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ORIGINAL RESEARCH

Protective Effects of Irisin Against LPS-Induced Intestinal Injury and Its Influence on Gut Microbiota in Septic Mice

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Background: Irisin, a novel myokine, has garnered significant attention for its roles in metabolic regulation and anti-inflammatory responses. Sepsis disrupts the intestinal microenvironment, exacerbating its progression and highlighting the need for novel therapeutic approaches. This study aims to investigate whether irisin exerts protective effects against lipopolysaccharide (LPS)-induced intestinal injury in septic conditions and to explore the underlying mechanisms involving the gut microbiota.

Methods: To induce sepsis, C57BL/6 mice were injected intraperitoneally with LPS at a dose of 10 mg/kg, and then administered with 1 μg/kg of irisin. The Activity levels and 7-day survival rate were recorded. The intestinal expression of irisin/FNDC5 was assessed using Western blotting and immunofluorescence staining. Inflammatory factors were measured using enzyme-linked immunosorbent assay (ELISA). Peripheral blood bacteria were cultured on blood agar plates. Intestinal histomorphology was analyzed via hematoxylin and eosin (H&E) staining. The expression of occludin and apoptotic-related proteins was determined by Western blot, and apoptotic cells were detected using the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) method. The intestinal microbiota was analyzed through 16S rRNA amplicon sequencing.

Results: Irisin improved the survival state and rate of LPS-induced septic mice. It restored endogenous irisin/FNDC5 levels in intestinal tissues, mitigated intestinal barrier injury, and alleviated bacteremia following sepsis treatment. Furthermore, irisin exhibited anti-inflammatory properties by increasing the levels of IL-22 while decreasing those of TNF-α and IL-6, as well as anti-apoptotic effects by increasing levels of pro-caspase-3 and Bcl-2 while decreasing cleaved caspase-3, Bax, and the positive density of apoptotic cells. Additionally, it regulated intestinal microbiota dysfunction.

Conclusion: Irisin effectively treats septic acute intestinal injury by reducing apoptosis and inflammation, with the intestinal microbiota likely playing a crucial role. This finding offers a novel approach to clinical management of sepsis.

Keywords: irisin/FNDC5, sepsis, anti-inflammation, anti-apoptosis, intestinal microbiota

Introduction

Irisin is a hormone derived from the cleavage of fibronectin type III domain-containing protein 5 (FNDC5), a membrane protein recently identified as primarily produced by skeletal and cardiac muscles, and secreted into circulation in response to endurance exercise.[1] Lower expression levels have also been observed in other tissues, including adipose tissue, the brain and liver.[2] Studies have demonstrated that irisin enhances glucose uptake, reduces insulin resistance, promotes adipose tissue browning, and increases energy expenditure.[2,3] It plays a significant role in mitigating metabolic disorders, including type 2 diabetes mellitus, obesity, metabolic syndrome, metabolic bone disease, cardiovascular disease and polycystic ovary syndrome.[3,4]

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Interestingly, apart from the aforementioned functions, irisin also exhibits anti-inflammatory effects by modulating various signaling pathways, including TLR4, Mst1/JNK, JNK-LATS2, SIRT1/Nrf2, Nrf2/GPX4, and NF-κB pathways. It inhibits the production of pro-inflammatory factors and chemokines while promoting the generation of anti-inflammatory cytokines, accompanied by changes in the immune system.[5–12] Furthermore, exercise alleviates organ dysfunction during sepsis by stabilizing intestinal microbiota,[13] suggesting a likely correlation between gut microbiota and exercise-induced hormone irisin in sepsis. Concurrently, by restoring the balance of the gut microbiota, irisin is considered protective against many different diseases, including myocardial ischemia-reperfusion injury, reproductive endocrine disorders, cognitive impairment, and type 1 diabetes.[14–18] Therefore, the effects of irisin in mitigating septic intestinal damage are potential associated with its anti-inflammatory properties and interactions with the gut microbiota.

Sepsis is a life-threatening condition resulting from infection, characterized by multiple organ dysfunction and metabolic abnormalities.[19] It involves an imbalance between pro-inflammatory and anti-inflammatory pathways, as well as immune system failure.[19,20] Moreover, Sepsis is the leading cause of death in intensive care units (ICU).[21] In mainland China, one in five intensive care unit (ICU) patients is infected with sepsis, with a 90-day mortality rate of 35.5%.[22] Although the mortality rate from sepsis has decreased in recent decades, its incidence, along with associated organ failures, continues to rise and requires more care, placing a significant burden on healthcare resources and society.[23,24] The pathogenesis of sepsis is complex and remains poorly understood.[25] Thus, further investigation into sepsis-related mechanisms and the ongoing search for new therapeutic options are essential.

The intestinal microenvironment plays a pivotal role in the development of sepsis and multiple organ dysfunction.[26] Damage to the intestinal epithelium, dysbiosis of the gut microbiota, inflammatory activation, and alterations in immune responses are consistently associated with the onset of sepsis, which further accelerates its progression.[26–28] Gut symbiotic bacteria and their metabolic derivatives are important for improving sepsis survival rates, as they invade pathogens in the intestine, alleviate inflammation and immunity.[28] However, in sepsis patients, the gut microbiota is frequently dominated by a single bacterial genus, typically consisting of pathogenic and antibiotic-resistant strains, along with a marked reduction in gut symbiotic bacteria, causally linked to the mortality of sepsis.[28,29] As a dynamic system, the gut becomes unable to prevent the translocation of pathogens or their derivatives into the mesenteric lymph, bloodstream and extraintestinal organs when its barrier function is compromised, thereby initiating or worsening sepsis and contributing to increased mortality.[28] Consequently, an effective strategy to combat sepsis involves not only reducing intestinal damage but also correcting the imbalance of gut microbiota.

Recent studies have shown that circulating irisin levels are reduced in sepsis patients, with a negative correlation observed between irisin levels and biomarkers of mortality, inflammation, and metabolic states.[30,31] Irisin is strongly related to the diagnosis and prognosis of sepsis. Furthermore, several studies suggest that irisin protects against sepsis-induced multiple organ dysfunction, including myocardial injury, encephalopathy, acute kidney injury, renal damage, and acute lung injury.[5–12] This protective effect is mediated through mechanisms such as anti-inflammatory, antioxidant, anti-ferroptotic and anti-apoptotic pathways.[6–12] However, the research exploring the relationship between irisin, sepsis-induced intestinal injury, and the gut microbiome remains limited. The irisin/FNDC5 proteins in humans and mice exhibit a high degree of homology.[32] In conclusion, the aim of our study is to test the hypothesis that irisin may ameliorate LPS-induced intestinal damage, enhance survival rates, and investigate the role of the gut microbiota in septic mice following irisin treatment.

Materials and Methods

Reagents and Materials

Irisin (Recombinant irisin, 100–65) was obtained from Pepro Tech. Lipopolysaccharide (LPS) was sourced from Sigma-Aldrich, USA. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG and goat anti-rabbit IgG were provided by Santa Cruz Biotechnology, USA. The TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling) assay kit for in situ detection of apoptosis was purchased from Biyuntian Biotechnology Research Institute. Rabbit anti-mouse FNDC5, occludin, caspase-3, and Bax antibodies were obtained from Wuhan Sanying Biotechnology

Co., Ltd. Rabbit anti-mouse Bcl-2 antibody was purchased from Abcam, UK. Mouse IL-6, IL-22, and TNF-α the enzyme-linked immunosorbent assay (ELISA) kits were sourced from Beijing Andy Huatai Technology Co., Ltd.

Animal Grouping and Sepsis Model

Healthy male C57BL/6 mice (8–12 weeks, 20–30g) were obtained from Chengdu Dashuo Experimental Co., Ltd. All animals were housed in the animal facility of the Affiliated Hospital of Southwest Medical University for one week to acclimate, with 3–4 mice per cage, under standard conditions (25 ± 2°C, 12-hour light/dark cycle, 50% humidity). All experimental procedures adhered to the Guidelines for the Ethical Review of Experimental Animal Welfare (GB/T 35892–2018) and were approved by the Animal Ethics Committee of Southwest Medical University (No:20231019–006). The mice were randomly assigned to three groups: (1) Control (Ctrl) group, (2) sepsis group (LPS group), and (3) sepsis + irisin group (L.Iri group). This study employed a double-blind design. One of our team members prepared LPS, irisin, and saline solutions in identical vials labeled with numbers. The administration personnel injected the mice intraperitoneally or via the tail vein according to these assigned numbers. Experimental observers and sample collectors recorded data and conducted measurements based on the same labeling system. Only after the data collection was completed did another team member responsible for statistics unlock the grouping table corresponding to the numbers for analysis. Sepsis was induced by intraperitoneal injection of lipopolysaccharide (10 mg/kg). In the LPS group, normal saline was administered, while 1 μg/kg of irisin was administered in the L.Iri group, both via tail vein injection. Mice in the Ctrl group were treated identically to those in the experimental groups but were injected with 0.9% NaCl instead of lipopolysaccharide and irisin. This section outlines the experimental procedures utilized in the study (Figure 1A).

Experiment 1

Mice were observed for appearance, behavior, and overall condition within 24 hours after injection, and the 7-day survival rate was recorded for each group (n=10/group).

Experiment 2

Twenty-four hours after modeling, mice were anesthetized and sacrificed for tissue collection (10 mice per group). Mice that died during the experiment were replaced to ensure model consistency, resulting in a total of 50 mice being sacrificed.

