_Clostridiales
c_Clostridia
LDA SCORE (log 10)
J
Firmicutes/Bacteroidetes ratio
D0
AM
K
Proteobacteria
p = 0.0018
**
Relative abundance
D0
AM
L
Lactobacillus
p = 0.0166
*
Relative abundance
D0
AM
M
Allobaculum
p = 0.0268
*
Relative abundance
D0
AM


A
Serum FITC-dextran (ng/mL)
6000
4000
2000
0
p = 0.0025
**
p = 0.0012
**
D0
D2
AM
B
Expression of Zo-1 mRNA (fold induction)
6
4
2
0
p = 0.0069
**
p = 0.0078
**
D0
D2
AM
C
Expression of Claudin-3 mRNA (fold induction)
6
4
2
0
p < 0.001
***
p = 0.0009
***
D0
D2
AM
D
Expression of JAMA mRNA (fold induction)
6
4
2
0
p = 0.0015
**
p = 0.0003
***
D0
D2
AM
E
Expression of Claudin-2 mRNA (fold induction)
600
400
200
0
-200
p < 0.001
***
D0
D2
AM
F
Expression of Mucin-2 mRNA (fold induction)
4
3
2
1
0
p = 0.0139
*
p = 0.0003
***
D0
D2
AM


A
EUB 338
DAPI
Merge
Enlarge
D0
D2
AM
100 μm
B
Number of positive bacteria/field
-50
0
50
100
150
D0
D2
AM
***


A
Water treatment
Abx treatment
FMT
Con
Abx
FMT
D0
D10
D17
D19
16S rRNA sequencing
B
Observed species
p < 0.001
***
p = 0.0134
*
C
Chao1
p < 0.001
***
p = 0.0116
*
D
Simpson index
p = 0.0188
*
p = 0.0372
*
E
Shannon index
p = 0.0004
***
p = 0.0256
*
F
Relative abundance
Others
Deferribacterota
Euryarchaeota
Campylobacterota
Desulfobacterota
Patescibacteria
Actinobacteriota
Spirochaetota
Firmicutes
Bacteroidota
Proteobacteria
G
Con
Abx
FMT
Relative abundance
Bacteroidota
Firmicutes
Proteobacteria
H
Water treatment
Abx treatment
FMT
PBS or leptospires
(i.p.)
I
Survival (%)
Time (days)
J
Leptospires (μg DNA)-1
p < 0.001
***
p < 0.001
***
K
TNF-α
mRNA (fold induction)
p = 0.007
**
p = 0.0543
p < 0.001
***
p = 0.0007
***
L
IL-1β
mRNA (fold induction)
p = 0.0034
**
p = 0.0392
*
p = 0.001
***
p = 0.0054
**
M
IL-10
mRNA (fold induction)
ns
ns
Abx
FMT
Lep


A
uninfected
AM
Direct transfer into dish
or
pretreatment with polymyxin B at 37°C, 2 h
co-culture with macrophages
37°C, 4 h
qPCR
Feces for B-E
FFU: fecal filtration of uninfected hamsters
FFA: fecal filtration of AM hamsters
Sera for F-I
SU: serum of uninfected hamsters
SA: serum of AM hamsters
B
Expression of TNF-α mRNA (fold induction)
p < 0.001
p < 0.001
untreated
5% FFU
5% FFA
20% FFU
20% FFA
C
Expression of IL-1β mRNA (fold induction)
p = 0.0013
D
Expression of IL-10 mRNA (fold induction)
p = 0.0089
E
Expression of TLR4 mRNA (fold induction)
p = 0.0032
F
Expression of TNF-α mRNA (fold induction)
p = 0.002
untreated
5% SU
5% SA
20% SU
20% SA
G
Expression of IL-1β mRNA (fold induction)
p = 0.0004
p = 0.0079
H
Expression of IL-10 mRNA (fold induction)
p = 0.0018
p = 0.0003
I
Expression of TLR4 mRNA (fold induction)
p = 0.009
p = 0.0136
Feces for J-M
FFA: fecal filtration of AM hamsters
PMB: polymyxin B
sera for N-Q
SA: serum of AM hamsters
J
Expression of TNF-α mRNA (fold induction)
p = 0.0017
PMB
FFA
K
Expression of IL-1β mRNA (fold induction)
p = 0.0002
L
Expression of IL-10 mRNA (fold induction)
p = 0.0017
M
Expression of TLR4 mRNA (fold induction)
p = 0.0039
N
Expression of TNF-α mRNA (fold induction)
p = 0.0009
PMB
SA
O
Expression of IL-1β mRNA (fold induction)
p = 0.029
P
Expression of IL-10 mRNA (fold induction)
p = 0.0013
Q
Expression of TLR4 mRNA (fold induction)
p = 0.0217


A
Saline
PMB
Ab
Dox
PMB + Ab
PMB + Dox
10^7 leptospires
(i.p.)
D0
D4 D5 D6
D21
B
Survival (%)
days
Saline
PMB
Ab
Dox
PMB + Ab
PMB + Dox
C
Leptospires (μg DNA)^-1
PMB
Ab
Dox
D
Expression of TNF-α
mRNA (fold induction)
ns
E
Expression of IL-1β
mRNA (fold induction)
F
Expression of IL-10
mRNA (fold induction)