

Synergistic effect of polymyxin B with baicalein against mcr-1 positive *Escherichia coli*

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Abstract

The frequent emergence of multi-drug resistance *Escherichia coli* (*E. coli*) in both poultry and human health has facilitated the demand of seeking a novel therapeutic alternative. Traditional Chinese medicine has become a recent hotspot in drug development due to its efficacious assistance with existing antibiotics against drug-resistant pathogens. Herein, we estimated the synergistic effect of polymyxin B and baicalein (the major integrant of a Chinese herb, *Scutellaria baicalensis*) against *mcr-1* positive *E. coli* HP144 through checkerboard assays accompanying the time-killing curve, and found that baicalein significantly amplified antimicrobial activity of polymyxin B. *E. coli* treated with polymyxin B and baicalein also showed an obviously enhanced destruction of bacterial outer membranes through scanning electron microscopy. Furthermore, transcriptome and metabolome analysis were implemented to reveal the molecular mechanism. Various differentially expressed genes were identified between polymyxin B and the drug combined group, especially lipopolysaccharide (LPS) core synthesis genes (*rfaQ*, *rfbB* and *rfaP*) and efflux pump correlative genes (*evgA*, *arcA* and *mgrB*). Metabolome results also illustrated significant alterations of multiple metabolites, including nicotinamide adenine dinucleotide (NAD⁺) and D-Alanyl-Dalanine dipeptide, which are closely associated with bacterial invasion and outer membrane integrity. Subsequently, mice infection models were utilized to discuss the efficacy of synergistic therapy *in vivo*. After the co-treatment of polymyxin B and baicalein, bacteria loads in mice abdomens were significantly diminished and the survival rate was remarkably increased by ameliorating inflammatory reactions in mice. In conclusion, by increasing the antibiotic permeability of outer membranes, baicalein could efficiently improve the bactericidal effects of polymyxin B *in vitro* and *in vivo*, demonstrating its promising potential as a polymyxin B adjuvant and antibiotic alternative.

1 Background

As one of the most dominant and abundant opportunistic bacterial pathogens that are inhabited the animal intestinal tract, *E. coli* could cause multiple symptoms such as peritonitis, cholecystitis, cystitis, diarrhea, vomiting and fever by invading vital organs of host animals [1]. More seriously, various strains of *E. coli*, including enterohemorrhagic *E. coli*, Shiga toxin-producing *E. coli*, and others, could transmit from animals to humans through food or direct contact and cause severe zoonotic diseases [2, 3]. Due to the unrestrained misuse of antibiotics for the treatment of infection and prophylaxis of some infections in farm animals, the emergence of multi-drug resistant (MDR) bacteria has become increasingly frequent [4, 5]. *E. coli* can operate as a donor and recipient of resistance genes and an intermediary for horizontal gene transfer among bacterial species. Therefore, the treatment of *E. coli* infections is jeopardized by the advent of highly resistant strains, resulting in prolonged hospitalization, raised costs, and forced applications of the latest generation of antibiotics to retain maximal efficacy [6].

Because of the significant rise in infections caused by MDR *E. coli*, colistin (polymyxin E) has been widely utilized in both veterinary and human medicine for over 50 years [7, 8]. However, the long-term clinical use of colistin expedited the emergence and dissemination of colistin resistance in *E. coli*. For instance, Shen and his colleague first reported the plasmid-borne colistin resistance gene *mcr-1* in 2015, which facilitates

the transmission of resistance genes through frequent horizontal transfers [9]. Polymyxin B and colistin share an almost identical primary structure that is composed of cyclic lipopeptides with only one amino acid difference [10]. Polymyxin B has a unique mechanism of action involving disruption of the outer membrane integrity of gram-negative bacteria via selectively binding with LPSs on bacterial outer membranes and replacement of the divalent cation on lipids that in addition to providing rapid bactericidal activity may enhance the activity of other antibiotic classes [11–13]. By means of reducing negative charges on the membrane and the permeability of exogenous drugs, *mcr-1* positive *E. coli* (MCRPEC) exhibits an extremely high resistance to polymyxin B, thus imposing grave threats to the last resort of antibiotics [14]. To date, ten *mcr* types (*mcr-1* to *mcr-10*) have been identified from animals and humans, and various MCRPEC isolates have also been reported in more than 50 countries [15]. Therefore, novel patterns of antibacterial agents and alternative strategies are urgently required to fill the void caused by the attenuated efficacy of existing antibiotics.

During the past two decades, bioactive molecules of plant origins have received enormous attention owing to their preferable bactericidal and prophylactic properties [4, 16]. Massive reports have verified the prominent antibacterial and anti-inflammatory characteristics of natural products and their successful combination use with exhibit conventional antibiotics, which is consistent with the tendency of reducing antibiotics use in the worldwide [17–19]. EPrevious investigations have reported that baicalein shows a significant inhibition to numerous types of pathogenic bacteria and fungi, therefore can be applied in multiple aspects of clinical medicine, such as neurogenesis, antibacterial, anti-tumor, anti-hyperglycemic, cardio protection, anti-inflammatory and wound healing [20, 21]. On the other hand, as a flavonoid derivative, baicalein can efficiently disrupt biofilm formation, inhibit bacterial DNA, RNA and protein synthesis, and degrade endotoxins [22, 23].