Specimen Collection and Handing

After 24 hours of model development, 10 mice from each group were euthanized under deep anesthesia, and peripheral blood, intestinal colon tissues, and intestinal contents were collected. Blood samples, anticoagulated with heparin, were taken for bacterial culture, while the supernatant of the remaining blood samples was used to detect serum inflammatory markers (IL-22, TNF-α, and IL-6). Intestine tissues, 2 cm in length, were harvested 1 cm from the ileocecal junction in each group for H&E staining, immunofluorescence, TUNEL analysis using the TUNEL detection kit, ELISA and Western blotting. The following proteins were analyzed: irisin/FNDC5 (dilution: 1:750), pro caspase-3, cleaved caspase-3, Bax, Bcl-2, and occludin (dilution: 1:1000). The intestinal contents were collected and squeezed into 1.5 mL sterile EP tubes for 16S rRNA gene sequencing of the intestinal microbiota.

Survival Status Observation

The mice were monitored for appearance, behavior, and general condition within 24 hours, as well as for their 7-day survival rate.

The Bacterial Culture of Peripheral Blood

After 24 hours of sepsis model induction, the mice were euthanized under deep anesthesia, and peripheral blood was collected. Part of the blood, after heparin anticoagulation, was diluted 10-fold and 100-fold with sterile normal saline,

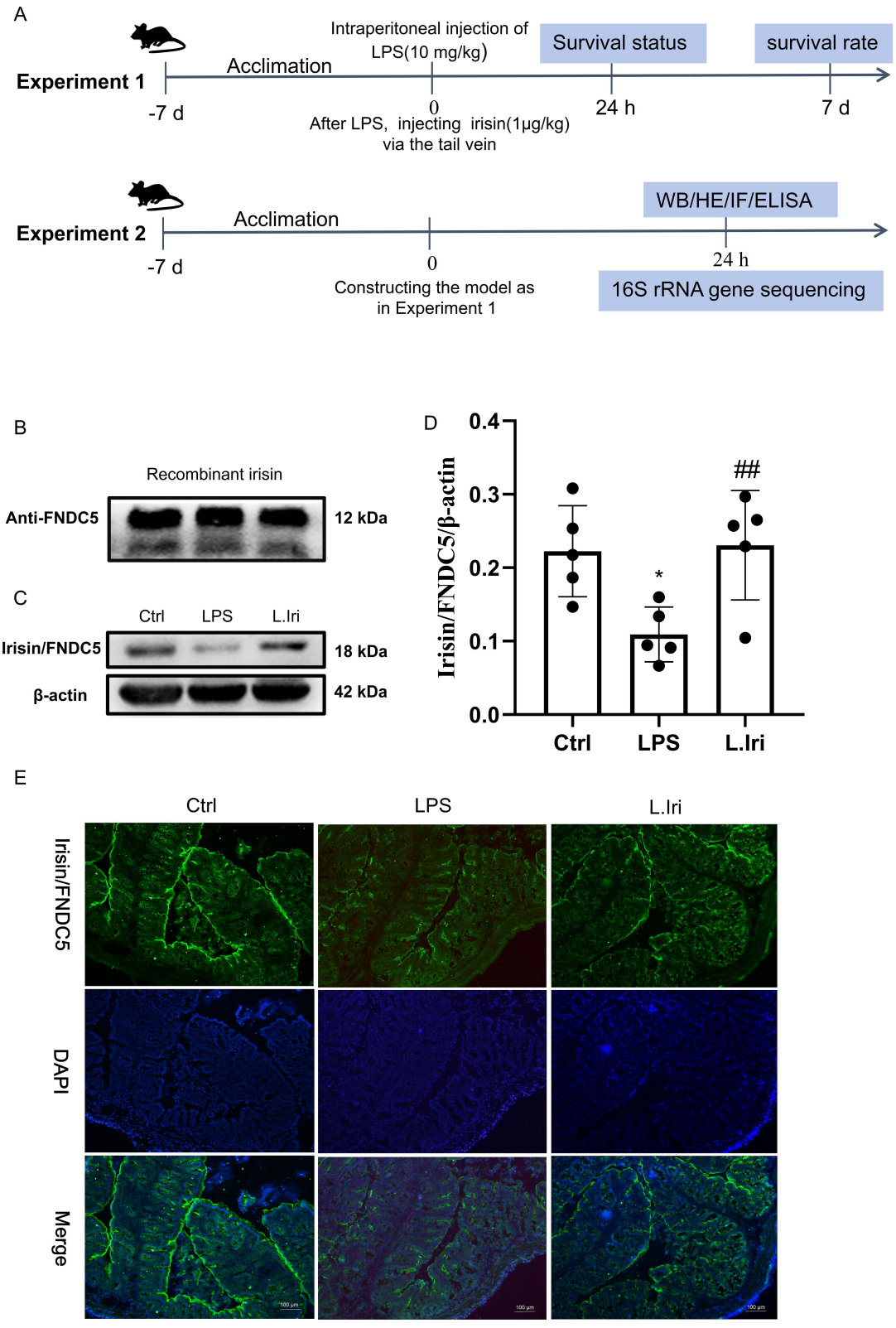

Figure 1 (**A**) The experimental procedure employed in this study. The intestinal expression of irisin/FNDC5 in each group. (**B**) Western blot band of recombinant irisin and Anti-FNDC5 antibody binding, the samples are triplicates. (**C** and **D**) Western blot and its analysis of the expression irisin/FNDC5. (**E**) Representative photos of frozen section immunofluorescence staining of the expression irisin/FNDC5 (green) and corresponding nuclear counterstaining (blue) in the intestine. Scale bar = 100 μm. $^{*}P < 0.05$, vs Ctrl group; $^{\#\#}P < 0.01$, vs LPS group.

and 0.4 mL of each dilution was cultured on Columbia blood agar plates (5% sheep blood) at 37°C for 24 hours. All samples were observed, and CFU (Colony-Forming Units) were counted on the blood plates.

Detection of Serum and Intestine Inflammatory Factors

The remaining peripheral blood and intestinal colon tissues were allowed to coagulate and then centrifuged at 3000 rpm for 15 minutes. The supernatant was collected, and the levels of IL-22, TNF-α, and IL-6 in both the serum and intestine were measured by ELISA according to the manufacturer's instructions.

Detection of Gut Microbiota (16S rRNA Gene Sequencing)

After euthanizing the mice, intestine near the ileocecal area was excised using sterile scissors. The intestinal contents were then squeezed into 1.5 mL sterile EP tubes, immediately frozen in liquid nitrogen, and stored at −80°C. DNA was extracted from the samples using the CTAB (Cetyltrimethylammonium Bromide) / SDS (Sodium Dodecyl Sulfate) method, and its concentration and purity were assessed using 1% agarose gel electrophoresis. The extracted DNA was diluted to 1 ng/μL with sterile water and served as a template for amplifying different regions of the 16S rRNA gene (16S V3-V4) using specific primers (341F-CCTAYGGGRBGCASCAG and 806R-GGACTACNNGGGTATCTAAT). The amplified PCR products were electrophoresed on a 2% agarose gel, and the products were purified using the GeneJET™ Gel Extraction Kit (Thermo Scientific). Following the manufacturer's instructions, sequencing libraries were prepared using the Ion Plus Fragment Library Kit (48 reactions, Thermo Scientific). The quality of the library was evaluated with a Qubit™ 2.0 fluorometer (Thermo Scientific). Sequencing was conducted on an Ion S5™ XL platform, generating single-end reads of 400 bp and 600 bp lengths. Subsequently, the amplified PCR products underwent splitting, filtration, and chimera removal. The final clean reads were utilized for sequence analysis using Uparse software (Uparse v7.0.1001) and uploaded to the public database (NCBI, SRR35569561). Sequences with ≥97% similarity were grouped into the same operational taxonomic units (OTUs). The abundance data of OTUs were normalized based on the sequence count of the sample with the lowest number of sequences, referencing the platform phase of the rarefaction curve as well. Taxonomic information for each representative sequence was annotated using the Silva database based on the Mothur algorithm. The species diversities of our samples were calculated with quantitative insights into microbial ecology (QIIME, Version1.7.0).

H&E Staining

The intestinal colon tissues were 2 cm in length and were collected 1 cm from the ileocecal junction. The collected tissues were divided into two pieces. The 1 cm-long sections were placed in 4% formaldehyde for 24 hours and then embedded, sectioned (at a thickness of 3 μm) for H&E staining. The remaining tissues were placed in Eppendorf tubes and immediately stored at −80°C for subsequent experiments.

Immunofluorescence

The remaining 1 cm-long tissue sections were cut into 8 μm-thick frozen pieces, which were randomly divided into two parts. One part was incubated with Anti-FNDC5 antibody at 4°C overnight. The corresponding fluorescein-labeled antibody was then applied dropwise and incubated at 37°C for 30 minutes. DAPI was subsequently added to stain the nuclei. Finally, the sections were examined under a fluorescent microscope.

TUNEL

The remaining frozen sections were used to detect apoptotic cells according to the instructions of the TUNEL detection kit under a fluorescence microscope. Sections with a 1 mm^2 area each were randomly selected from each group to calculate the apoptotic cell density.

Western Blot

The intestinal tissues were weighed, mixed with RIPA lysis buffer and protease inhibitor, and then homogenized by ultrasound to extract proteins. After centrifugation for 15 minutes (13,000 rpm, 4°C), the supernatants were collected.

