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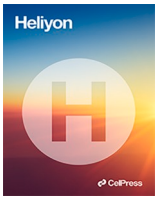

Research article

Huang Lian Jie Du decoction attenuates inflammation in septic rats by activating autophagy and altering the intestinal microbiome

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ABSTRACT

Aims: The aim of this study was to investigate the protective effect of HLJDD on septic rats and the underlying mechanisms.
Materials and methods: Adult male Sprague–Dawley (SD) adult rats (150–180 g) were randomly divided into the following 5 groups (n = 7 per group): the Sham group, caecal ligation and puncture (CLP) group, HLJDD + CLP (Huang Lian Jie Du Decoction, HLJDD) group (1 g/mL/100 g), HLJDD + Rap + CLP (H. Rap) group (Rap: 3 mg/kg), and HLJDD+3-MA + CLP (H. 3-MA) group (3-MA: 30 mg/kg). Rapamycin (Rap) and 3-methyladenosine (3-MA) were used to activate and inhibit autophagy, respectively. HLJDD was purchased from Beijing Tong Ren Tang Guiyang Branch and verified by experts as a genuine product. We used CLP to establish an animal model of sepsis in the last four groups. Survival was analysed by the Kaplan−Meier method. Then, we examined autophagy-related genes (*Atgs*) and proteins using real-time PCR and Western blotting, respectively. The microstructure of the ileum and the number of autophagosomes were observed by transmission electron microscopy (TEM). Analyses of HE-stained pathological ileum and inflammatory factor levels were examined to assess the extent of septic injury. The effect of HLJDD on the gut microbiota was analysed by 16S rRNA gene sequencing of faeces.
Results: In this study, we identified the protective effects of HLJDD on mortality and inflammation in septic rats. Several key proteins, including LC3-II, Beclin-1 and p62, were examined and showed that HLJDD could effectively reverse the sepsis-induced decrease in autophagy. TEM was performed and the expression of *Atgs* was assessed to evaluate fluctuations in autophagy. Then, we examined the intestinal tight junction protein zona occludens (ZO-1), lipopolysaccharide (LPS) and inflammatory factors, and found that HLJDD effectively alleviated the increase in *ZO-1* gene expression, the level of LPS and serum level of inflammatory factors caused by sepsis. These results were consistent with those obtained from pathological sectioning and TEM analysis.

Abbreviations: HLJDD, Huang Lian Jie Du decoction; SD, Sprague-Dawley; CLP, caecal ligation and puncture; Rap, rapamycin; 3-MA, 3-methyladenosine; TEM, transmission electron microscope; HE, histopathological examination; LPS, lipopolysaccharide; ZO-1, zona occludens; TNF-α, tumour necrosis factor-α; IL-6, Interleukin-6; IL-1β, Interleukin-1β; IL-10, Interleukin-10; Atgs, autophagy-related genes; ATG, autophagy-related protein; ASV, Amplicon Sequence Variants; LEfSe, LDA Effect Size.

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Moreover, autophagy activation effectively ameliorated sepsis, and autophagy inhibition exacerbated the systemic symptoms caused by infection. By examining the expression of key proteins upstream of the autophagy pathway, we found that HLJDD inhibited mTOR via the MAPK/PI3K signalling pathway to promote autophagy in septic rats. 16S rRNA sequencing revealed that HLJDD significantly affected the diversity and physiological function of the gut microbiota in septic rats.

Conclusions: The results of this study indicate that autophagy activation is a potential mechanism underlying the protective effect of HLJDD on the intestine in septic rats.

1. Introduction

As a life-threatening organ dysfunction, sepsis has been newly defined in the latest "Sepsis-3.0″ consensus definition [1]. Unfortunately, sepsis affects approximately 19 million people worldwide each year, and due to its high death rate, it remains one of the most problematic conditions in the intensive care units [2]. The early stages of sepsis are characterized by a hyperinflammatory syndrome caused by the release of large amounts of proinflammatory cytokines, interleukin-6 and tumour necrosis factor-α (IL-6 and TNF-α), which is described as a "cytokine storm" [3]. Autophagy is a type of adaptive protective mechanism in organisms that is induced by triggers, such as inflammation, starvation, and reduced ATP levels [4]. It not only removes damaged proteins or organelles from the cells of an organism but also eliminates bacteria and pathogens that have invaded the cells [5]. A growing number of studies have identified a potential role for autophagy in sepsis [4]. One convincing study revealed that LPS increased the expression of LC3-II in the mouse heart in mice and that overexpression of Beclin-1 in the heart inhibited mTOR signalling, improved cardiac function and alleviated LPS-induced inflammation and fibrosis [6]. Another study demonstrated that hydrogen-rich saline attenuated lung injury by inhibiting autophagy in alveolar macrophages in septic rats [7].

The microbiome plays a significant role in the pathogenesis of various inflammatory diseases including inflammatory bowel disease (IBD), allergies, autoimmunity, and neurodegeneration [8–10]. Sepsis also induces the inflammatory and oxidative stress pathways in the intestine, causing dysbiosis of the intestinal gut microbiota. Changes in the gut microbiota severely reduce beneficial anaerobic bacteria and damage intestinal epithelial integrity [11]. Autophagy impairment in intestinal epithelial cells also alters the gut microbiota and reduces alpha diversity resulting in dysregulation of the immune response [12]. Further studies are needed to identify better therapeutic molecules to treat sepsis by targeting autophagy and the gut microbiota [13].

The clinical symptoms and development of sepsis are similar to those of "warm fever disease", as discussed in the classical Chinese Medicine works "Treatise on Typhoid" and "Treatise on Warm Diseases" [14]. Heat toxicity is present during sepsis and plays a leading role in the development of this condition. Huang Lian Jie Du Decoction (HLJDD) was first described by the Tang Dynasty physician Wang Tao in his book "*Secret Essentials of Wai Tai*" [15]. HLJDD is a representative prescription in Chinese medicine for clearing heat and detoxifying toxins and consists of Copptidis Rhizoma, Scutellaria Radix, Phelodendri Chinensis Cortex and Gardenia Fructus, which are combined at a ratio of 3:2:2:3 [15–17]. Because of its significant anti-inflammatory and anti-infective benefits, it is widely used in China as a clinical adjunct for treating inflammation in sepsis [18]. HLJDD could ameliorate the LPS-induced inflammatory response by inhibiting the MAPK signalling pathway and suppressing IL-6 and TNF-α expression, thereby exerting protective anti-inflammatory effects against LPS-induced acute kidney injury [19]. Modern pharmacological studies have also confirmed the significant anti-inflammatory and antibacterial effects of HLJDD [16,20]. In a previous study, we found that serum containing HLJDD had a significant effect on the expression of *Atgs* and significantly reduced the levels of inflammatory factors in a LPS-induced sepsis cell model [21,22]. Currently, the mechanism by which HLJDD protects animal models of sepsis is still unclear. Based on this research background and foundation, we conducted a further study.

In the present study, we investigated the effects of HLJDD on intestinal autophagy in septic rats. After administering autophagy inducers and inhibitors, we showed that HLJDD protected against sepsis in the ileum by regulating autophagy. Then, we focused on examining the autophagy signalling pathway, including the classic mTOR pathway and upstream MAPK/PI3K pathways. Finally, 16S rRNA gene sequencing was used to investigate the effect of HLJDD on the gut microbiota.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley (SD) adult rats weighing 150–180 g were purchased from the Hubei Experimental Animal Research Center (Hubei, China.) (animal certificate of conformity no. 42000600042413). The animals were acclimatized to the experimental environment for 1 week before the experiment. The animals had free access to food and water, none of the animals died before the start of the experiment. The experimental protocols were approved by the Animal Care and Use Committee of Guizhou University of Traditional Chinese Medicine (medical ethics no: 20,210,175).