In this study, we evaluated the synergistic effect of polymyxin B and baicalein against clinical MCRPEC HP144 originated from pig farms. The integrity of the bacterial outer membrane was observed by SEM under different drug conditions. Furthermore, transcriptome and metabolome analysis demonstrated that compared with polymyxin B alone, genes related to bacterial structure and antibiotic resistance were significantly down-regulated when baicalein was applied at the same time. *In vivo*, the mice infection model was also constructed to analyze pharmacodynamics and evaluate the mortality rate. Bacterial loads in abdominal cavity and inflammatory indicators of infected mice was obviously decreased after the co-treatment of polymyxin B and baicalein. In conclusion, we speculated that baicalein could efficiently amplify the antimicrobial capacity of polymyxin B, illustrating a robust and versatile therapeutic alternative for the clinical treatment of MCRPEC.

2 Materials and methods

2.1 Bacterial strains and chemical agents

Fifteen strains of *E. coli*, which were isolated from anal swabs of pig farms around Harbin, Heilongjiang, were provided by the Pharmacology Laboratory of Northeast Agricultural University as a gift. *mcr-1*

positive E. coli were identified by PCR and nominated as HP144. Baicalein, polymyxin B, and other chemical reagents were purchased from Aladdin (Shanghai, China)

2.2 Antibiotic susceptibility assays

Minimum inhibitory concentration (MICs) of HP144 were determined by the broth microdilution method according to the American Clinical and Laboratory Standards[24]. Polymyxin B and baicalein were twofold diluted in cation adjusted Mueller–Hinton Broth (CAMHB) and mixed with an equal volume of bacterial CAMHB suspensions containing approximately 1.5×10^6 CFUs mL⁻¹ in clear UV sterilized 96 well plates. After incubation at 37 °C for 18 h, the lowest concentrations of antibiotics with no visible growth of bacteria were determined as the MICs.

2.3 Checkerboard assays

Fractional inhibitory concentrations (FICs) were determined by checkerboard assays as described previously [25]. First, 100 µL CAMHB medium was dispensed into a 96-well plate. Baicalein was added to the last row and diluted along the ordinate. Subsequently, polymyxin B was added to the first column and diluted along the abscissa. Finally, in addition to the negative control, approximately 1×10^6 CFUs mL⁻¹ bacterial suspensions were added. After culturing at 37°C for 18 h, the MICs were recorded and the FIC was calculated according to the formula to evaluate the synergistic effect.

$$\text{FIC}_{\text{index}} = \frac{\text{FIC}_a + \text{FIC}_b}{\text{MIC}_{ab}/\text{MIC}_a + \text{MIC}_{ab}/\text{MIC}_b}$$

MIC a is the MIC of compound a alone; MIC ab is the MIC of compound a in combination with compound b; MIC b is the MIC of compound b alone; MIC ba is the MIC of compound b in combination with compound a; FIC a is the FIC of compound a; FIC b is the FIC of compound b. The synergy or additive was defined according to standard criteria: $\text{FICI} \leq 0.5$ was defined as synergistic; $0.5 < \text{FICI} \leq 1$ was defined as additive; $1 < \text{FICI} \leq 4$ was defined as indifference; $\text{FICI} > 4$ was defined as antagonism [26]. All experiments were performed as three biologically independent experiments.

2.4 The time-killing curve assays

The time-killing curve was determined in a previously described method [27]. HP144 was incubated in CAMHB for approximately 16 h at 37°C with 200 rpm shaking speed until the exponential period. The overnight cultures were adjusted to approximately 1×10^8 CFUs mL⁻¹. 200 µL of the diluted bacterial suspensions were mixed with 19.8 mL CAMHB, then added with 1 µg/mL polymyxin B, 80 µg/mL baicalein, or a combination with the same dose, respectively. After being cultured at 37°C with shaking at 200 rpm for 24 h, 100 µL aliquots were removed at 0h, 3h, 6h, 9h, 12h, and 24h, and then mixed with 1000 µL CAMHB. Subsequently, bacterial suspensions were diluted in tenfold series and inoculated on MacConkey medium plates. The time-killing curve was constructed according to CFUs after incubation at 37°C for 18 h. All experiments were replicated three times.

2.5 Scanning electron microscopy assays

Exponential phase bacteria were treated with absolute alcohol (negative control), polymyxin B, baicalein and polymyxin B accompanied with baicalein at 37°C for 4 h, respectively. Morphological changes of bacteria were examined by SEM (Hitachi, Japan).

2.6 Transcriptomics analysis and reverse transcription–quantitative PCR

Bacteria were cultured in CAMHB until it reached the early stage of exponential phase. Bacterial samples were disposed by polymyxin B (1 µg/mL), baicalein (80 µg/mL) or polymyxin B (1 µg/mL) coupled with baicalein (80 µg/mL) for 4h, then the total RNA was extracted by Trizol reagent (Thermo Fisher Science, Carlsbad, Canada). After the quality of extracted RNA had been evaluated by the ultraviolet-visible spectrophotometer (Metash Instruments, Shanghai, China), the transcriptome library was constructed according to the instruction of TruSeq RNA sample preparation Kit (San Diego, CA). After the library was qualified and optimized to achieve standards, paired-end libraries were sequenced by Illumina NovaSeq in Biozeron Biotechnology Co., Ltd (Shanghai, China). The distribution of error rate of the original sequencing outcomes was calculated and the sequencing data was filtered to qualify data. Bowtie2 was utilized to locate the filtered sequence according to the reference genome (*E. Coli* K-12) and HTSeq software was used to analyze the gene expression level of each sample. The differentiated expression genes were analyzed according to the FPKM (fragments per kilobase of exon per million fragments mapped) value.