Protein quantification was performed using the Bradford method, with BSA as the standard. The membrane was incubated with primary antibodies at 4°C overnight, including irisin/FNDC5 (dilution: 1:750), pro caspase-3, cleaved caspase-3, Bax, Bcl-2, and occludin (dilution: 1:1000), followed by incubation with secondary IgG-HRP antibodies. The bands were developed using a digital gel image analysis system after washing the membrane. The absorbance values of the bands represent the protein expression levels, which were measured using Image J software and normalized to β-actin intensity.

Statistical Analysis

All data in this experiment are presented as mean ± standard deviation ($\bar{x} \pm s$). Statistical analyses were performed using GraphPad Prism 8 and SPSS 21 software. The Kruskal–Wallis test was used to assess differences in the relative abundance of gut microbiota between groups. One-way analysis of variance (ANOVA) was employed for pairwise comparisons of other data. Spearman correlation analysis was used to evaluate the relationship between two variables. A P-value of less than 0.05 ($P < 0.05$) was considered statistically significant.

Results

The Expression of Endogenous Irisin/FNDC5 in Intestinal Tissues and Its Modulation Following Treatment with LPS or Exogenous Irisin

Previous studies have reported that the basal serum irisin concentration in mice ranges from 5 to 15 ng/mL.[33,34] Remote limb ischemic preconditioning (RIPC) exerts a protective effect against lung ischemic injury by increasing endogenous serum irisin levels threefold. Following intravenous injection of exogenous recombinant irisin (1 μg/kg), serum irisin levels in mice rise by 20 ng/mL, approximately 2–3 times higher than the baseline concentration, similar to the effect of RIPC.[35] Additionally, RIPC was shown to ameliorate organ damage, including liver damage, in sepsis.[36] Consequently, 1 μg/kg of exogenous recombinant irisin was selected for our study.

There was no specific antibody available to clearly distinguish FNDC5 from irisin. However, the Anti-FNDC5 antibody could bind both recombinant irisin and FNDC5, as demonstrated by Western blot analysis, which showed a protein band (Figure 1B), confirming that the Anti-FNDC5 antibody used in this experiment detected both FNDC5 and irisin simultaneously. Therefore, the Anti-FNDC5 antibody was employed to measure the expression levels of irisin/FNDC5 in mouse intestinal tissues by Western blot and immunofluorescence. Western blot analysis revealed that irisin/FNDC5 was expressed in the intestinal tissues of the Ctrl group, but expression in the LPS group was significantly lower compared to the Ctrl and L.Iri groups ($P < 0.05$) (Figure 1C and D). The immunofluorescence results were consistent with the Western blot data (Figure 1E). Furthermore, immunofluorescence imaging showed that irisin/FNDC5 was predominantly localized in intestinal epithelial cell membrane.

Irisin Improved the Survival State of LPS-Induced Septic Mice and Increased the 7-Day Survival Rate

The activities of mice in each group were monitored within 24 hours after establishing the sepsis model. The results showed that the mice in the Ctrl group exhibited normal behavior, indicating good overall health. In contrast, mice in the LPS group were observed lying prone in the cage, with weak limbs and low energy. Mice in the L.Iri group were curled up in the cage, demonstrating better vitality, activity, and coat condition compared to the LPS group (Figure 2A). By the seventh day after modeling, all mice in the Ctrl group survived, while only 2 of 10 mice in the LPS group and 5 of 10 mice in the L.Iri group survived. Compared to the Ctrl group, the 7-day survival rates of the LPS and L.Iri groups were significantly reduced, with the survival rate in the L.Iri group notably higher than that in the LPS group (Figure 2B) ($P < 0.05$ or $P < 0.01$).

The Effect of Irisin on Serum and Intestinal Levels of Inflammatory Factors IL-22, TNF-α and IL-6 in Septic Mice

IL-22, IL-6, and TNF-α were selected as key inflammatory indicators for assessing septic intestinal injury and prognosis. IL-22 functions as a central "anti-inflammatory repair factor" in intestinal inflammation, exhibiting a high degree of tissue specificity for the intestine.[37] It regulates the balance of the inflammatory response and is a crucial regulator in the

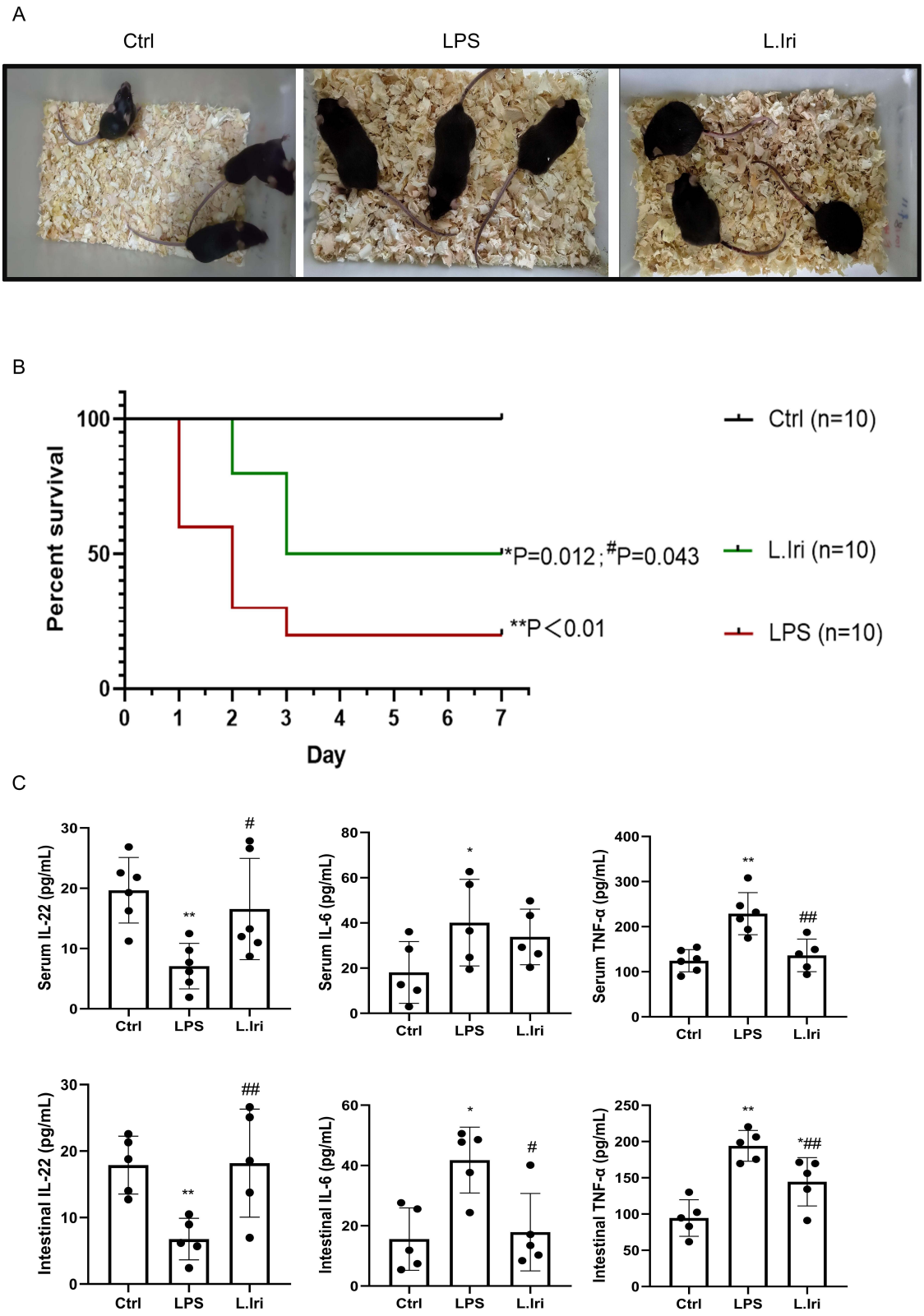

Figure 2 Irisin improved the survival, and decreased both systemic and local inflammatory response in LPS-induced septic mice. (**A**) The activity of the mice in the three groups within 24h of the model. (**B**) The 7-day survival rate of mice in each group after different treatment. 2 of 10 mice in the LPS group survived, 5 of 10 mice in the L.Iri group survived. (**C**) The detection of serum and intestinal IL-22, IL-6 and TNF-α expression in each group of mice by ELISA method. $^{*}P < 0.05$, $^{**}P < 0.01$, vs Ctrl group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, vs LPS.

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restoration of intestinal barrier integrity.[38,39] TNF-α and IL-6 are not only core mediators of the systemic inflammatory response; they also directly or indirectly affect the intestinal mucosal barrier, immune cells, and gut microbiota, thereby destroying intestinal structure and function through multiple targets and pathways.[40–42] Compared to the Ctrl group, the LPS group exhibited decreased levels of IL-22 and increased levels of TNF-α and IL-6 in both serum and intestine. In the L.Iri group, TNF-α levels were elevated in the intestine, while no significant differences were observed for the other cytokines compared with the Ctrl group. In contrast to the LPS group, IL-22 levels increased, while TNF-α levels decreased in both the serum and intestine, along with a decrease in IL-6 levels in the intestine, but no significant change in serum IL-6 levels in the L.Iri group (Figure 2C) ($P < 0.05$ or $P < 0.01$).