2.2. Preparation of HLJDD

The Chinese herbal formula used in this study was HLJDD, which consists of four herbs: Copptidis Rhizoma (*Coptis chinensis* Franch.

rhizome), Scutellaria Radix (*Scutellaria baicalensis* Georgi., Radix), Phelodendri Chinensis Cortex (*Phellodendron chinense* Schneid., cortex) and Gardenia Fructus (*Gardenia jasminoides* Ellis., fructus) [17]. The plant names were checked with a specific website (http://www.theplantlist.org). The plants were purchased from Beijing Tong Ren Tang Guiyang Branch and identified by experts from the University's School of Pharmacy to be genuine products. The aqueous decoction was prepared according to a previous study [15]. HLJDD was prepared with ultrapure water using the proportions in the original formula "The Secret of Wai Tai" (9 g of Copptidis Rhizoma, 6 g of Scutellaria Radix, 6 g of Phelodendri Chinensis Cortex, and 9 g of Gardenia Fructus). The herbs were cut into small pieces, mixed well, steeped in ultrapure water for 40 min, boiled for 1.5 h, filtered and collected. Then, 300 mL of water was added and the mixture was boiled for 40 min before being filtered. The two filtrates were combined and concentrated to a final concentration of 1 g/mL using a rotary evaporator (model: RE5203, Shanghai Yarong Biochemical Instrument Factory, Shanghai, China). The concentrated filtrate was stored in at 4 °C. Several active components, such as geniposide, baicalin, coptisine, berberine, and phellodendrine, in the HLJDD extract were identified using high-performance liquid chromatography and standards (Agilent 1260, United States) as previously reported [23].

2.3. Establishment of an animal model of polymicrobial sepsis

An animal model of polymicrobial sepsis was induced in rats by caecal ligation and puncture (CLP), as previously described [24]. Briefly, the animals were fasted for 12 h before the procedure. Deep anaesthesia was induced with an intraperitoneal injection of 50 mg/kg sodium pentobarbital, and the caecum was exposed and ligated with a 3-0 silk wire just below the distal caecum to a quarter of the ileocaecal flap. Two caecal punctures were performed with a 20-gauge needle in the distal quarter of the caecum. A small drop of faeces was extruded from the puncture site into the peritoneum, the exposed caecum was placed back into the abdominal cavity, and the abdominal wall was closed in layers with sterile 6-0 surgical sutures. After surgery, fluid resuscitation was performed by subcutaneous administration of 3 mL of prewarmed sterile saline, and the rats received standard food and water and were allowed to move freely. The same procedure was performed on sham-operated rats, except for ligation and puncture. The status and survival rates of the rats in each group were observed and recorded every 12 h for 72 h.

2.4. Experimental protocol

Thirty-five SD rats were randomly divided into five groups: (1) Sham group (n = 7); (2) CLP group (n = 7); (3) HLJDD + CLP (HLJDD) group (n = 7); (4) HLJDD + Rap + CLP (H. Rap) group (n = 7); and (5) HLJDD+3-MA + CLP (H. 3-MA) group (n = 7). Additionally, 42 rats were used for survival rate analysis with the same groupings as (1)–(3), with 14 rats in each group. Survival rates were analysed by the Kaplan–Meier method using log-rank tests until 72 h after CLP. One week before CLP surgery, HLJDD at a concentration of 1 g/mL/100 g body weight was administered by gavage daily for 7 days. An equal volume of saline gavage was administered to the sham-operated and CLP groups by gavage. Rapamycin (Abcam, Cambridge, UK; Rap, 3 mg/kg) and 3-methyladenosine (Cayman, Ann Arbor. MI, USA; 3-MA, 30 mg/kg) were intraperitoneally injected within 1 h after CLP surgery [25,26]. An equal volume of saline was intraperitoneally injected into rats in the sham, CLP, and HLJDD + CLP groups. The rats were administered intraperitoneal injections of Rap or 3-MA to induce or inhibit autophagy, respectively. Rap was dissolved in dimethyl sulfoxide (Sigma–Aldrich, USA), and 3-MA was dissolved in warm saline. After the animals were anesthetized, blood was collected from the abdominal aorta, and the intestine was removed for further analysis. Then, serum and intestinal tissues were obtained and stored at −80 °C until analysis 24 h after surgery.

2.5. Histopathological examination (HE)

Intestinal tissues were fixed in 4 % paraformaldehyde, embedded in paraffin, and sectioned at a thickness of 5 μm. After they were deparaffinized and dehydrated, the sections were stained with haematoxylin and eosin. Histopathological changes were observed under a light microscope (Leica microscope DM4B).

2.6. Enzyme-linked immunosorbent assay for cytokines

Serum levels of Interleukin-10 (IL-10), TNF-α, IL-6, Interleukin-1β (IL-1β) and LPS were measured by ELISA kits (Elabscience, Wuhan, China) according to the instructions. Abdominal aortic blood was collected 24 h after modelling, centrifuged for 10 min and analysed by ELISA.

2.7. Western blot analysis

Intestinal tissues were collected from each group. The samples were lysed using immunoprecipitation (IP) lysis buffer (P0013, Beyotime, Beijing, China) containing phenylmethanesulfonyl fluoride (PMSF). The protein concentrations of the samples were determined using a BCA Protein Assay Kit (Beyotime, P0012S) according to the manufacturer's instructions. The clarified lysates were centrifuged and boiled in 5 × SDS–PAGE loading buffer for 10 min. The protein samples were then separated by 12 % SDS–PAGE and electrotransferred onto polyvinylidene fluoride (PVDF) membranes. The Blue PlusTM protein marker (14–160 KD) (TransGene Biotech, Beijing, China) was used. The membranes were blocked with 5 % skim milk for 1 h at room temperature (RT). The membranes were then incubated with primary antibodies (LC-3, 1:1000, AF5402, Affinity, China; Beclin-1, 1:1000, AF5128, Affinity; P62, 1:1000,

AF5384, Affinity; *p*-mTOR, 1:800, AF3309, Affinity; *p*-AKT, 1:1000, AF0016, Affinity; *p*-ERK, 1:1000, AF1015, Affinity; *p*-JNK, 1:1000, AF3318, Affinity; p-P38, 1:800, AF3455, Affinity; GAPDH, 1:1000, AB-P-R 001, Hangzhou Xianzhi Biotechnology Co., Ltd) at 4 °C overnight. The membranes were then incubated with goat anti-rabbit HRP secondary antibodies (Boster, China) for 1 h at RT. Finally, the density of the bands was analysed by Image Lab 5.2.1 (Bio-Rad, Inc. USA) [27,28].

2.8. Real-time PCR

Total RNA was extracted from intestinal tissues using TRIzol reagent. We measured the OD260, OD280, and OD260/OD280 values using a NanoDrop 8000 (Thermo, USA) and calculated the purity and concentration of the RNA. Based on the OD260/OD280 ratio, the RNA quality was estimated to meet the experimental requirements at a ratio between 1.8 and 2.0. A total of 3 μg of RNA and reverse transcribed into cDNA using the Superscript First-Strand Synthesis System (Thermo, USA). The cDNA was amplified using real-time quantitative PCR with SYBR Green Master Mix (Vazyme, China) for real-time quantitative PCR using the following specific primers: *ZO-1* (F: 5′-CGCCTCTGTCCAACTCTTCT-3′; R: 5′-GGTGTGAATCGGTTGTATGCTG-3′; 265 bp product), *Atg5* (F: 5′-GAGCCATACTATTTGCTTTTGCC-3′; R: 5′-TTTCAGGGGTGTGCCTTCAT-3′; 138 bp product), *Atg7* (F: 5′-GACGAGGCACCCAAAGACATCAAGG-3′; R: 5′-ACAGAGGGCAGGGCAGCAAAA-3′; 342 bp product), *Atg9* (F: 5′-TTACTGTGGAGGTCGTCGGTGTGGG-3′; R: 5′-GGATGTGAAAAGGGCATTCTCTGGG-3′; 299 bp product) and *GAPDH* (F: 5′-ACAGCAACAGGGTGGTGGAC-3′; R: 5′-TTTGAGGGTGCAGCGAACTT-3′; 253 bp product). The primers were obtained from Sangon Biotech (Shanghai, China).