The protocol of RT-qPCR was described previously [27]. Briefly, the total RNA was extracted by Trizol reagent and reversely transcribed into cDNA. All specific primers (Table 1) were designed according to the genome sequence of *E. coli* GW-AmxH19 (NCBI reference sequence: GCF_010706435.1). Real-time fluorescence PCR based on the SYBR Green I method was performed to evaluate the expression of specific genes following the instructions of the full gold quantitative PCR kit. 16S rRNA was used as a housekeeping gene to standardize the expression levels, and the data of mRNA relative expression were analyzed by the $2^{-\Delta\Delta Ct}$ method.

Table 1
Primer sequences for RT-qPCR

Gene	Forward primer	Reverse primer
16S rRNA	CGGTGGAGCATGTGGTTT	AGCCATGCAGCACCTGTCT
<i>rfaQ</i>	TGGCGTAATAGTTTCACTCATTTG	ACTCATGGTTGTCTGCTTCAC
<i>rfaP</i>	ACCACCGTGACTGTTATATCTGT	GGGGCGACAAAATACACTTTCA
<i>evgA</i>	AACGGTATCCAGGTGTTAGAA	TTACTCACGAAACCATTAGCG
<i>argT</i>	TCTGGATGCTGCGTTACAAG	CACCGTAGACATTAAAGTCGAAATA
<i>ptsI</i>	GTTGACCGTGGTAATGATATGATT	TGCCAGTCCATTTGCCTTC
<i>fimA</i>	ACATTTAGTTCAGAAACAACCCT	TCGCATCCGCATTAGCAG
<i>yehE</i>	TTGTGGTCAGTTAACGTATAATGT	CGTTTGCTATCGCTATCTGC
<i>mgrB</i>	GATGGGTCGCTCTGGTTG	AAACTGGTTAATGGCACAA
<i>arcA</i>	CCTCGAAATCGGTGCAGATG	CGGCTGTTGATGTCCAGTTC

2.7 Metabolomics analysis

Bacteria samples were treated under the same condition as transcriptomics analysis. Then, the sample preparation for metabolomics UHPLC-MS/MS analysis was performed as previously described [28]. 1 mL of bacterial samples were freeze-dried and resuspended with pre-chilled 80% methanol and 0.1% formic acid by well vortex. Then the samples were incubated on ice for 5 min and centrifuged at 15000 g, 4°C for 15 min. Supernatants were diluted to a final concentration containing 53% methanol by LC-MS grade water, followed by 15 min centrifugation at 15000 g, 4°C.

UHPLC-MS/MS analysis was performed by a Vanquish UHPLC system (Thermo Fisher, Germany) coupled with an Orbitrap Q Exactive TMHF-X mass spectrometer (Thermo Fisher, Germany) in Biozeron Biotechnology Co., Ltd. (Shanghai, China). Q ExactiveTMHF-X mass spectrometer was operated in positive/negative polarity mode with a spray voltage of 3.2 kV, capillary temperature of 320°C.

The raw data were processed by the Compound Discoverer 3.1 (Thermo Fisher) to perform peak alignment, peak picking and quantitation for each metabolite. After that, peak intensities were normalized to the total spectral intensity. The normalized data were used to predict the molecular formula based on additive ions, molecular ion peaks, and fragment ions. Then peaks were matched with the mzCloud (<https://www.mzcloud.org/>) database to obtain accurate qualitative and relative quantitative results. Differential metabolites were screened out if OPLS-DA_VIP ≥ 1 , fold change ≥ 2 , and P 0.05. Ultimately, metabolic pathways were annotated based on KEGG database by MetaboAnalyst 5.0 online software.

2.8 Mice peritoneal infection model

Male ICR mice aged 6–8 weeks and weighing ≈ 20 g from Animal Experiment Centre of Anhui Medical University were used in this study. Mice were adapted to standardized environmental conditions (temperature = $23^{\circ}\text{C} \pm 2^{\circ}\text{C}$; humidity = $55\% \pm 10\%$) for one week before infection in Anhui Agricultural University to minimize potential confounders. Mice were adapted to standardized environmental conditions for one week and fasted for 12 h before the infection. According to the minimum lethal dose (MLD), each group ($n = 10$) was injected intraperitoneally with 0.1 mL bacterial suspensions containing 1.5×10^8 CFU mL^{-1} HP144.

2.8.1 Mice survival experiments

At 1 h post infection, mice were treated with 0.9% saline (negative control), polymyxin B (1 $\mu\text{g}/\text{ml}$), baicalein (80 $\mu\text{g}/\text{ml}$) and polymyxin B (2 $\mu\text{g}/\text{ml}$) coupled with baicalein (80 $\mu\text{g}/\text{ml}$) by intraperitoneal injection, respectively. The survival rate and physiological states of the mice were observed and recorded.

2.8.2 Bacterial loads assays

At 120 h post treatment, mice were euthanized by acute blood loss after anesthesia. 2 mL of saline was injected into the abdomen followed by the extraction of 1 mL ascites. Extracted ascites was diluted in tenfold and 100 μL bacterial suspensions were inoculated on MacConkey medium plates to count the bacterial loads in mice.

2.8.3 Histology and immune assays

Visceral organ were collected from euthanized mice and preserved in 4% paraformaldehyde. Slices of organs were stained by hematoxylin and eosin (H&E) and examined by microscope. For the immunoprecipitation assay, extracted serum was thawed after centrifugation and incubated with Mouse IL-1 β Elisa Kit, Mouse IL-6 β Elisa Kit and Mouse TNF- α Elisa Kit (Shanghai, China) according to the manual instruction. The results were observed by an enzyme marker and recorded.