Irisin Mitigated Intestinal Barrier Injury by Reducing Intestinal Injury in Septic Mice

The intestinal H&E staining (Figure 3A) revealed that the intestinal colon mucosal epithelium in the Ctrl group was intact, with regularly arranged goblet cells and glands, and no inflammatory cell infiltration. In contrast, mice in the LPS group exhibited damage to the intestinal mucosal epithelium, with disruption of the tight junctions between cells, extensive inflammatory cell infiltration, a reduction in goblet cells, and deformed, disorganized glands. In the L.Iri group, the intestinal epithelium remained largely intact, with the arrangement of goblet cells and glands appearing normal, and a reduction in inflammatory cell infiltration compared to the LPS group. These results suggest that sepsis caused damage to the intestinal mucosa, which was mitigated by exogenous irisin treatment in septic mice.

Irisin Enhanced Intestinal Barrier Function by Increasing the Expression of the Tight Junction Protein Occludin in Septic Mice

Occludin, a tight junction protein, is commonly used to assess the barrier function of the intestinal mucosa.[43] Western blot analysis was employed to measure the expression level of occludin in intestinal tissues (Figure 3B). The expression of occludin in the LPS group was lower than that in the Ctrl group, while the expression in the L.Iri group was higher than that in the LPS group (Figure 3C) ($P < 0.05$ or $P < 0.01$). These results suggest that irisin increased the expression of the tight junction protein occludin in the intestinal mucosa of septic mice, thereby ameliorating damage to the intestinal mucosal barrier.

Irisin Reduced Intestinal Apoptosis in Septic Mice

Those proteins including caspase-3, Bcl-2, Bax are closely related to the process of apoptosis.[44] The expression levels of anti-apoptotic proteins, including pro caspase-3 (Figure 3D and E) and Bcl-2 (Figure 3F and G) were lower in the LPS group than in the L.Iri group ($P < 0.05$ or $P < 0.01$). Conversely, the expression levels of pro-apoptotic proteins assessed by Western blot, including cleaved caspase-3 (Figure 3D and H) and Bax (Figure 3I and J), were higher in the LPS group compared to the Ctrl and L.Iri groups ($P < 0.01$ or $P < 0.05$). No statistically significant differences in the expression of these proteins were observed between the Ctrl and L.Iri groups. Additionally, TUNEL staining (Figure 4A and B) showed that the positive density of apoptotic cells in the intestinal tissue was significantly higher in the LPS group compared to both the Ctrl and L.Iri groups ($P < 0.05$ or $P < 0.01$). No statistically significant difference was found between the Ctrl and L.Iri groups. These results suggest that irisin protects against intestinal tissue apoptosis in septic mice.

Irisin Alleviated LPS-Induced Bacteremia in Septic Mice

Bacterial colonies cultured from peripheral blood were used to assess the level of bacteremia. The number of bacterial colonies in the blood of the LPS group was significantly higher than that in both the Ctrl and L.Iri groups ($P < 0.05$ or $P < 0.01$), with no statistically significant difference observed between the Ctrl and L.Iri groups (Figure 4C and D). These results suggest that intraperitoneal injection of LPS to induce sepsis caused intestinal mucosal damage, disruption of intestinal barrier function, bacterial translocation, and bacteremia in septic mice. Furthermore, treatment with exogenous irisin alleviated bacteremia in these mice.

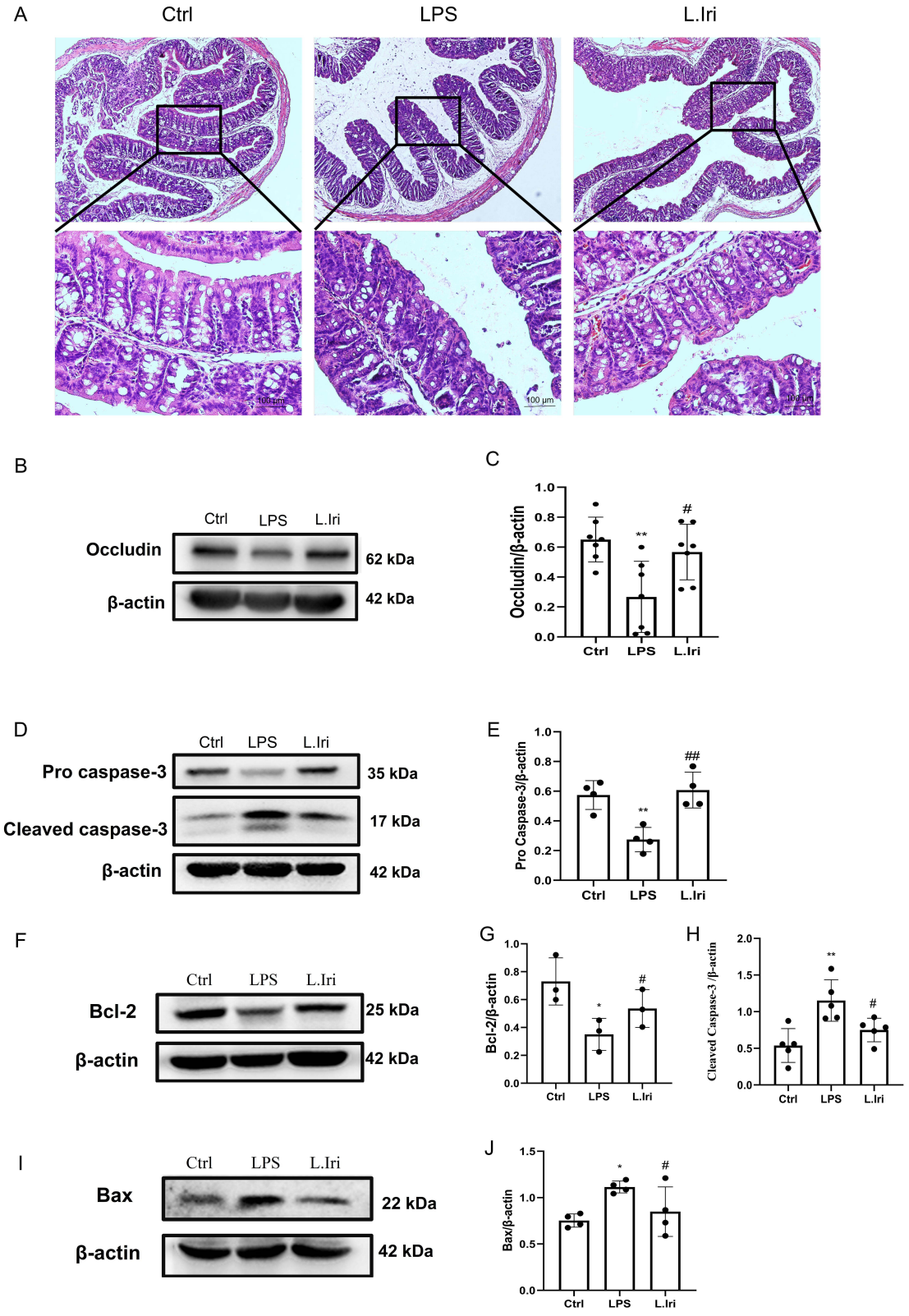

Figure 3 Irisin alleviated LPS-induced intestinal injury in septic mice. (**A**) Pathological changes of intestinal tissues were observed with H&E staining in each group. Scale bar = 100 μm. (**B** and **C**) The expression levels of tight junction protein occludin were detected by Western blot and the statistical analysis of results data were shown. Irisin also protected intestinal tissues from apoptosis in septic mice. The intestinal tissue apoptosis-related proteins such as pro caspase-3 (**D** and **E**), Bcl-2 (**F** and **G**), cleaved caspase-3 (**D** and **H**), Bax (**I** and **J**) and were detected by Western blot and analyzed with quantification. $^{*}P < 0.05$, $^{**}P < 0.01$, vs Ctrl group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, vs LPS group.