2.9. Transmission electron microscopy analysis

Fresh rat ileal tissues less than 1 mm × 1 mm × 1 mm in volume were removed to minimize mechanical damage and fixed at 4 °C in electron microscope fixative. The samples were rinsed with 0.1 M phosphate buffer for 3 min, and the wash steps were repeated 3 times. Then, 1 % osmium acid prepared with 0.1 M phosphate buffer was added and incubated at room temperature for 2 h. The samples were again rinsed with 0.1 M phosphate buffer 3 times for 15 min each. The ileal tissue was dehydrated using a gradient alcohol series (30%-50%-70%-80%-95%-100%–100 % for 20 min each time) and 100 % acetone twice for 15 min each. The sample was then inserted into the embedding plate with acetone and 812 embedding agent at a 1:1 ratio at 37 °C for 2 h, at a 1:2 ratio at 37 °C overnight, and in pure 812 embedding agent at 37 °C for 5 h. Then, the sample was placed on the plate with pure embedding agent and heated in an oven at 37 °C overnight. Finally, the embedding plate was placed in a 60 °C oven to polymerize for 48 h, and the resin block was removed and set aside. The resin block was sliced with an ultrathin 70-nm slicer and mounted on 150-mesh copper with Fang Hua film. The copper mesh was stained in 2 % uranyl acetate saturated alcohol solution for 8 min, washed 3 times with 70 % alcohol, washed 3 times with ultrapure water, stained with 2.6 % lead citrate solution for 8 min, washed 3 times with ultrapure water, and lightly blotted with filter paper. The copper mesh sections were placed in a copper mesh box and dried at room temperature overnight. The cells were observed under a transmission electron microscope (TEM, Hitachi HT7800/HT7700), and images were collected for analysis.

2.10. 16S rRNA gene sequencing analysis

Faecal samples were collected at when the animals were euthanized and were frozen at −80 °C. Total DNA in the faeces was extracted using the QIAamp PowerFecal DNA Kit (Qiagen, USA) according to manufacturer's instructions. Subsequently, the PCR products were mixed in equal ratios. The PCR mixture was purified with a Qiagen Gel Extraction Kit (Qiagen, USA). The 16S rRNA V4 region was amplified using the following primers: 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTW TCTAAT-3′)Mix (New England Biolabs). Sequencing libraries were generated using a TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA) according to the manufacturer's recommendations, and index codes were added. The library quality was assessed on a Qubit 2.0 fluorometer (Thermo Scientific, USA). The libraries were ssequenced on a NovaSeq platform (Illumina, USA) by Novogene Bioinformatics Technology Co., Ltd [29]. DADA2 was mainly used for noise reduction, which no longer clustered with similarity, and only performed dereplication or equivalent to clustering with 100 % similarity. The processed sequences and alpha and beta diversity analyses were performed by RDP classifier software (version 2.11).

2.11. Statistical analysis

The statistical analyses were performed using SPSS version 18.0, and the results are expressed as the means ± SDs. Differences between groups were analysed by one-way analysis of variance and Tukey's tests. A *t*-test was used for comparisons between two groups. All experiments, except for the sequencing experiment, involved three biological replicates. $P < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. Effects of HLJDD on survival and inflammation in septic rats

After CLP, the rats exhibited an increased respiratory rate, reduced eating and drinking, loss of hair lustre, bloody discharge at the corner of the eye, and damp water-like stools. Compared to the CLP group, the HLJDD group had significantly higher survival rates

(Fig. 1 A). To study the effect of HLJDD on inflammation in septic rats, we further measured the levels of LPS and inflammatory factors in the serum by ELISA. We found that the serum LPS levels were increased significantly in the CLP group compared to the sham group and were markedly decreased in the HLJDD group (Fig. 1 B). To fully clarify the effect of HLJDD on inflammation in septic rats, we examined several cytokines, including anti-inflammatory cytokines (IL-10) and proinflammatory cytokines (IL-6, TNF-α, and IL-1β). The level of the anti-inflammatory and immunosuppressive factor IL-10 was decreased significantly in the CLP group but was significantly restored after pharmacological intervention (Fig. 1C). This result suggested that HLJDD could reactivate the immune activity of monocytes and macrophages by inducing multiple immune cells to secrete IL-10. The changes in IL-6, TNF-α and IL-1β were consistent with those of LPS, and the opposite effect was observed for IL-10 (Fig. 1D–F). These results showed that HLJDD could effectively alleviate inflammation and reduce the mortality in septic rats.

3.2. Effect of HLJDD on autophagy in septic rats

To study the effect of HLJDD on autophagy in the ileum in septic rats, we investigated changes in the expression of key autophagy-related proteins (ATGs). The results showed that the expression of LC3-II and Beclin-1 was significantly increased in the HLJDD group compared to the CLP group (Fig. 2 A, C and D). The levels of these two ATGs were further increased in the H. Rap group compared to the HLJDD group and the H. 3-MA group showed the opposite effects (Fig. 2 A, C and D). These results indicated that HLJDD increased autophagic activity in septic rats. In addition, we investigated the effect of HLJDD on autophagic flux in septic rats. We found that p62 expression was significantly reduced in the HLJDD group compared to the CLP group (Fig. 2 B and E). This result suggested that HLJDD increased autophagy in septic rats and that autophagic flux was intact. The protein expression of p62 was significantly lower in the H. Rap group than in the HLJDD group, suggesting that the autophagic flux was further enhanced by the addition of autophagy agonists (Fig. 2 B and E). In contrast, the H.3-MA group exhibited significantly increased protein expression of p62, suggesting that autophagy was blocked (Fig. 2 B and E). These findings suggested that the levels of autophagy and autophagic flux were decreased significantly in septic rats and HLJDD effectively ameliorated the reduction in autophagic flux.

3.3. Effects of HLJDD on Atg5, Atg7, and Atg9 in the ileum of CLP-treated septic rats and transmission electron microscopy analysis

We observed typical double-membrane autophagosomes and autophagolysosomes in intestinal epithelial cells by transmission electron microscopy. The results showed that the number of autophagosomes and autophagolysosomes was significantly decreased in the CLP group compared to the sham group and HLJDD reversed the reduction in autophagy caused by infection (Fig. 3 A). Furthermore, rapamycin-induced autophagy further enhanced the effect of HLJDD on intestinal autophagy during sepsis (Fig. 3 A). The *Atg5*, *Atg7* and *Atg9* are involved in autophagy initiation. Our results showed that the expression levels of *Atg5*, *Atg7* and *Atg9* were markedly increased in the ileum in the HLJDD group compared to the CLP group (Fig. 3B–D). The autophagy inducer Rap mimicked and enhanced this effect of HLJDD, while the autophagy inhibitor 3-MA exerted the opposite effect (Fig. 3B–D). These results were