2.9 Statistical data analysis

GraphPad Prism 9 (GraphPad Software, Inc., San Diego, CA, USA) was used for statistical analysis. The values reported were shown as mean $\pm$ standard deviation. For *in vitro* assays, P values were calculated using an unpaired t-test between two groups (normally distributed data) or a one-way ANOVA between multiple groups. For *in vivo* assays, P values were determined by a log-rank test. Results with p values of < 0.05 were considered significant.

3 Results

3.1 Synergistic effect of baicalein and polymyxin B against HP144

The MIC of polymyxin B and baicalein is 4 µg/mL and 320µg/mL, respectively, verifying that HP144 exhibits an obvious resistance to polymyxin B and other drugs as *mcr-1* positive *E. coli*. To evaluate the synergistic effect of baicalein and polymyxin B, the FICI was calculated by checkerboard dilution assays. A fourfold decrease of polymyxin B resistance was observed after the combination treatment of baicalein, illustrating a remarkable synergistic effect (Fig. 1A).

In addition, the time-killing curves during different growth state were established to evaluate the synergistic efficacy more thoroughly. Neither baicalein nor polymyxin B could inhibit bacterial growth completely, while a significantly decreased amount of bacterial loads (8 logs) were observed in the combined group (Fig. 1B). As previous studies have authenticated, a synergistic effect could be defined when bacterial loads decrease more than 2 logs between a single drug and the combination group.

3.2 Baicalein increases the destruction of bacterial outer membranes caused by polymyxin B

In order to discuss how baicalein facilitate the antibacterial activity of polymyxin B, SEM was applied to distinguish the alteration of cellular structure when *E. coli* HP144 were administered by polymyxin B, baicalein or both (Fig. 1C). SEM images demonstrated that baicalein has no influence on the outer membrane, and polymyxin B could cause inconspicuous damage such as mildly irregular protrusion and slight depression. However, extremely remarkable morphology change and membrane disruption were observed in the drug-combined group. Hence, we speculated that baicalein could be unitized as an efficacious adjuvant through increasing the membrane interference capacity of polymyxin B.

3.3 Transcriptomics analysis revealed obvious changes in gene expressive levels which were enriched in multiple pathways

To further elucidate the molecular mechanism of synergistic effects, gene expression levels were evaluated by transcriptome analysis. Compared with baicalein treatment, 88 differentially expressed genes were identified when HP144 were disposed of by baicalein and Polymyxin B, including 2 up-regulated genes and 84 down-regulated genes (Fig. 2A). Meanwhile, a total number of 2249 differentially expressed genes were identified between polymyxin B and the drug-combined group, including 817 up-regulated genes and 1432 down-regulated genes (Fig. 2B).

Subsequently, Gene Ontology (GO) was performed to annotate differentially expressed genes into biological functional pathways. As shown in Fig. 2C, differentially expressed genes between polymyxin B and the combined group were primarily enriched in 18 pathways, including biological processes (biological regulation, cellular processes, and metabolic processes), cellular components (cellular anatomical entity, intracellular components and protein-containing complex) and molecular function (binding, catalytic activity and transporter activity). Furthermore, Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis was applied to annotate these differentially expressed genes into 142 pathways, which are chiefly correlated to ABC transporters, two – component system, LPS

biosynthesis and bacterial metabolism related pathways (Fig. 2D). It is notable that core genes related to LPS and flagella synthesis were significantly down-regulated in the combination group. Since LPS is a major component of cell walls in gram-negative bacteria, we speculated that the synergistic effect of polymyxin B and baicalein could inhibit LPS synthesis, thus destroying the outer membrane of MDR bacteria and increasing drug permeability.

Furthermore, genes relevant to the bacterial structure and antibiotic resistance in *E. coli* were scanned out among the differentially expressed genes, and a significant variation of expression level in several genes were observed, including *rfaQ*, *rfbB*, *rfaP*, *yehE*, *evgA*, *arcA*, *ptsI*, *mgrB*, *ybtX*, *fimA*, *gltI*, *argT* and others (Table 2). To validate alterations of the above identified genes, RT-qPCR were performed for nine selected genes. Consistent with outcomes of transcriptome analysis, genes in combined group that participate in the formation of antibiotic resistance were significantly down-regulated following an analogous pattern, illustrating that the synergistic effect of baicalein efficiently enhanced the therapeutic efficacy of polymyxin B against MDR bacterial infections (Fig. 3).

Table 2
Polymyxin resistance-associated differentially expressed genes (in part)

Gene	Product function	log ₂ FC
<i>rfaQ</i>	lipopolysaccharide core heptosyltransferase	-15.14
<i>rfaP</i>	lipopolysaccharide core heptose (I) kinase	-14.86
<i>evgA</i>	acid-sensing system DNA-binding response regulator	-2.08
<i>argT</i>	lysine/arginine/ornithine ABC transporter substrate-binding protein ArgT	-3.57
<i>ptsI</i>	phosphoenolpyruvate-protein phosphotransferase PtsI	-1.58
<i>fimA</i>	metal ABC transporter substrate-binding lipoprotein/fibrin-binding adhesin	-11.67
<i>yehE</i>	family protein	-2.59
<i>mgrB</i>	PhoP/PhoQ regulator	-1.43
<i>arcA</i>	two-component system response regulator	-1.87