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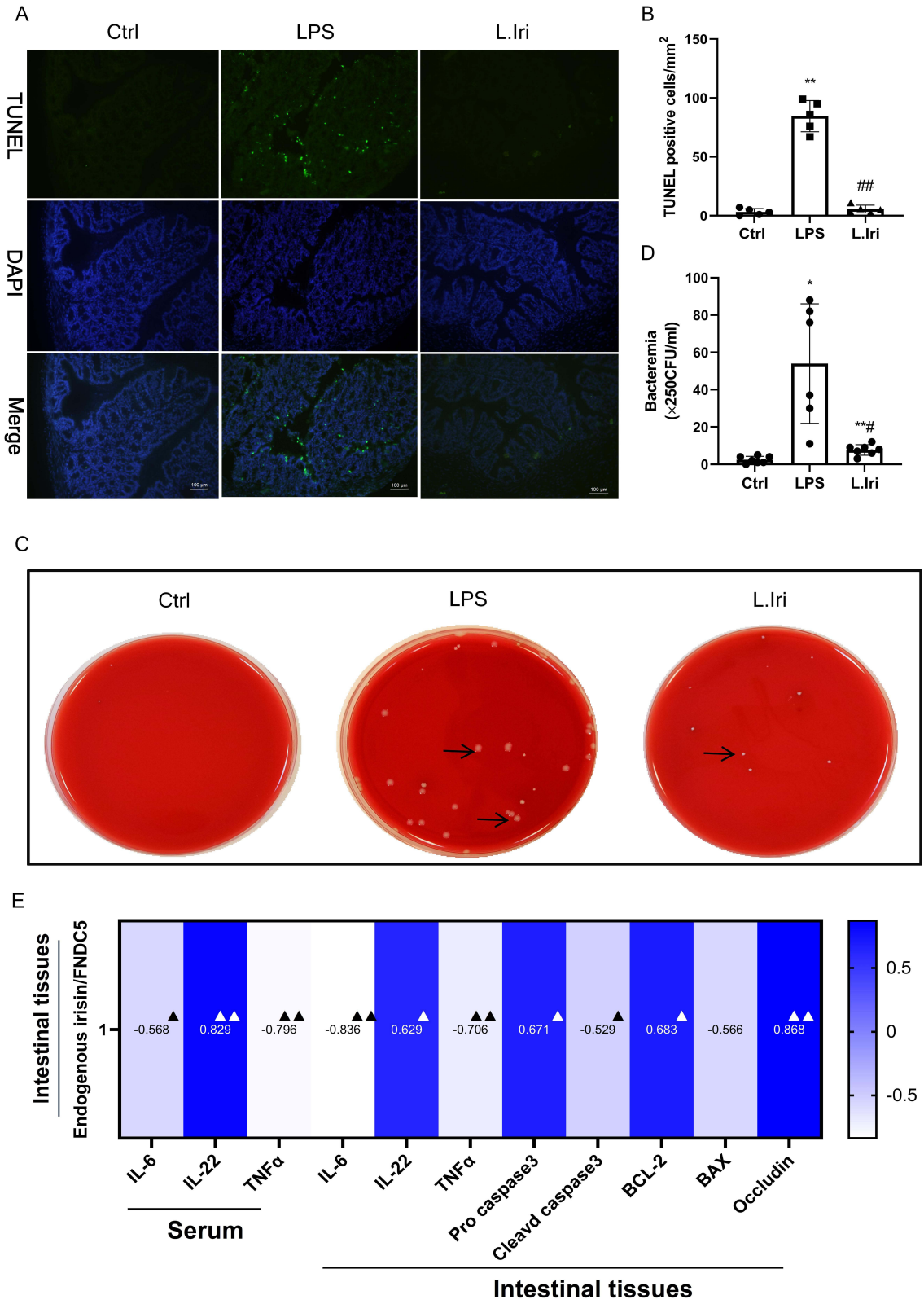

Figure 4 (**A**) The intestinal apoptosis of mice in each group was detected by TUNEL fluorescent staining. Nuclei were labeled with blue fluorescence (DAPI), and cell apoptosis was labeled with green fluorescence. Scale bar = 100 μm. (**B**) The density of TUNEL-positive cells was analyzed. (**C**) Irisin alleviated bacteremia in septic mice, as assessed by incubating peripheral blood on a blood agar plate. The black arrows in the figure indicate the bacterial colonies. (**D**) The CFU of bacteremia were count and analyzed (CFU/mL). $^{*}P < 0.05$, $^{**}P < 0.01$, vs Ctrl group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, vs LPS group. (**E**) The correlation between endogenous irisin/FNDC5 and various biomarkers in serum and intestinal tissue was evaluated. The Spearman correlation coefficient, which ranges from −1 to 1, is depicted in varying shades of blue, transitioning from light to dark. Specific values are marked in the respective areas, indicating the direction and strength of the correlation between the two variables. The triangles, both black and white, signify that the P-values for these correlations are statistically significant ($^{\blacktriangle}P < 0.05$, $^{\blacktriangle\blacktriangle}P < 0.01$).

The Correlation Between Endogenous Irisin/FNDC5 and Inflammatory Factors, Apoptosis-Related Proteins, and the Tight Junction Protein Occludin

The correlation between endogenous irisin/FNDC5 and various biomarkers, including inflammatory factors (IL-22, TNF-α, and IL-6) in serum and intestinal tissue, apoptosis-related proteins (pro caspase-3, cleaved caspase-3, Bcl-2, and Bax), and the tight junction protein occludin in intestinal tissue, was evaluated (Figure 4E). The results revealed that intestinal endogenous irisin/FNDC5 showed a positive correlation with IL-22 in serum and a negative correlation with IL-6, TNF-α ($P < 0.05$ or $P < 0.01$). In intestinal tissue, it was positively correlated with IL-22, pro caspase-3, Bcl-2 and occludin but negatively correlated with TNF-α and cleaved caspase-3 ($P < 0.05$ or $P < 0.01$). Therefore, it is further demonstrated that intestinal endogenous irisin/FNDC5 is significantly associated with inflammation and immune apoptosis in the process of septic intestinal injury.

Irisin Regulated Intestinal Microbiota Dysfunction in Septic Mice

Irisin was found to inhibit intestinal microbiota dysfunction in septic mice. Intestinal contents from all three groups were randomly selected for 16S rRNA sequencing of the gut microbiota. The rarefaction curve confirmed the validity of the sequencing data, as it leveled off, indicating sufficient species richness and uniformity in each group, stabilizing at 50,000 sequences, with the dilution depth also established at 50,000 (Figure 5A). The rank abundance curve visually displayed the species richness and uniformity in the samples.[45] Higher species richness corresponds to a greater span of the curve along the horizontal axis, while a flatter curve indicates a more uniform species distribution (Figure 5B). No significant differences were observed between the two curves across the treatment groups. Non-Metric Multi-Dimensional Scaling (NMDS) is a statistical ordination technique particularly well-suited for ecological research, which reveals both inter-group and intra-group differences by representing the distances between points.[46] The distances in the NMDS was shorter between the L.Iri group and the Ctrl group, indicating that the intestinal microbiota composition in the L.Iri group was more similar to that of the Ctrl group, compared to the LPS group (Figure 5C). Sequences with ≥97% similarity were grouped into the same operational taxonomic units (OTUs). The Venn diagram illustrating the shared and unique OTUs among different groups suggested that the L.Iri group possessed the highest total number of OTUs, followed by the Ctrl group, with the LPS group having the fewest. In comparison to the LPS group, the L.Iri group demonstrated a greater overlap of shared OTUs with the Ctrl group (Figure 5D). The alpha diversity indices, specifically observed species (Figure 5E) and Shannon (Figure 5F), are visually represented by box plots that illustrate the median, dispersion, maximum, minimum, and outliers of species diversity within each group. The analysis revealed no significant statistical differences in inter-group species diversity.

Subsequently, we examined changes in the relative abundance of intestinal microbiota represented by the top 10 phyla across five taxonomic levels: phylum, class, order, family, and genus. Specifically, at the phylum level (Figure 6A and B), *Bacteroidetes* and *Firmicutes* were identified as the dominant groups, comprising the majority of the microbiota in the experimental mouse groups. A species abundance clustering heatmap was generated based on species annotation and abundance data at the phylum level across all samples. The top 12 phyla, ranked by abundance, were selected for clustering analyses of both species and samples, resulting in a heatmap that that facilitates the identification of species with varying abundance levels in specific samples, including *Verrucomicrobia, Deferribacteres, Proteobacteria, Cyanobacteria, Acidobacteria* (Figures 6C). Compared to the Ctrl group, the LPS group exhibited higher relative abundance at the phylum level for *Proteobacteria, Verrucomicrobia*, and *Deferribacteres* (Figure 6D) ($P < 0.05$). At the class level, the top 10 phyla are shown (Figure 6E), with the LPS group demonstrating significantly higher levels of *Gammaproteobacteria, Verrucomicrobiae*, and *unidentified Deferribacteres*, while *Bacilli* were less abundant (Figure 6F) ($P < 0.05$). At the order level, the top 10 phyla are presented (Figure 7A), *Enterobacteriales, Campylobacterales*, and *Verrucomicrobiales* were more abundant, whereas *Lactobacillales* exhibited a significant reduction (Figure 7B) ($P < 0.05$). At the family level, the top 10 phyla are displayed (Figure 7C), with *Enterobacteriaceae, Helicobacteraceae*, and *Akkermansiaceae* significantly increased, while *Lactobacillaceae* significantly decreased (Figure 7D) ($P < 0.05$). At the genus level, the top 10 phyla are indicated (Figure 7E), *Helicobacter, Allobaculum*, and *Akkermansia* were significantly elevated, while *Lactobacillus* was significantly reduced (Figure 7F) ($P < 0.05$). No significant differences in other