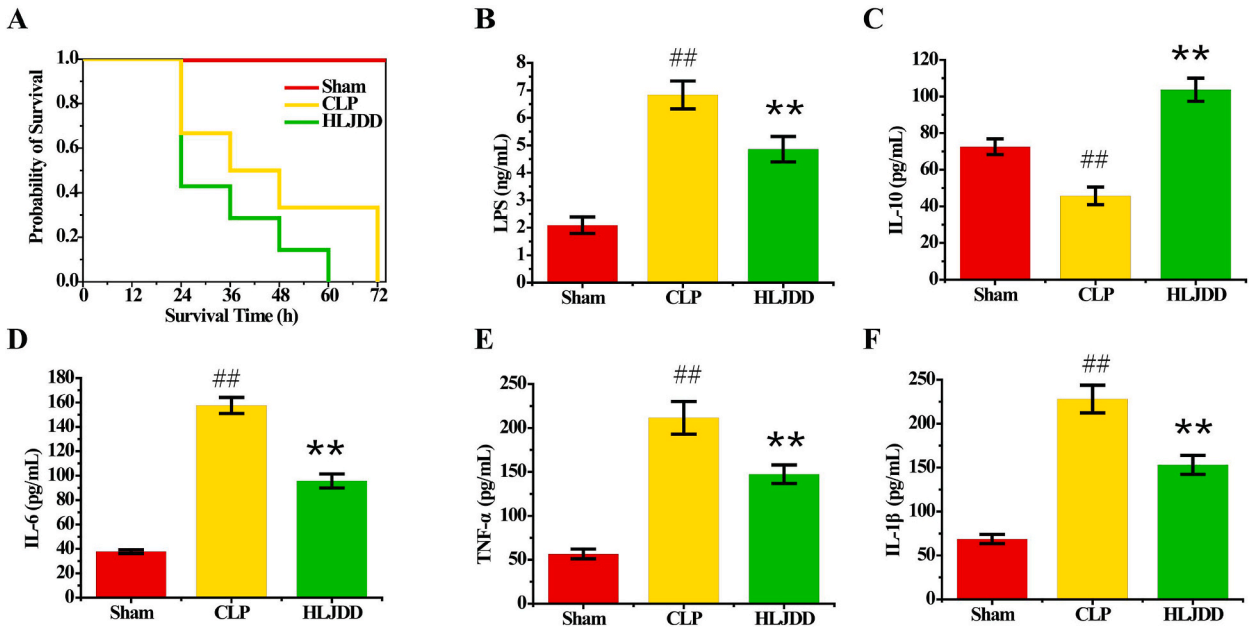

Fig. 1. Effects of HLJDD on survival and inflammation in septic rats. (A) Survival statistics (n = 14). (B) Serum levels of LPS in septic rats. (C–F) Serum levels of IL-10, IL-6, TNF-α and IL-1β in septic rats. The values in B–F are expressed as the means ± SEMs (n = 7). Statistical significance was analysed with Student's *t*-test ($^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ vs. the sham group; $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. the CLP group).

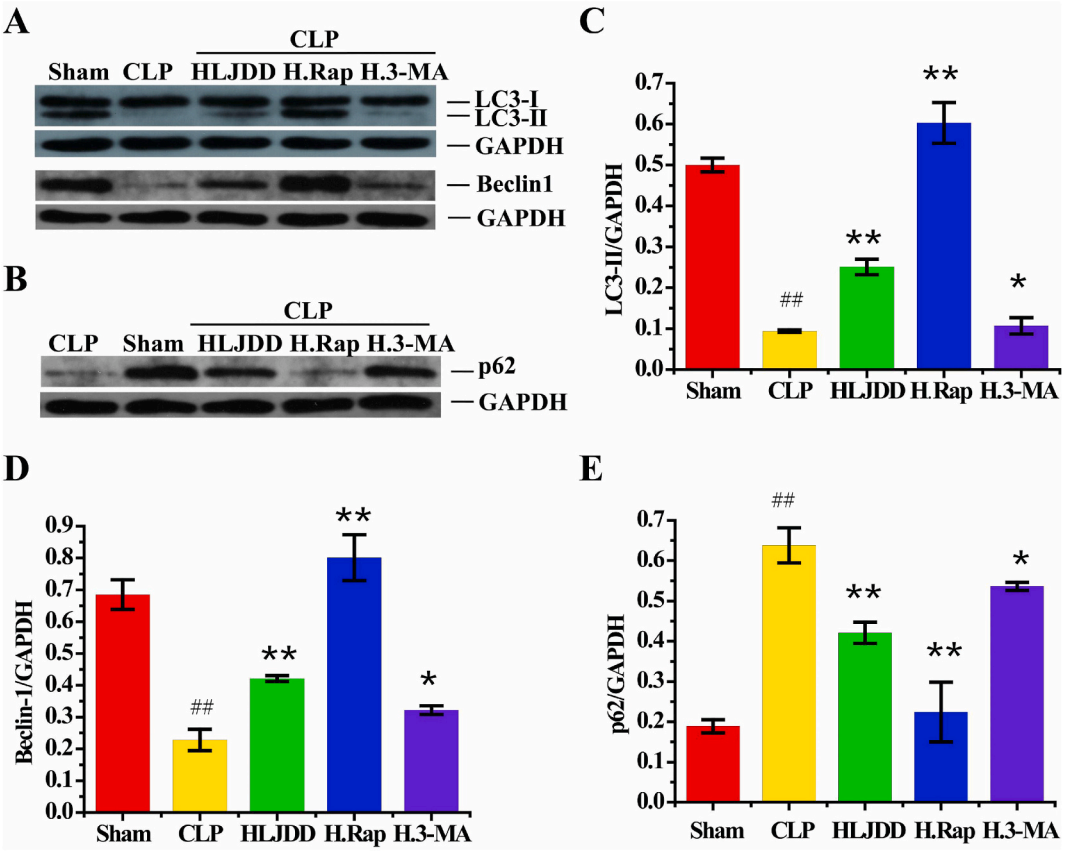

Fig. 2. Effect of HLJDD on CLP-induced autophagy inhibition in septic rats. (A) Western blot analysis of the key autophagy proteins LC3 and Beclin-1. (B) Western blot analysis of the autophagic flux marker protein p62. (C–E) Representative results in A-B are shown in the graphs. The values are expressed as the means ± SEMs (n = 7). Statistical significance was analysed with Student's *t*-test ($^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ vs. the sham group; $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. the CLP group).

consistent with previous results (Fig. 2).

3.4. Autophagy-mediated protective effect of HLJDD on the ileum in septic rats

We observed structural changes in the ileum in each group by HE staining. Pathological ileum sections from the Sham group revealed a clear structure of intestinal epithelial cells, neat arrangement of villi (black arrows), and no infiltration of inflammatory cells. In contrast, histopathological sections from the CLP group showed intestinal mucosal congestion and oedema (red arrowheads) necrosis and shedding of some villi epithelial cells; damage, collapse, and disordered arrangement of villi; and the infiltration of inflammatory cells, mainly monocytes, macrophages, and lymphocytes (red arrows) (Fig. 4 A). The HLJDD group showed some degree of villous structure restoration with an intact lamina propria, smaller mucosal gaps, and relatively less inflammatory tissue infiltration (Fig. 4 A). The H. Rap group showed better recovery of damaged villi structures, intact lamina propria, smaller mucosal gaps, and less inflammatory infiltration than the HLJDD group (Fig. 4 A). However, compared to those in the CLP group, rats in the H. 3-MA group showed worse ileal injury, as evidenced by damaged and collapsed intestinal mucosal villi, a congested intestinal mucosa, and robust infiltration of inflammatory cells (Fig. 4 A). Similarly, the ultrastructures of the ileal epithelial villi of the rats in each group were observed by transmission electron microscopy (Fig. 3 A). As indicated by the black arrow, ileal epithelial villi in the HLJDD group were more intact and the degree of damage was notably weaken than in the CLP group (Fig. 3 A). The H. Rap group exhibited better results (Fig. 3 A). These results suggested that HLJDD could alleviate pathological damage in the ileum in septic rats, and autophagy activation could further enhance this effect.

Normal intestinal barrier function is closely related to the degree of intestinal injury and is mediated by tight junction proteins such as ZO-1. We found that the expression of *ZO-1* was increased in the HLJDD group compared to the CLP group, and further increased in the H. Rap group (Fig. 4 B). In the H.3-MA group, the *ZO-1* expression level showed the opposite trend (Fig. 4 B). These results suggested that HLJDD could significantly protect against intestinal barrier damage in septic rats.