3.5 Metabolomics analysis revealed obvious changes in metabolites levels which were enriched in multiple pathways

Discriminant metabolites were identified by metabolomics and uploaded to KEGG ontology for functional analysis. There were 61 discriminant metabolites between baicalein and drug-combined group, containing 32 up-regulated genes and 29 down-regulated genes. As shown in Fig. 4A, these metabolites were chiefly enriched in riboflavin metabolism, carbon metabolism, oxidative phosphorylation and part of secondary metabolites synthesis. On the other hand, 201 altered metabolites were found out from

polymyxin B and drug combined group, including 99 up-regulated 102 down-regulated metabolites. KEGG analysis annotated monobactam biosynthesis, cholesterol metabolism, AMPK (Adenosine 5'-monophosphate activated protein kinase) signaling pathway, primary bile acid biosynthesis and secondary bile acid biosynthesis as the most abundant functional pathways (Fig. 4B).

Furthermore, changes of certain metabolites relevant to antibiotics resistance from the comparison of polymyxin B and drug combined group were available in Table 3. NAD⁺ is considered as an essential coenzyme for multiple biochemical and physiological processes, thus the stable level of NAD⁺ works crucially for cell homeostasis [29]. Meanwhile, it also functions as a signaling molecule in regulating inflammation, immune responses, cell division, energy metabolism, DNA repair and stress resistance, which are intimately correlated to pathogenic infection and host cell survival [30, 31]. The log₂FC value of NAD⁺ in the combination group decreased in a remarkable trend, while the level of D-alanyl-D-alanine dipeptide (D-Ala-D-Ala) increased significantly. D-Ala-D-Ala is an essential component of the intracellular peptidoglycan precursor, which is involved in the cross-link of peptidoglycan chains to maintain the integrity of bacterial cell walls and promote the survival of pathogens [32, 33]. In conclusion, all these results demonstrated that baicalein might affect various vital metabolism pathways to exert its adjuvant effect on polymyxin B.

Table 3
Changes of some discriminant metabolites between Polyxmyxin B and drug combined group.

Name	Log ₂ FC	VIP	Trend
NAD ⁺	-30.68	1.44235	Down
D-Alanyl-Dalanine	33.54	1.44235	Up
Adenosine 5'-monophosphate	-1.9	1.44235	Down

3.5 Baicalein enhances the therapeutic efficacy of polymyxin B in mice infection model

Since baicalein and polymyxin B could exhibit a significantly synergistic effect against antibiotic-resistant bacteria *in vitro*, we supposed whether baicalein could reverse polymyxin B resistance *in vivo*. Mice infection model was established by peritoneal injection of *mcr-1* postive *E. coli*. One day post injection, the natural mortality rate of mice is more than 50% and infection symptoms such as inflammation, organ damage and positive blood culture were observed, confirming the successful construction of mice infection model. As shown in Fig. 5A, all the mice died within 40 hours in control and polymyxin B. Meanwhile, 40% and 70% survival rate were observed in baicalein and drug combined group, respectively. Furthermore, a significant decrease of bacterial loads was observed in the drug combined group (Fig. 5B).

Moreover, H&E-stained slices of duodenum and spleen stained were scanned to evaluate therapeutic efficacy. As shown in Fig. 5C, the margin zone between the red and white pulp of infected mice spleen was indistinct. An obvious deterioration was also observed in the duodenum, including disintegrated intestinal villi, massive cellulosic exudation, inflammatory cell infiltration and the invasion of red blood cells into vessels. However, after co-treatments of polymyxin B and baicalein, spleen and duodenum of euthanized mice retained an integrity and orderly structure without no destruction and hemorrhage. Finally, the levels of inflammatory factors in extracted serum were detected by enzyme-linked immunosorbent assays (ELISA). Compared with the negative control and single drug groups, an obvious alleviated levels of IL-1B, IL-6B and TNF- α could be observed in the co-treatment group (Figs. 5D-F). Collectively, all these results corroborated that baicalein has a prominent capacity to restore polymyxin B resistance in mice, thus mitigating inflammation of the infected host and improving the survival rate.

4 Discussion

The increasing emergence of MDR Gram-negative bacteria has imposed enormous threats to human health and livestock husbandry in modern society. Since novel antibiotics development and drug repurposing lag so far behind the prompt variation and dissemination of antibiotic resistant genes, particularly the plasmid-mediated polymyxin resistant family *mcr*, there is an urgent need to identify new antibiotic targets or efficacious adjuvants of existing antibiotics [34, 35]. Previous reports have verified that polymyxin B could collaborate with traditional Chinese medicines to inhibit the growth of *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* [36–39]. Baicalein, which is identified as a flavonoid derivative from *Scutellaria baicalensis* has been successfully applied in clinic due to its excellent properties of antipyretic, anti-tumor and anti-inflammatory [18, 40]. Another investigation have reported that baicalein could effectively alleviate the damage of Shiga toxin secreted by *Escherichia coli* Shiga to Vero cells and improve cell activity in enterhemorrhagic patients [20]. Moreover, baicalein-loaded nanoparticles have the capacity to enhance elimination of *S. aureus* biofilm both *in vivo* and *in vitro* [41]. Nevertheless, the synergetic effect and correlated mechanism of baicalein with polymyxin B still remain obscure. In this paper, we verified that baicalein could efficiently restore polymyxin B efficacy against *mcr-1* positive *E. coli* by enhancing the outer membrane destruction capacity of polymyxin B. Furthermore, when infected mice were co-treated by baicalein and polymyxin B, bacterial infections were significantly alleviated, including reduced inflammatory and toxin factors, mitigation of organic dysfunctions, and elevated body recovery. The final survival rate of infected mice were increased by 70% compared with only the polymyxin B treatment.