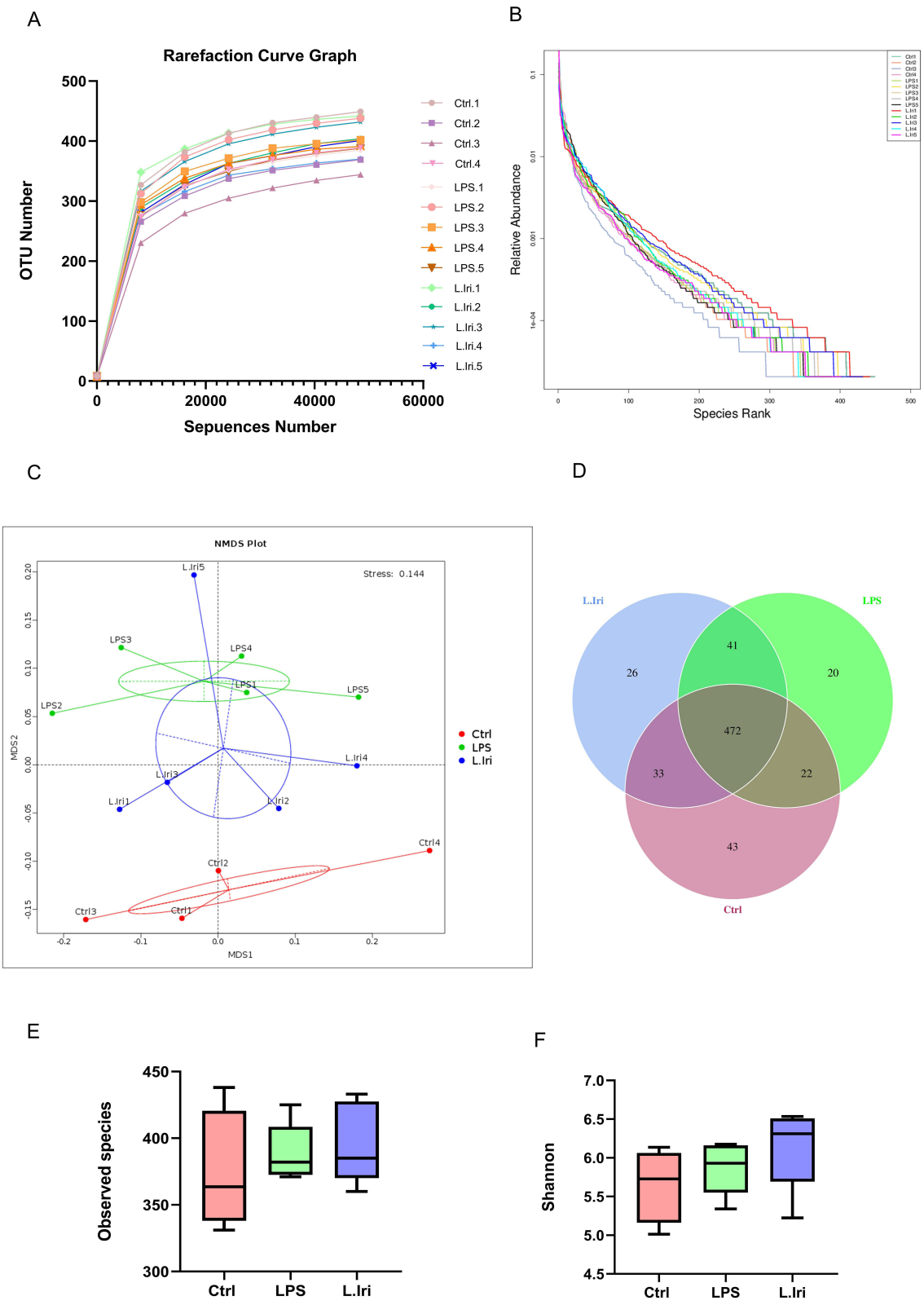

Figure 5 Changes in the gut microbiota of LPS-induced septic mice following irisin treatment. (**A**) Rarefaction curve graph. (**B**) Rank abundance graph. (**C**) Principal component analysis of the similarity of samples among groups (NMDS). (**D**) Venn graph. Boxplots illustrating the inter-group differences in the alpha diversity indices, including observed_species (**E**) and Shannon (**F**).

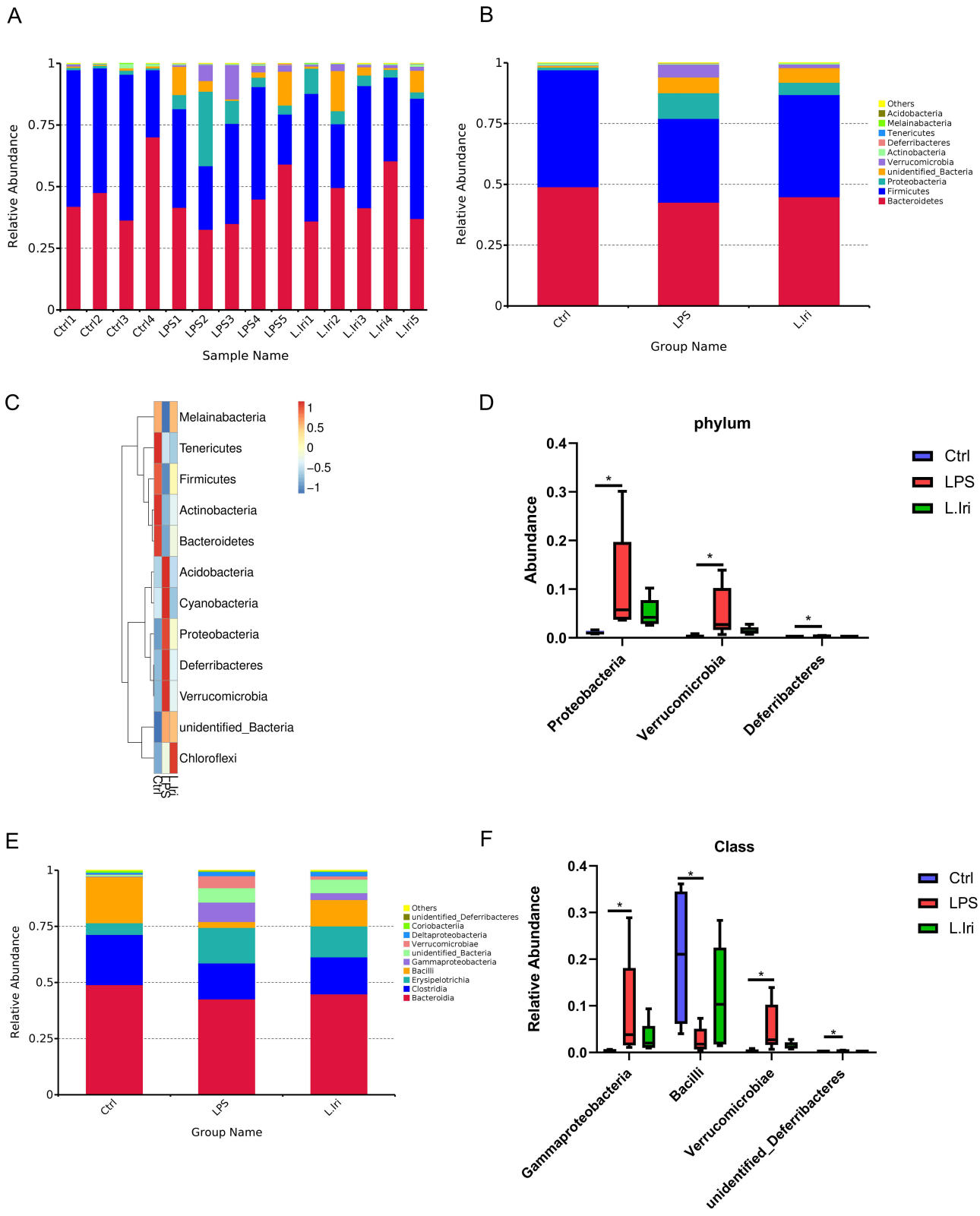

Figure 6 Irisin influenced the intestinal microbiota in LPS-induced septic mice. (**A, B**) Variation in the top 10 relative abundance of group and each sample at the phylum level. (**C**) Species abundance clustering heatmap at the phylum level. (**D**) Comparison of the relative abundance of samples in each group at the phylum. Variation in the top 10 relative abundance (**E**) and comparison of the relative abundance (**F**) of group at the class. *$P < 0.05$ vs Ctrl group.

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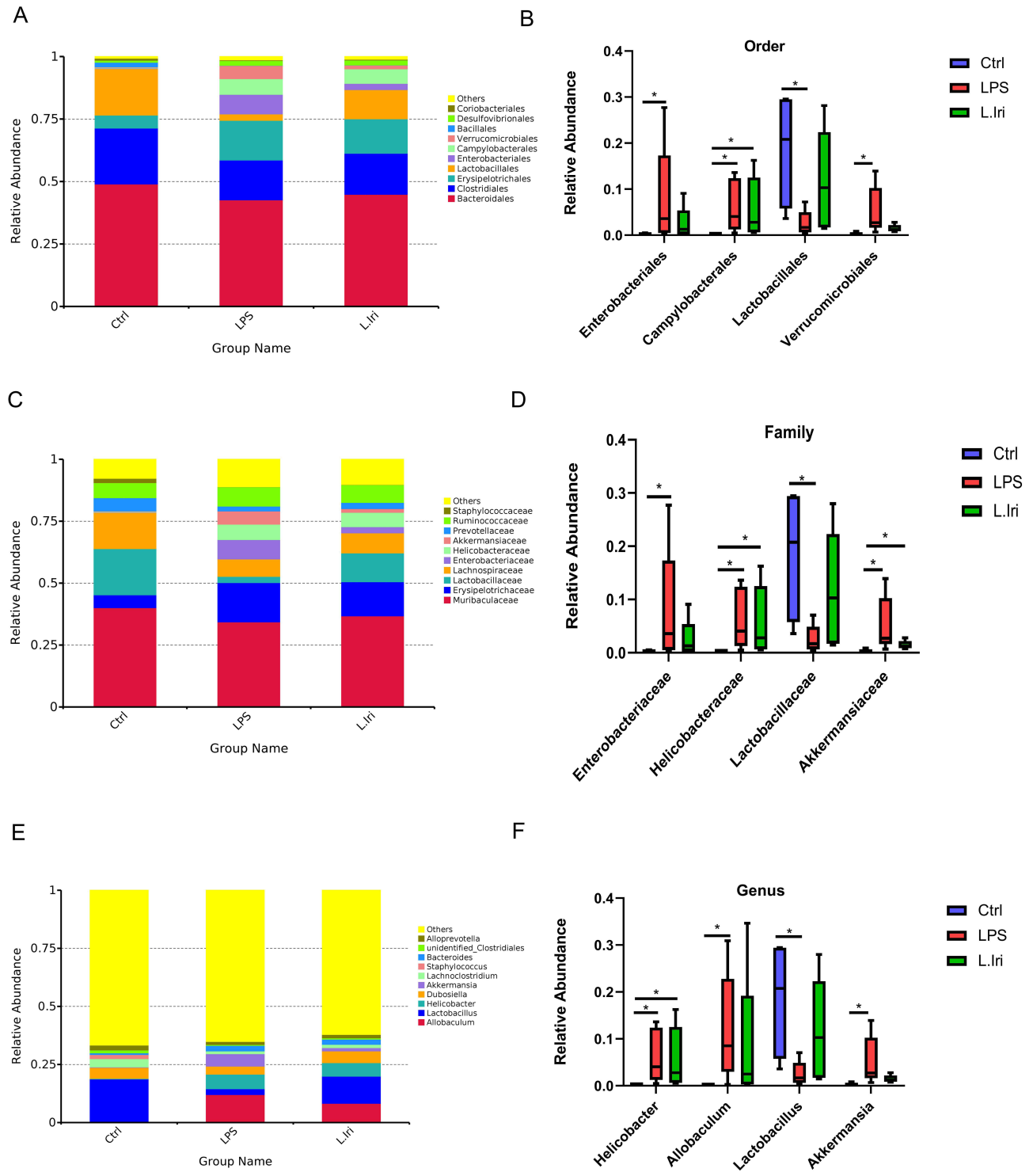