We further measured serum levels of LPS and inflammatory factors (TNF-α and IL-6) by ELISA. The results showed that serum LPS concentrations in the HLJDD group were significantly lower than those in the CLP group (Fig. 4C). Compared to those in the HLJDD

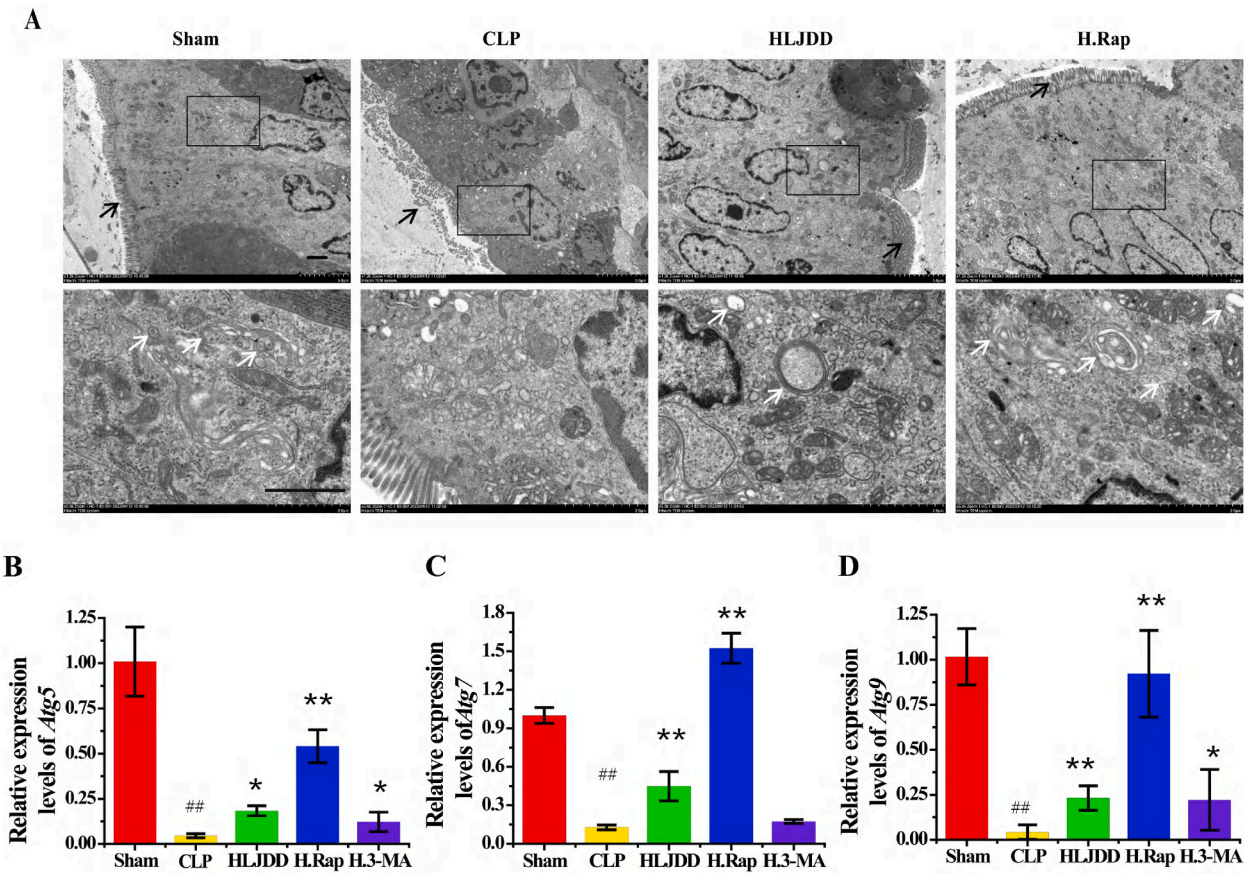

Fig. 3. Effect of HLJDD on autophagosomes and the expression of the autophagy-related genes *Atg5, Atg7,* and *Atg9* in the ileum in septic rats. (A) Autophagy was observed by transmission electron microscopy. The areas in the black frame are further enlarged. The black arrows indicate ileal villi ultrastructures; the white arrows indicate autophagosomes or autophagolysosomes. The scale bar indicates 2 μm. (B–D) Relative expression levels of *Atg5, Atg7* and *Atg9* in the ileum in septic rats. The values are expressed as the means ± SEMs (n = 7). Statistical significance was analysed with Student's *t*-test ($^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ vs. the sham group; $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. the CLP group).

group, LPS levels in the H. Rap group were significantly decreased but the level in the H. 3-MA was significantly increased (Fig. 4C). To further clarify the effect of HLJDD-induced autophagy on inflammation in septic rats, we examined several inflammatory factors, including TNF-α and IL-6. The changes in serum TNF-α and IL-6 levels in septic rats were consistent with the LPS results (Fig. 4 D and E). These results showed that HLJDD-induced autophagy effectively reduced LPS levels and the levels of inflammatory factors in septic rats to reduced the severity of sepsis, and autophagy activation further alleviated sepsis (Fig. 4). Combined analysis of pathological sections, *ZO-1* expression, inflammatory factor expression and ileal TEM data showed that HLJDD had a significant protective effect by inducing autophagy in the ileum in septic rats.

3.5. HLJDD inhibits mTOR via the MAPK/PI3K signalling pathway to promote autophagy in septic rats

To determine the signalling pathway involved in HLJDD-mediated regulation of autophagy and protection in septic rats, we examined the classic mTOR signalling pathway. We found that the level of *p*-mTOR was significantly increased in the CLP group compared to the sham group and was markedly lower in the HLJDD group than in the CLP group (Fig. 5 A and B). In comparison, the H. Rap group exhibited a more significant decrease in *p*-mTOR levels. In contrast, *p*-mTOR was significantly increased in the H.3-MA group compared to the HLJDD group (Fig. 5 A and B). As we know, mTOR is an inhibitory signal upstream of autophagy. Based on these results, we hypothesize that HLJDD could partially activate autophagy by inhibiting the excessive phosphorylation of mTOR in septic rats, thereby protecting against multiple organ damage during sepsis.

To clarify how HLJDD suppresses mTOR, we further investigated the pathways upstream of the mTOR pathway, including the PI3K/AKT and MAPK pathways. We measured the protein expression levels of p-P38, *p*-ERK, *p*-AKT, and *p*-JNK. The levels of these phosphorylated proteins were significantly increased in the CLP group compared to the sham group. Compared to that in the CLP group, the expression of p-P38, *p*-ERK, *p*-AKT and *p*-JNK in the HLJDD group was significantly decreased (Fig. 5C–F). The expression of these proteins was significantly inhibited in the H. Rap group compared to the HLJDD group (Fig. 5C–F). However, the level of the