In order to explore the unique molecular mechanism of prokaryotes, transcriptome and metabolome technology has become a potent tool in the exploration of prokaryotic molecular mechanism during past ten years. For instance, in *Salmonella* serotype C1, after mutations of two homologous genes that required for flagella motility and cell invasion, an oblivious down-regulation were observed in lipopolysaccharide core synthesis-related genes in double mutants by transcriptome analysis, including *rfaB*, *rfaI*, *rfaQ*, *rfaY*, *rfaK* and *rfaZ* [42]. The comparative transcriptome analysis also successfully identified a significantly altered expression of the two-component system EvgS/EvgA in colistin resistant

E. coli with a substitution of PmrB amino acid [43]. Moreover, gut metabolite difference between intrauterine infected patients and healthy women were evaluated by metabolome analysis, and N-formyl-L-methionine was identified as the most discriminant metabolite. After the treatment of antibiotics, alterations in cholesterol metabolism, arginine biosynthesis and bile secretion pathways were observed [44].

Herein, the transcriptome analysis was performed for *mcr-1 positive E. coli* after been cultured with polymyxin B or polymyxin B plus baicalein and over 2000 differentially expressed genes were identified. It is notable that we observed a remarkable variation in the expression of genes related to LPS synthesis, ABC transporter and two-component system, which have been reported as critical factors responsible for bacterial antibiotic resistance. Besides, results of RT-qPCR further validated the expression alterations of the above genes, illuminating that baicalein might participate in such functional pathways to exert the synergistic effect.

Bile acids can regulate the metabolism of lipids, glucose, and energy expenditure [45]. Our metabolome analysis detected the enrichment of the primary bile acid biosynthesis and secondary bile acid biosynthesis pathways after the co-treatment of polymyxin B and baicalein. Since the metabolite levels of antibiotic targets such as NAD⁺ and D-Alanyl-Dalanine dipeptide significantly changed as well, we speculated that baicalein could act on the above pathways and targets to enhance the antimicrobial efficacy of Polymyxin B. Apparently, more exhaustive researches are required to elaborate the synergy mechanism of polymyxin B and baicalein from the alteration of transcriptional and metabolic products.

5 Conclusion

Baicalein effectively enhanced the interference of polymyxin B on the outer membrane of *mcr-1 positive E. coli* to change the permeability of membranes, thus resulting in an increased intracellular drug absorption and antibiotics sensitivity. Transcriptome results and RT-qPCR further affirmed our speculation, as the expression of genes related to LPS core synthesis was significantly down-regulated. The synergy of baicalein and polymyxin B efficaciously inhibit bacterial growth and inflammatory factors to ameliorate clinical symptoms and increase survival of the infected mice. To sum up, as a common Chinese herbal medicine, baicalein could be applied as a feasible adjuvant of polymyxin B to counteract MCRPEC infection.

Declarations

Ethics approval and consent to participate

All animal experiments were performed in strict accordance with the relevant Chinese laws and were approved by Anhui Agriculture University Committee on Animal Care and Use. The experimental animal license number is SYXK (Anhui) 2016-007, certified by the Department of Science and Technology of Anhui Province.

Data Availability Statement

The datasets used and/or analyzed during the current study are available to the corresponding author on request.

Competing interests

The authors declare that they have no competing interests.

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Author Contributions

LL and LW: Conceptualization, data curation, methodology and original draft. ZL and YG: revising it critically for important intellectual content. QJ and LF: analysis and interpretation of data. FS: revise figures and tables. SQ: Supervision, conceptualization and funding acquisition. All authors have read and approved of the manuscript.