Figure 7 Following treatment with irisin, the imbalance of gut microbiota in septic mice was improved. Variation in the top 10 relative abundance and comparison of the relative abundance of group at the order (**A** and **B**), family (**C** and **D**) and genus (**E** and **F**) level. *$P < 0.05$ vs Ctrl group.

intestinal microbiota were detected among the experimental groups. Following the irisin intervention, the changes observed in the LPS group were largely reversed.

In summary, the intestinal microbiota structure in septic mice from the LPS group was disrupted, with a reduction in beneficial bacteria such as *Lactobacillus* and an increase in pathogenic bacteria such as *Proteus* and *Enterobacter*, which

are closely associated with sepsis. After irisin treatment, these microbiota changes were reversed, and intestinal microbiota dysfunction was stabilized, resulting in a composition more akin to that of healthy mice.

Discussion

Sepsis is associated with high mortality rates and substantial medical costs, primarily due to intestinal damage and disruption of the gut microbiota.[22,23,26] This study demonstrated that LPS treatment significantly reduced the expression of endogenous irisin/FNDC5 in the intestine, while exogenous irisin effectively reversed this downregulation varying with some biomarkers of inflammation and apoptosis. Furthermore, the administration of exogenous irisin alleviated gut disorders, restored intestinal microbiota balance, diminished inflammatory responses, and significantly improved the survival rate of LPS-induced septic mice. These findings suggest that endogenous irisin/FNDC5 in the intestine may serve as a clinical target for the diagnosis and treatment of sepsis. Additionally, exogenous irisin may protect against intestinal injury in septic mice by suppressing inflammation and apoptosis, with the modulation of gut microbiota composition dysfunction, as illustrated in the experimental mechanism diagram (Figure 8).

Serum and intestinal inflammatory factors IL-22, TNF-α, and IL-6 were assessed to evaluate both systemic and local intestinal inflammation induced by LPS in sepsis. IL-22 is a key repair regulator of the intestinal barrier, influencing intestinal stem cell-mediated intestinal epithelial regeneration, ameliorating inflammation, restoring goblet cell function, promoting mucus secretion, and participating in microbial defense.[39,47] TNF-α is significantly upregulated during the early stages of intestinal inflammation and activates various inflammatory pathways, leading to an "inflammatory cascade" that serves as a key mediator in the inflammatory response.[41] IL-6 acts as an amplifier of intestinal inflammation with its expression level effectively reflecting the "dynamic balance" of intestinal inflammation, and inhibits the synthesis of tight junction proteins and worsens barrier permeability.[42] These factors are critical inflammatory cytokines that mediate inflammatory responses, affecting intestinal structure and function, and they play essential roles in LPS-induced intestinal injury and repair. Our results indicated that exogenous irisin treatment increased IL-22 levels while reducing TNF-α and IL-6 levels in both serum and intestine. Meanwhile, it also alleviated intestinal damage and improved survival status and the 7-day survival rate. Overall, exogenous irisin may benefit septic mice by mitigating acute intestinal injury through anti-inflammatory effects.

Intestinal damage plays an important role in the onset and progression of sepsis.[26] The tight junction-related protein occludin stabilizes the barrier structure and permeability of the intestinal epithelium.[43] During the experiment, the intestinal tissue barrier was assessed using H&E staining, measurement of intestinal occludin levels, and markers of apoptosis. Our results showed that irisin ameliorated intestinal mucosa damage, as indicated by H&E staining, upregulated the expression of occludin and anti-apoptotic proteins such as pro caspase-3 and Bcl-2, and downregulated pro-apoptotic proteins like cleaved caspase-3, Bax, and the density of apoptotic cells. Consistent with these findings, irisin reduced blood culture colonies in LPS-induced mice, further supporting the integrity of the intestinal barrier. Thus, exogenous irisin mitigated intestinal apoptosis in LPS-induced mice, exerting a protective effect on the intestinal epithelial barrier and reducing microbiota translocation. This highlights irisin's potential as a treatment for sepsis.

In addition to being derived from muscle tissues, the liver, brain and adipose tissue, irisin is also partially detected in various areas of the body, including the skin, testis, pancreas, liver, spleen, stomach.[48] To date, few studies have investigated the presence of irisin in intestinal tissues. However, this study identified the expression of endogenous irisin/FNDC5 in the intestinal epithelial cells in healthy mice. Previous studies have reported a decrease in circulating irisin levels in sepsis patients, which is positively correlated with patient survival and negatively correlated with pro-inflammatory cytokines, oxidative stress markers, and mortality.[30,31] Furthermore, our findings demonstrated that endogenous irisin/FNDC5 levels, which were positively correlated with serum and intestinal anti-inflammatory cytokines, anti-apoptotic markers, and occludin, but negatively correlated with pro-inflammatory and pro-apoptotic cytokines, decreased in LPS-induced intestinal injury. These levels increased following irisin injection, which was associated with reduced intestinal injury, attenuated inflammation, and improved survival rates. Overall, endogenous intestinal irisin/FNDC5 may serve as a novel biomarker for septic gut injury, and supplementation with exogenous irisin could represent a promising therapeutic strategy.

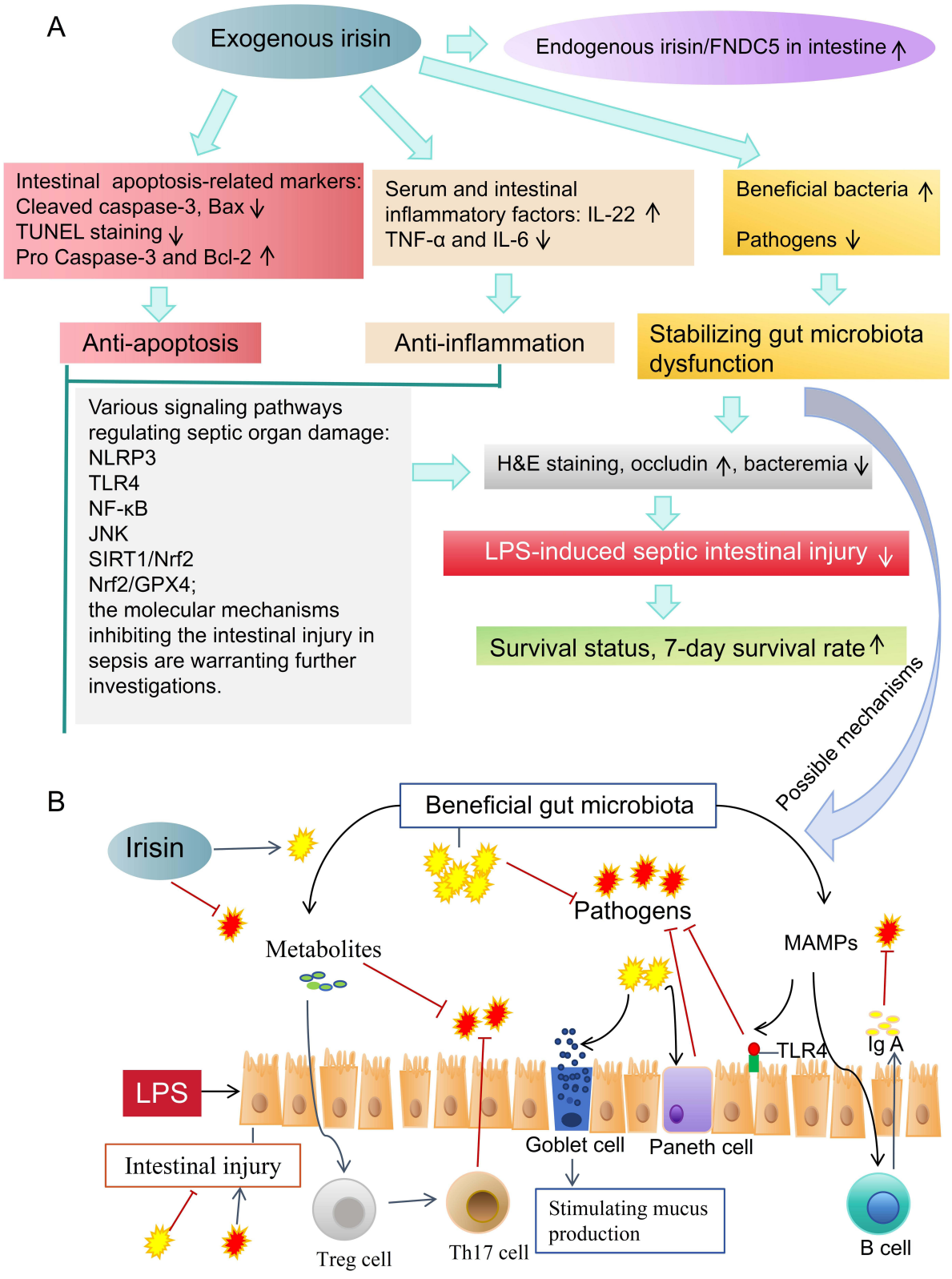

Figure 8 (**A**) The schematic representation of the mechanism involving exogenous irisin, gut microbiota, and septic intestinal injury. Irisin mitigates septic intestinal injury by restoring gut microbiota dysbiosis, with its specific protective effects potentially depicted in the (**B**) according to previous studies, though further research is needed. In the (**A**), the black arrows indicate the direction of action (upward denotes an increase, downward denotes a decrease); in the (**B**), black arrows represent promoting effects, while the red indicate inhibitory effects.