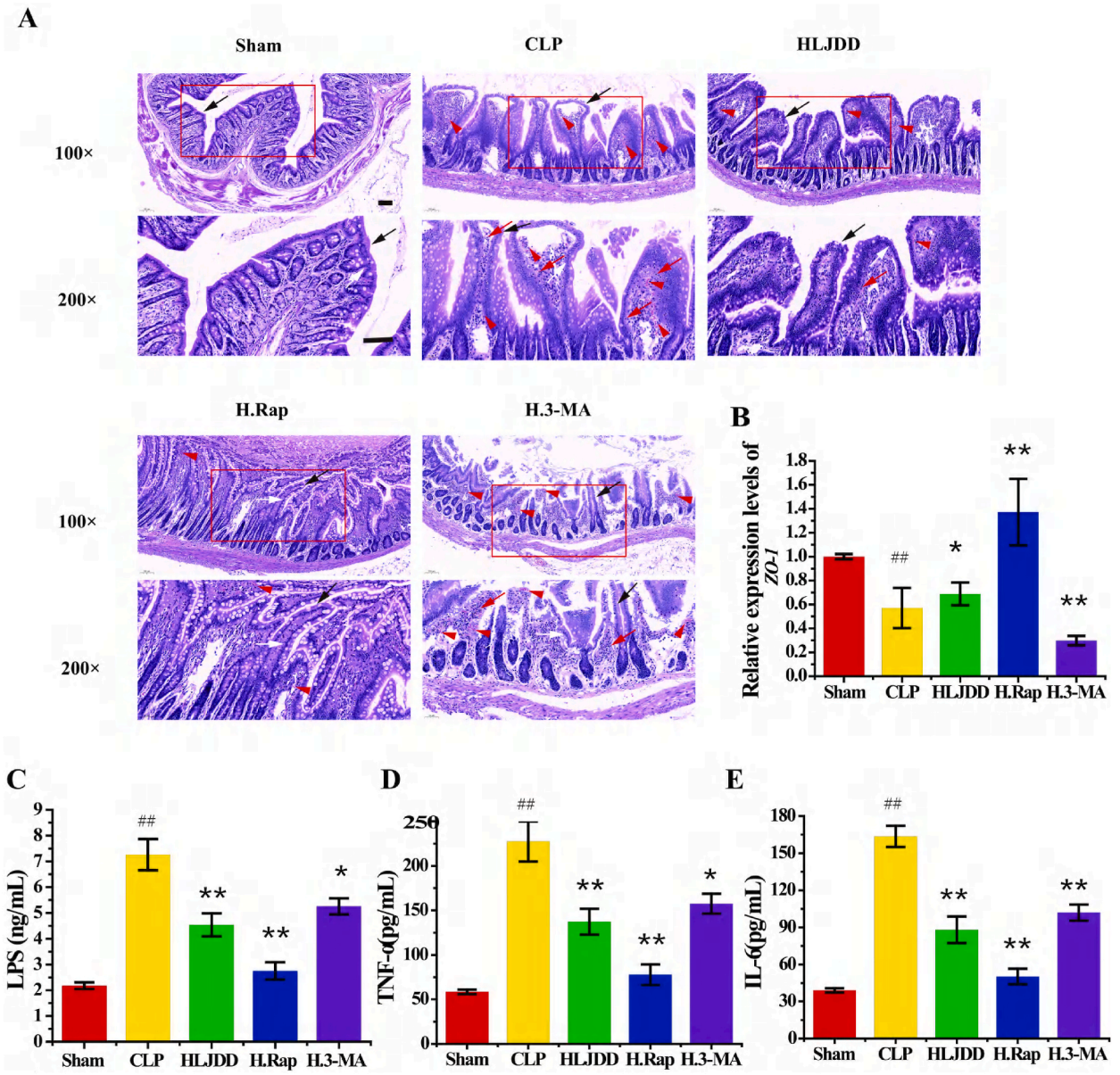

Fig. 4. HLJDD protects the ileum in septic rats. (A) Histopathological examination of the ileum in septic rats. The areas in the red frame are further enlarged. The black arrows indicate ileal microvilli; the white arrows indicate intestinal epithelial cells; the red arrows indicate inflammatory cell infiltration; and the red arrowheads indicate mucosal layer congestion. The scale bar indicates 50 μm. (B) Expression levels of *ZO-1* in the ileal tissue of septic rats. (C) Serum levels of LPS in septic rats. (D–E) Serum levels of TNF-α and IL-6 in septic rats. The values are expressed as the means ± SEMs (n = 7). Statistical significance was analysed with Student's *t*-test ($^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ vs. the sham group; $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. the CLP group). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

phosphorylated proteins were significantly increased in the H. 3-MA group (Fig. 5C–F). These results showed that HLJDD exerted its protective effect by inhibiting the expression of p-P38, *p*-ERK, *p*-AKT and *p*-JNK in the PI3K/AKT and MAPK pathways to activate autophagy.

3.6. HLJDD affected gut microbial richness and diversity in septic rats

To examine the effect of HLJDD on the gut microbiota during sepsis, we collected faeces samples and analysed the microbiome by deep sequencing. An alpha rarefaction curve was constructed to determine whether the sample size was sufficient (Fig. 6 A). The richness of the microbial community according to the Chao1 index showed that CLP significantly decreased community richness, and the HLJDD and autophagy intervention reversed this effect to some extent (Table 1). Alpha diversity was evaluated by the Simpson and

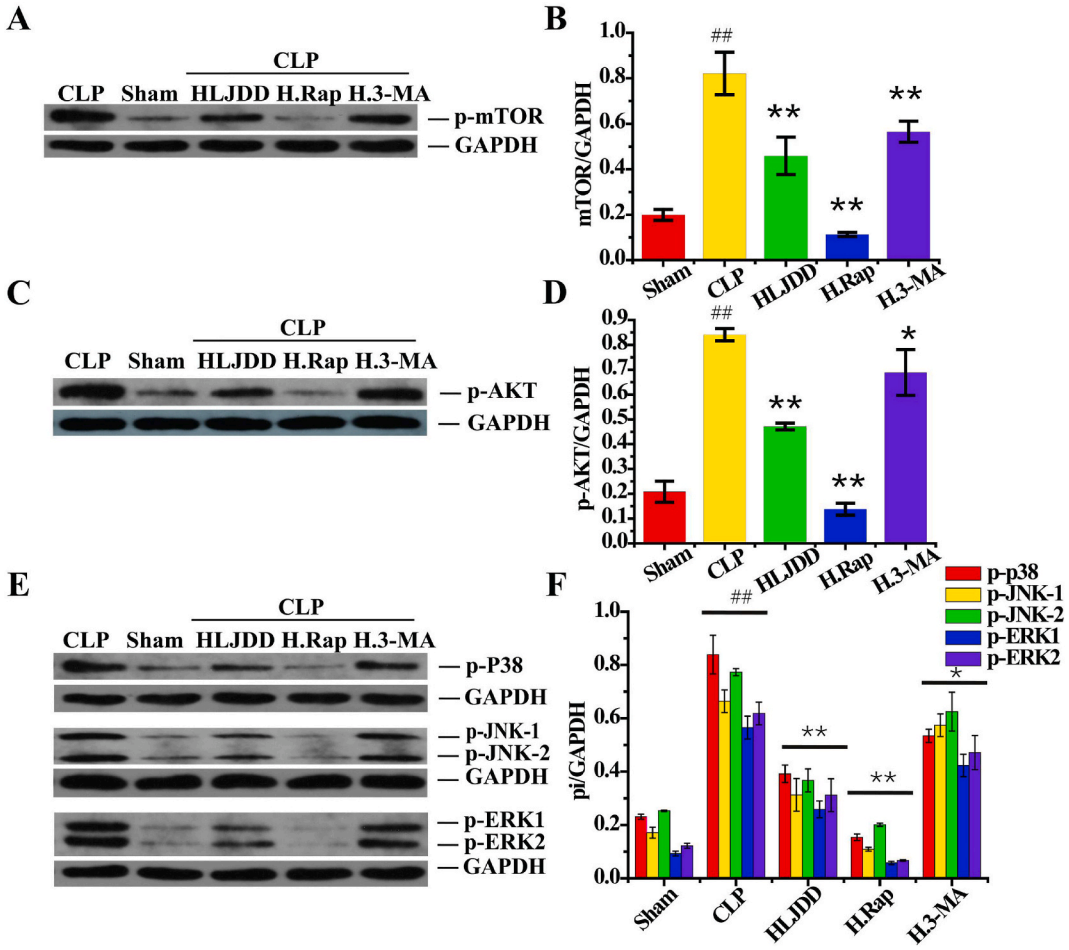

Fig. 5. Effect of HLJDD on phosphorylated MAPK/PI3K/mTOR signalling pathway proteins in the ileum in septic rats. (A) Western blot analysis of phosphorylated *p*-mTOR in the ileum. (B) Representative results in A are shown in the graphs. (C) Western blot analysis of *p*-AKT in the ileum. (D) Representative results in C are shown in the graphs. (E) Western blot analysis of p-P38, *p*-JNK and *p*-ERK in the ileum. (F) Representative results in E are shown in the graphs. The values are expressed as the means ± SEMs (n = 7). Statistical significance was analysed with Student's *t*-test ($^{\#}p < 0.01$ and $^{\#\#}p < 0.01$ vs. the sham group; $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. the CLP group).

Shannon indices. The two indices were significantly lower in the CLP group than in the sham group (Table 1). Although there was no significant difference, the Shannon and Simpson indices of the HLJDD and autophagy intervention groups were increased compared to those in the CLP group (Table 1). Interestingly, the richness and diversity of the gut microbiota in the HLJDD group were lower than those in the CLP group, which was inconsistent with our expectations (Table 1).