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Figures

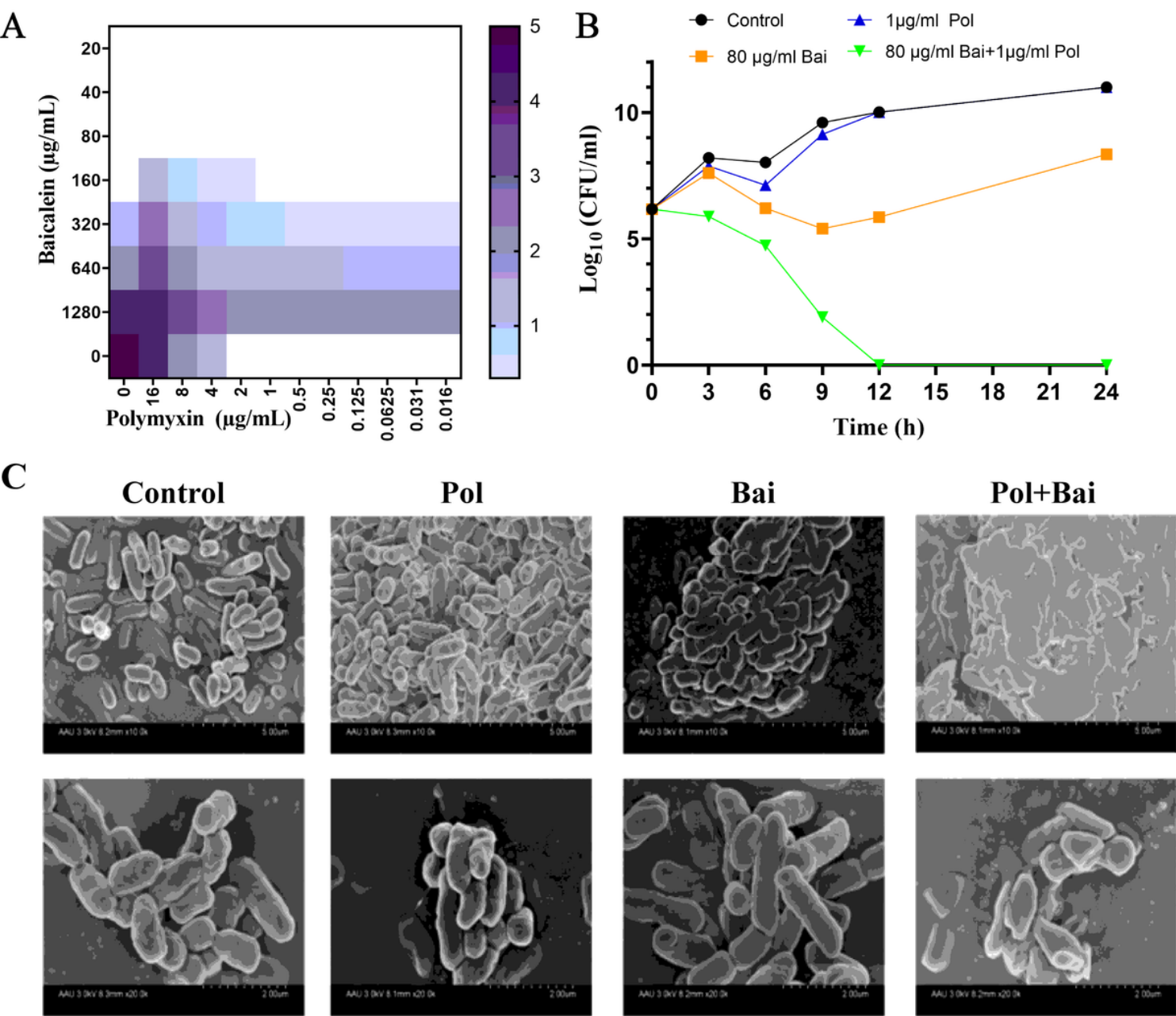


Figure 1

Synergistic effect of polymyxin B and baicalein against *mcr-1* positive *E. coli*. (A) MICs and FICI of polymyxin B and baicalein. (B) Time-killing curves under different therapeutic conditions. (C) Changes in cell membrane morphology of *Escherichia coli* treated with sub-MIC concentrations of polymyxin B and baicalein. Pol: Polymyxin B; Bai: baicalein.