In Caco-2 cells subjected to ischemia/reperfusion (H/R) treatment—which can differentiate into intestinal epithelial cells in vitro—exogenous irisin co-localizes with integrin αVβ5 proteins, indicating that irisin binds to the integrin αVβ5 receptor in the intestine.[49] Exogenous irisin may be transported to intestinal tissues via circulation binding to the integrin αVβ5 receptor or potentially restore the expression of endogenous irisin/FNDC5 to protect septic mice from LPS-induced gut injury. Recent studies emphasize that, besides its established role in metabolic regulation, irisin modulates various signaling pathways to elicit antioxidant, anti-apoptotic, anti-ferroptotic, anti-pyroptotic, and anti-inflammatory effects, thereby mitigating organ damage associated with sepsis.[5–12] The specific mechanisms underlying these effects are discussed below. In the LPS-induced model of septic cardiomyopathy, irisin inhibits DRP1-associated mitochondrial fission by downregulating the JNK-LATS2 signaling pathway.[5] Furthermore, irisin reduces inflammation, apoptosis, and pyroptosis by blocking the TLR4 and NLRP3 inflammasome pathways.[6,11] The combination of irisin and melatonin effectively inhibits the Mst1-JNK pathway, mitigates oxidative stress, and maintains mitochondrial homeostasis.[7] Consequently, irisin reduces myocardial injury. Research has also shown that irisin attenuates inflammation in the models of sepsis-associated encephalopathy induced by CLP or LPS, a process linked to the suppression of hippocampal ferroptosis through the Nrf2/GPX4 pathway.[8] In studies of LPS-induced septic alveolar injury, irisin ameliorates dysfunction of the alveolar epithelial barrier by decreasing inflammation and apoptosis via activation of the AMPK/SIRT1 pathway.[12] Additional studies on sepsis-induced kidney injury have demonstrated that irisin alleviates renal damage by activating the SIRT1/Nrf2 pathway to suppress ferroptosis or by inhibiting NF-κB-mediated inflammation and apoptosis in renal cells.[9,10] However, studies investigating the effects of irisin on LPS-induced acute intestinal injury in sepsis remain scarce. In our study, exogenous irisin treatment upregulated the expression of endogenous irisin/FNDC5 in the intestine and potentially conferred protective effects against acute intestinal injury in septic mice (Figure 8A). Moreover, the precise molecular mechanisms driving the protective role in LPS-induced intestinal damage remain unclear, warranting further investigation. The pathways associated with inflammation include NLRP3, TLR4, and NF-κB, while those linked to apoptosis include Mst1/JNK and JNK-LATS2.[5–12] Therefore, our next research will concentrate on the changes in the four pathways—NLRP3, TLR4, NF-κB, and JNK—after irisin intervention in septic mice with acute intestinal injury to identify significantly altered pathways. Subsequently, the specific molecular targets will be elucidated through which irisin regulates these pathways, thereby establishing a comprehensive mechanism chain of "irisin → [target] → [pathway] → improvement in septic acute intestinal injury."

Growing evidence suggests that exercise-induced irisin may be closely linked to disease outcomes through its effects on the intestinal microbiota.[14–18] Gut microbiota dysfunction is characterized by an increase in harmful bacteria, a decrease in beneficial bacteria, and alterations in bacterial metabolites, all of which vitally impacts the onset and progression of sepsis.[26,28] *Proteobacteria*, particularly *Gammaproteobacteria*, encompass numerous pathogenic bacteria, including *Escherichia coli, Helicobacter pylori, Salmonella, Vibrio, Yersinia pestis*, and *Pseudomonas aeruginosa*. These bacteria serve as markers of microbial dysbiosis, and their increased relative abundance exacerbates sepsis severity.[50] Conversely, *Lactobacillus*, a well-known probiotic, metabolizes sugars to produce lactic acid, inhibits the growth of pathogenic bacteria, upregulates tight junction protein expression, activates immune-related cells, stimulates the release of inflammatory factors, and enhances the production and secretion of immunoglobulin A (IgA).[51–53] Besides, it has also been shown to enhance the regenerative capacity of intestinal epithelial cells and restore the damaged intestinal mucosal barrier to mitigate severe intestinal leakage, bacteremia, and mortality in septic mouse.[54] In contrast, *Enterobacter*, which includes typical pathogens, significantly contributes to sepsis progression.[55] In our study, irisin administration restored the composition of gut microbiota in LPS-stimulated mice, reducing the relative abundance of *Gammaproteobacteria*, and *Enterobacter* while increasing *Lactobacillus*. The NMDS, OTU, and relative abundance assessments demonstrated that irisin treatment enhanced the prevalence of beneficial bacteria while diminishing harmful bacteria, thereby rendering the gut microbiota more similar to that of healthy controls. These findings indicated that irisin may mitigate sepsis-related injury through the normalization of gut microbiota imbalances.

Nevertheless, the precise mechanisms linking irisin, gut dysbiosis, and acute intestinal injury in sepsis remain poorly understood. Extensive research has revealed significant associations between specific gut microbiota, their metabolites, and sepsis. A reduction in bacterial genera such as *Faecalibacterium, Prevotella*, and *Coprococcus*, along with their metabolites—short-chain fatty acids (SCFAs) and butyrate—along with an increase in genera like *Clostridia* and

Enterococcus, as well as the metabolic product alpha-hydroxybutyrate, has been shown to negatively affect the functionality of gut epithelial cells, immunity, and inflammation in patients with sepsis.[56] Additionally, in microbe-associated molecular patterns (MAMPs), gut microbiota and their metabolites can activate Toll-like receptor 4 (TLR4), modulate the development of regulatory T (Treg) cells, enhance the production of IgA by B cells, induce the differentiation of T helper 17 (Th17) cells.[28] Meanwhile, They also compete for nutrients, inhibit the expression of pathogen virulence factors, activate the immune defenses of Paneth cells, and stimulate mucus production by goblet cells.[28,51,57] These processes are vital for maintaining intestinal homeostasis, thereby mitigating LPS-induced intestinal injury, thus providing insights and a theoretical foundation for future research (Figure 8B). Future research will verify whether irisin alleviates intestinal injury indirectly by regulating the gut microbiota through fecal microbiota transplantation. The phenotypic changes and cytokine profiles of immune cells in the intestinal mucosa will be examined following irisin intervention, alongside metagenomic sequencing of the intestinal microbiota, to determine whether the "specific microbiota or its metabolites regulated by irisin" indirectly suppress intestinal inflammation by "regulating immune cell function", thereby refining the complete regulatory chain of "irisin - microbiota - immune inflammation - intestinal injury".

Nonetheless, our research has primarily focused on the protective effects of irisin on LPS-induced gut damage and microbiota dysfunction. A comprehensive analysis of the specific bacterial species affected, their mechanisms of action, and the molecular pathways involved in irisin's protection against septic intestinal injury is beyond the scope of this study and warrants further investigation.

Conclusion

The protective effects of irisin against LPS-induced septic intestinal injury and its impact on gut microbiota in mice were investigated. First, we report the presence of endogenous irisin/FNDC5 in intestinal tissues, which may serve as a potential clinical target for sepsis treatment. The study further demonstrated that exogenous irisin treatment significantly alleviated LPS-induced intestinal damage. This included a reduction in inflammatory markers, improvement in intestinal permeability and apoptosis, inhibition of intestinal bacterial translocation, and enhancement of gut barrier function. Additionally, irisin was found to modulate the composition of the gut microbiota, promoting the growth of beneficial microbial populations while suppressing the proliferation of harmful bacteria. These findings suggest that irisin may represent a potential therapeutic strategy for managing septic intestinal injury by targeting anti-inflammatory and anti-apoptotic pathways, while also highlighting the important role of gut microbiota.

Author Contributions

All authors made significant contributions to the reported work, including conception, study design, execution, acquisition of data, analysis, and interpretation. Ling Yang, Mudi Liu, Beilin Hu, and Kangle Yong participated in drafting, revising, or critically reviewing the article. Jun Zhou and Ye Chen provided final approval of the published version and agreed on the journal to which the article has been submitted, taking accountability for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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