Based on the results of the species annotation, we selected the top 10 species with the greatest abundance in each sample or group at the phylum level (Fig. 6B and C). Community richness analysis and abundance clustering showed that *Firmicutes*, *Bacteroidota*, and *Proteobacteria* were among the three most abundant phyla among the groups (Fig. 6B and C). Grouping clustering revealed showed that the abundance of *Proteobacteria* was significantly higher in the CLP group than in the sham group, and the abundances of *Firmicutes* and *Bacteroidetes* were lower (Fig. 6C). HLJDD altered the abundance of *Proteobacteria* in response to CLP and autophagy activation further exacerbated this change (Fig. 6C). A flower diagram was drawn to compare the distribution of Amplicon Sequence Variants (ASVs). The diagram showed that there were 901, 310, 362, 242, and 212 unique ASVs among the sham group, CLP group, HLJDD group, H. Rap group, and H. 3-MA group, respectively (Fig. 6 D). These results reveal that HLJDD and host autophagy play vital roles in shaping the gut microbiome in septic rats.

3.7. HLJDD affected biomarkers of the gut microbiota in septic rats

A cladogram at the species level was reconstructed using LDA Effect Size (LEfSe) analysis. The results showed that there were 21 distinguishing components at different taxon levels with an LDA score >4.0, including 8 species enriched in the sham group, 2 species enriched in the HLJDD group, 2 species enriched in the H. Rap group, 3 species enriched in the H. 3-MA group and 1 species enriched in the CLP group ($p < 0.05$, Fig. 7). At the phylum level, the only species enriched in the CLP group belonged to *Bacteroidetes* and the 8

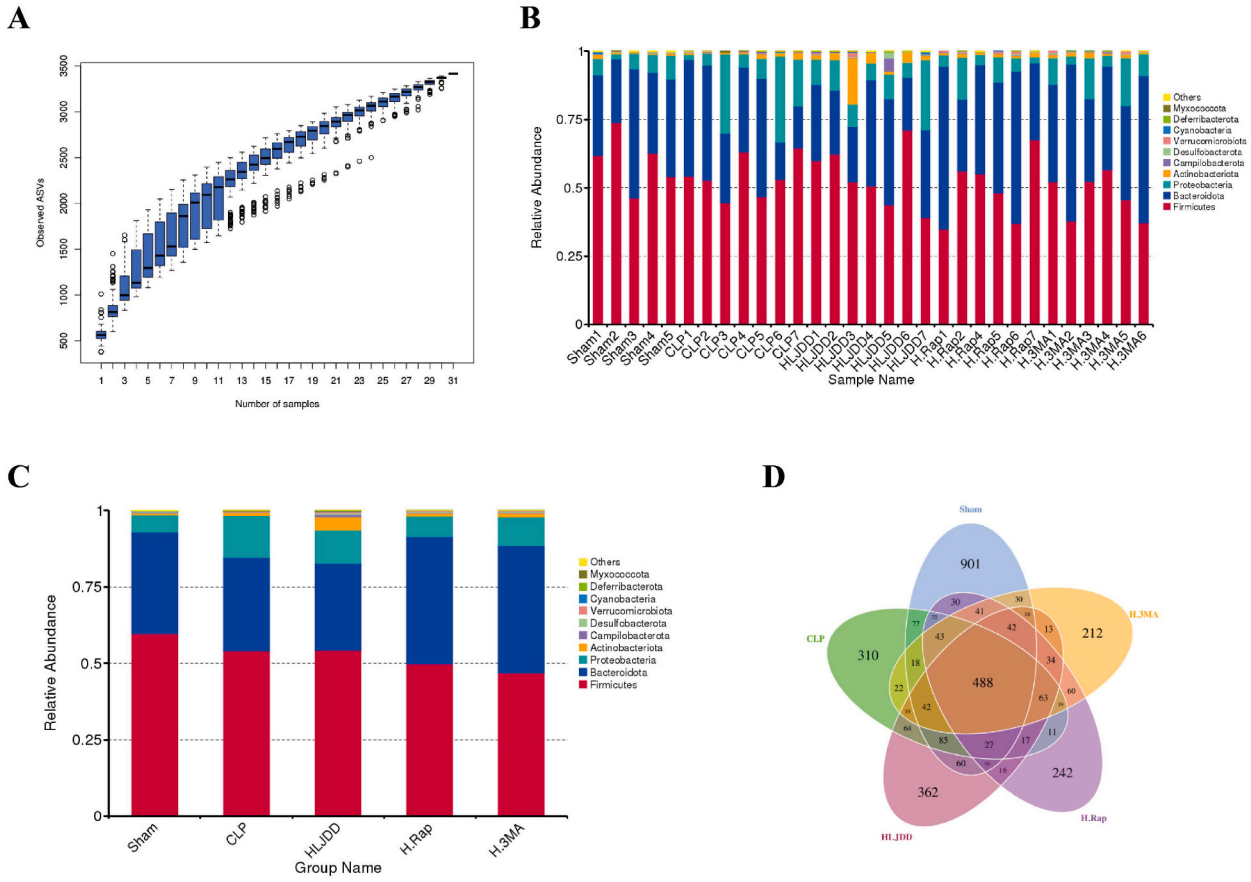

Fig. 6. HLJDD affected the richness and abundance of the microbial community (n = 7). (A) Species accumulation boxplot of alpha diversity. (B–C) The 10 species with the highest abundance in each sample (B) or group (C) at the phylum level. (D) Flower diagram of shared and unique ASVs among the different groups.

Table 1
Alpha diversity in the different groups (n = 7).

	Sham	CLP	CLP		
			HLJDD	H. Rap	H. 3-MA
Chao	820.79 ± 123.94	550.71 ± 34.25[a]	514.84 ± 84.12[a]	562.60 ± 35.61[a]	572.94 ± 47.77[a]
Shannon	7.11 ± 0.31	6.07 ± 0.53[a]	5.88 ± 0.48[a]	6.38 ± 0.32[a]	6.41 ± 0.25[a]
Simpson	0.97 ± 0.02	0.95 ± 0.02[a]	0.94 ± 0.02[a]	0.96 ± 0.01	0.96 ± 0.01
Observed-OTUs	818.80 ± 111.17	549.14 ± 34.25	513.71 ± 83.64	561.67 ± 35.89	571.50 ± 46.96

Kruskal–Wallis test, false discovery rate <0.05.
[a], vs. the sham group.
[b], vs. the CLP group.

species enriched in the sham group were *Firmicutes*. Interestingly, the 7 species enriched in the HLJDD group were *Actinobacteria* (n = 5) and *Bacteroidetes* (n = 2) and the 3 species enriched in the autophagy inhibition groups were all *Bacteroidetes* (Fig. 7).

4. Discussion

In this study, we confirmed that HLJDD protected against intestinal injury in septic rats. The mechanism underlying this protective effect involved the regulation of mTOR and upstream MAPK/PI3K pathways to activate autophagy, which was inhibited in septic animals. Moreover, drug intervention affected the richness, composition, and diversity of the gut microbiota. This study provides a new potential direction for TCM research and the treatment of sepsis and its complications.

Clinical observations show that females are less susceptible to sepsis and seem to recover more effectively than males [30]. Numerous preclinical studies have suggested that females may experience favourable outcomes compared with males [31]. Therefore,

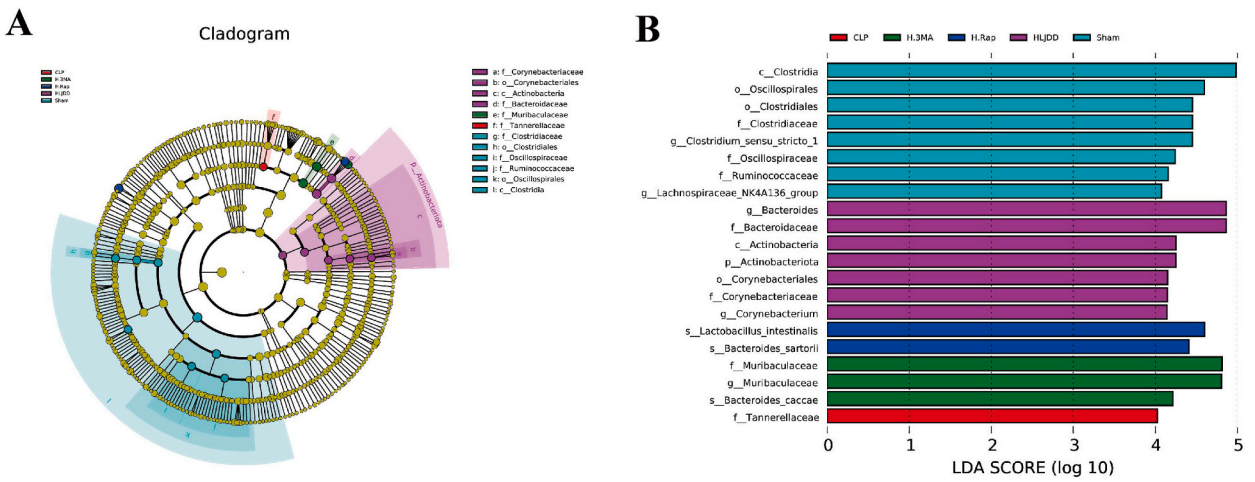

Fig. 7. HLJDD altered biomarkers (n = 7). (A) Cladogram of ASVs (LDA>4) at the species level. (B) Bar plot of ASVs (LDA>4) at the species level.

the vast majority of preclinical sepsis studies have been performed only on male animals to control for hormonal effects [32,33]. Thus, we also selected male rats for the experiment in this study.

Several studies have revealed that some drugs can regulate autophagy, which may offer new hope for the treatment of sepsis [34,35]. Currently, herbal medicine has been shown to exert significant therapeutic effects on sepsis [36,37]. Bai Hu Tang, which is a classical traditional Chinese medicine for clearing heat as HLJDD, significantly improved the survival rate and significantly decreased the expression levels of IL-6 and IL-10 in septic rats [37]. Modern researchers have shown that HLJDD exert anti-inflammatory effects and has been widely used in clinical practice in China for the treatment of inflammatory diseases [4,16,20]. Our study revealed that HLJDD effectively reduced the mortality rate, ameliorated pathological injury and change in tight junctions in the ileum in septic rats, decreased the serum levels of LPS, and reduced the level of inflammation. Our results are consistent with the effects of many herbal formulas for the treatment of sepsis. However, the exact mechanism by which HLJDD ameliorates ileal injury in septic rats remains unclear. Based on these studies and foundations, we conducted further study.

The intestine is known as the “engine of multiorgan failure”. Studies have shown that the overproduction of inflammatory cytokines induces intestinal epithelial barrier dysfunction and worsens the impaired permeability of tight junction proteins [38,39]. One study compared the effects of HLJDD and berberine alone or in combination on acute liver injury in septic rats and demonstrated that HLJDD had better anti-inflammatory, antibacterial and antioxidant effects than berberine [20]. However, there is still a research gap in this area regarding the mechanism by which HLJDD can treat intestinal injury caused by sepsis. We found that HLJDD could effectively alleviate ileal injury by upregulating the expression of tight junction proteins in septic rats through autophagy activation. Autophagy, which is an important mechanism for maintaining cellular homeostasis, plays an important role in the pathogenesis of sepsis and has been intensively studied [4]. In recent years, there has been increasing evidence that autophagy is involved in sepsis and plays different roles in different organs [6,7,40]. Autophagy activation significantly reduced serum levels of LPS and various inflammatory factors. However, the protective effect of HLJDD was greatly reduced when autophagy was inhibited. These results suggested that HLJDD protected against intestinal injury and reduced inflammation in septic rats via autophagy. Our results suggested that the occurrence of autophagy in the ileum under septic conditions is beneficial to the host. HLJDD alleviates the inhibition of autophagy in the septic ileum, and an increase in autophagy protects the ileum under septic conditions.

mTOR, which is a key molecule that regulates autophagy, is an important downstream effector of the PI3K/Akt and MAPK signalling pathways. Some compounds, such as astaxanthin, have been shown to attenuate organ damage in septic animals by modulating MAPK and PI3K/AKT/mTOR signalling [41,42]. The PI3K/Akt/mTOR and MAPK/mTOR signalling pathways are involved in important cellular activities, such as autophagy, survival, and apoptosis [25,43–45]. We demonstrated that HLJDD could effectively inhibit the phosphorylation of mTOR to activate autophagy and protect the ileum in septic rats. HLJDD significantly reduced the expression levels of *p*-AKT, *p*-ERK, *p*-JNK and p-P38 compared to those in the CLP group. In conclusion, HLJDD reduced the phosphorylation of mTOR through the PI3K/AKT and MAPK pathways and subsequently activated autophagy to protect against sepsis-induced intestinal injury, which was consistent with previous studies.

Gut microbiota dysbiosis is associated with susceptibility to sepsis, as many studies have reported [46–48]. Sepsis can disrupt the gut ecosystem, and gut dysbiosis may predispose individuals to sepsis. Gut barrier function plays essential roles in the host response to sepsis [49]. The gut microbiome affects host susceptibility and responses to sepsis through numerous pathways. To further investigate the regulatory effects of HLJDD on the gut microbiota, we analysed the structure of the intestinal bacteria with the greatest abundance in each sample or group at each classification level. The LEfSe (LDA >4) analysis showed that the abundance of *Proteobacteria*, *Myxococcota* and *Verrucomicrobiota* were significantly higher in the CLP group than in the sham group, whereas those of *Firmicutes* and *Bacteroidetes* were significantly lower. HLJDD significantly changed the abundance of *Proteobacteria* and *Actinobacteria*, and drug treatment plus autophagy intervention further affected these communities. *Proteobacteria* are common in various human diseases [50]. A subset of *Proteobacteria* with adherent-invasive capabilities can exploit the genetic defects in pathogen recognition and bacterial

clearance, allowing them to perpetuate unchecked and providing the trigger that drives inflammation [51]. We found that HLJDD or altering autophagy significantly reduced the abundance of *Proteobacteria*. These findings suggested that there was a close relationship between HLJDD, autophagy, the gut microbiota and sepsis-related inflammation, which deserves a further study.

However, there are still some shortcomings to our research. Sepsis causes inflammatory storms, autophagy inhibition, and changes in the gut microbiota. The relationships between HLJDD and these three factors are still unclear. Our experiments revealed that HLJDD could alleviate the inflammatory response in sepsis to a certain extent by activating autophagy and that the activation of this pathway was regulated by the upstream PI3K/MAPK-mTOR pathway. It is not clear whether HLJDD directly affects the composition of the gut microbiota or the alleviation of inflammation in the animals themselves. In conclusion, this study showed that HLJDD ameliorated sepsis-induced intestinal injury and inflammation by regulating autophagy and altering the intestinal microbiome. Overall, more experiments are needed to determine the specific molecular mechanisms involved.

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Data availability statement

Data will be made available on request.

Declarations

Ethics approval and consent to participate

All animal experiments were approved by the Ethics Committee of Gui-zhou University of Traditional Chinese Medicine and performed according to the guidelines of the Chinese National Institutes of Health.

Consent for publication

We declare that the publisher has the author's permission to publish the relevant contribution.

Competing interests

The authors declare no competing interests.

CRediT authorship contribution statement

La Wang: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **GuiTong Jiang:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **WenJia Wang:** Writing – review & editing, Methodology. **ZunLi Ke:** Writing – review & editing, Formal analysis. **RuiXi Luo:** Writing – review & editing, Software, Methodology, Formal analysis. **WeiYi Tian:** Writing – review & editing, Validation, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

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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Not applicable.

Appendix ASupplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e31607.

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