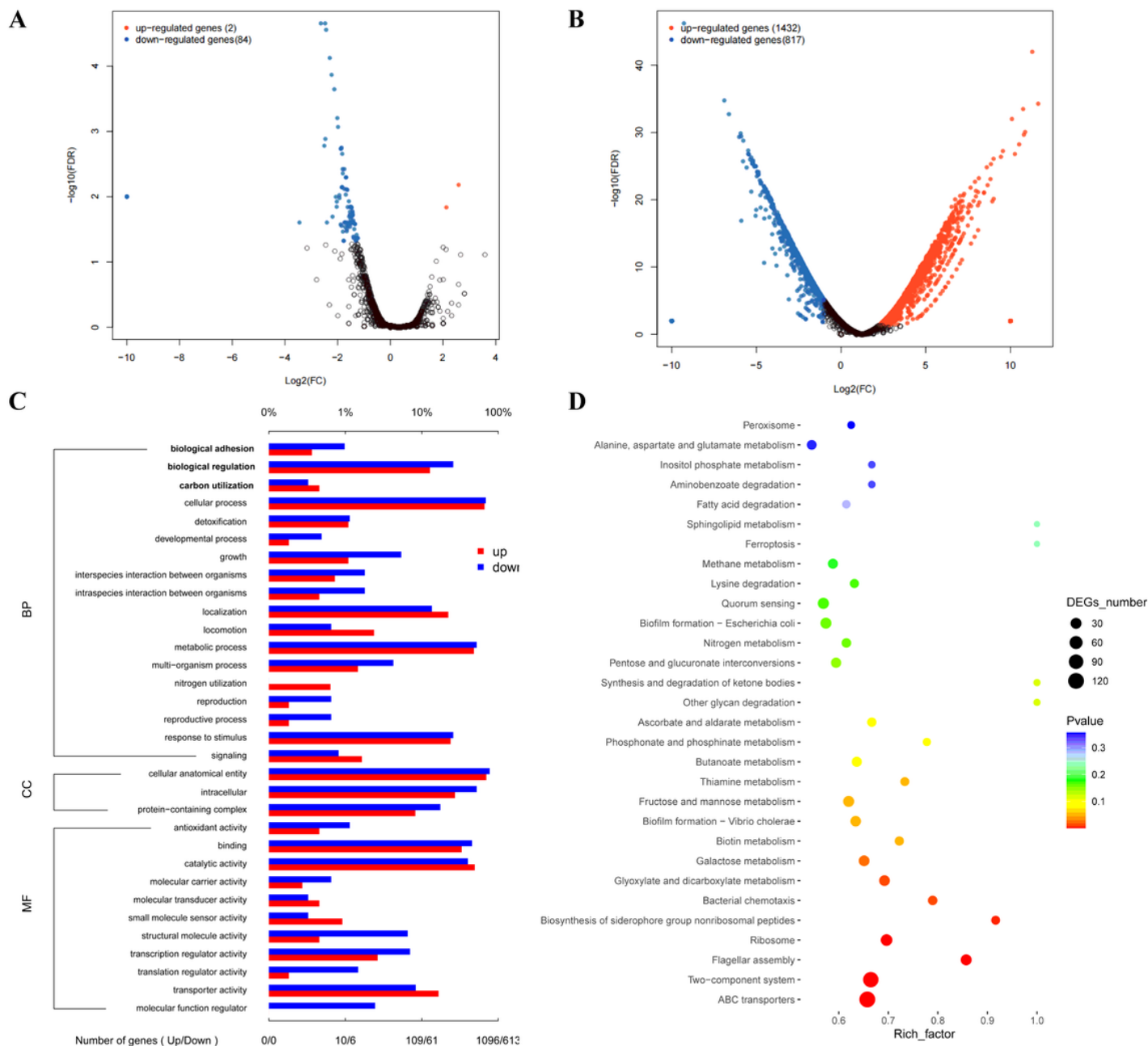


Figure 2

Transcriptomics analysis of *mcr-1* positive *Escherichia coli* treated by different drugs. (A) The volcano map of differentially expressed genes between baicalein and the combined group. (B) The volcano map of differentially expressed genes between polymyxin B and the combined group. (C) Enrichment of differentially expressed genes annotated by GO pathway (polymyxin B/polymyxin B plus baicalein). BP:

biological processes; CC: cellular components; MF: molecular function (D) Enrichment of differentially expressed genes by KEGG pathway (polymyxin B/polymyxin B plus baicalein).

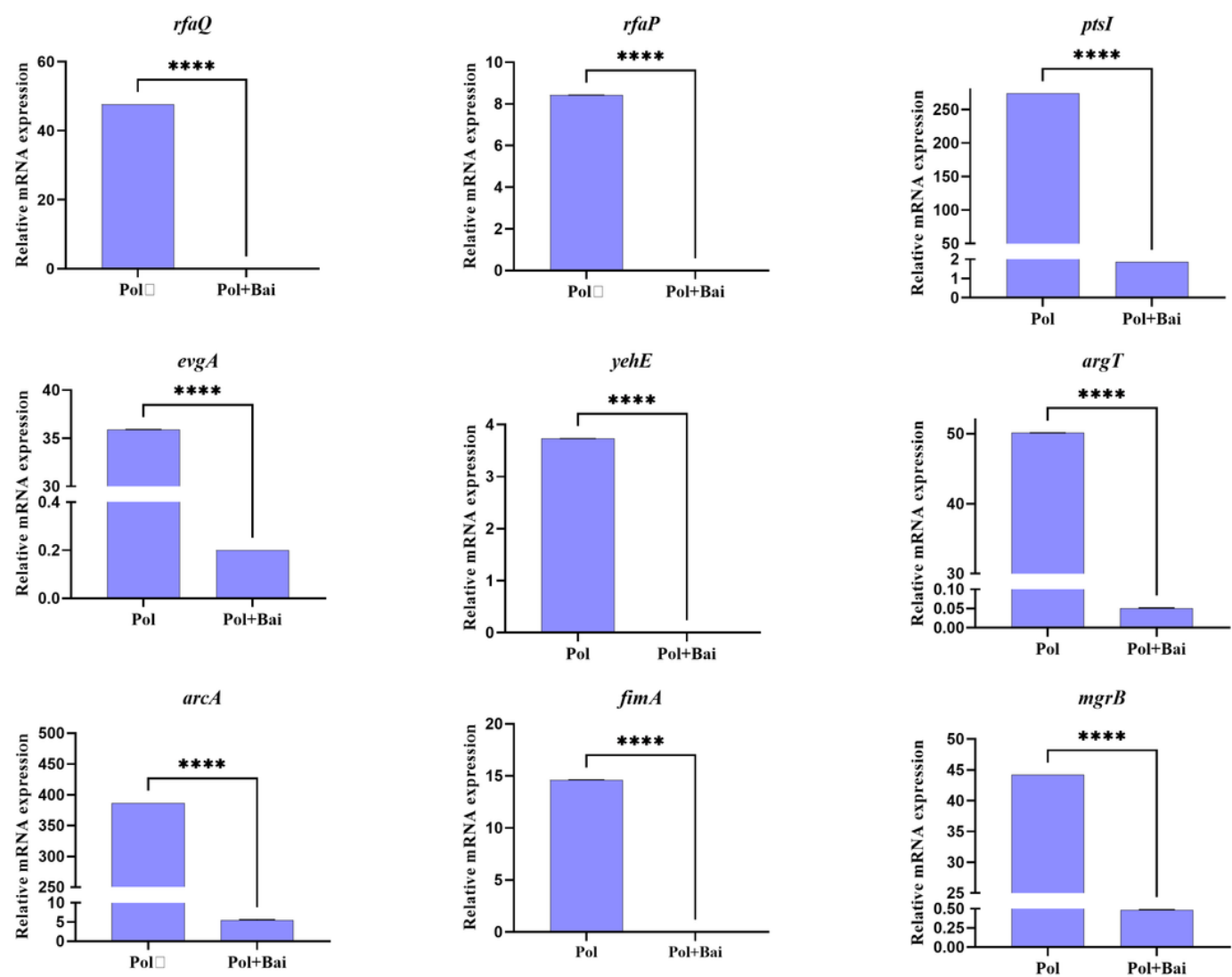


Figure 3

RT-qPCR validated alterations in the expression of genes related to bacterial structure and antibiotic resistance. Pol: Polymyxin B; Bai: baicalein.

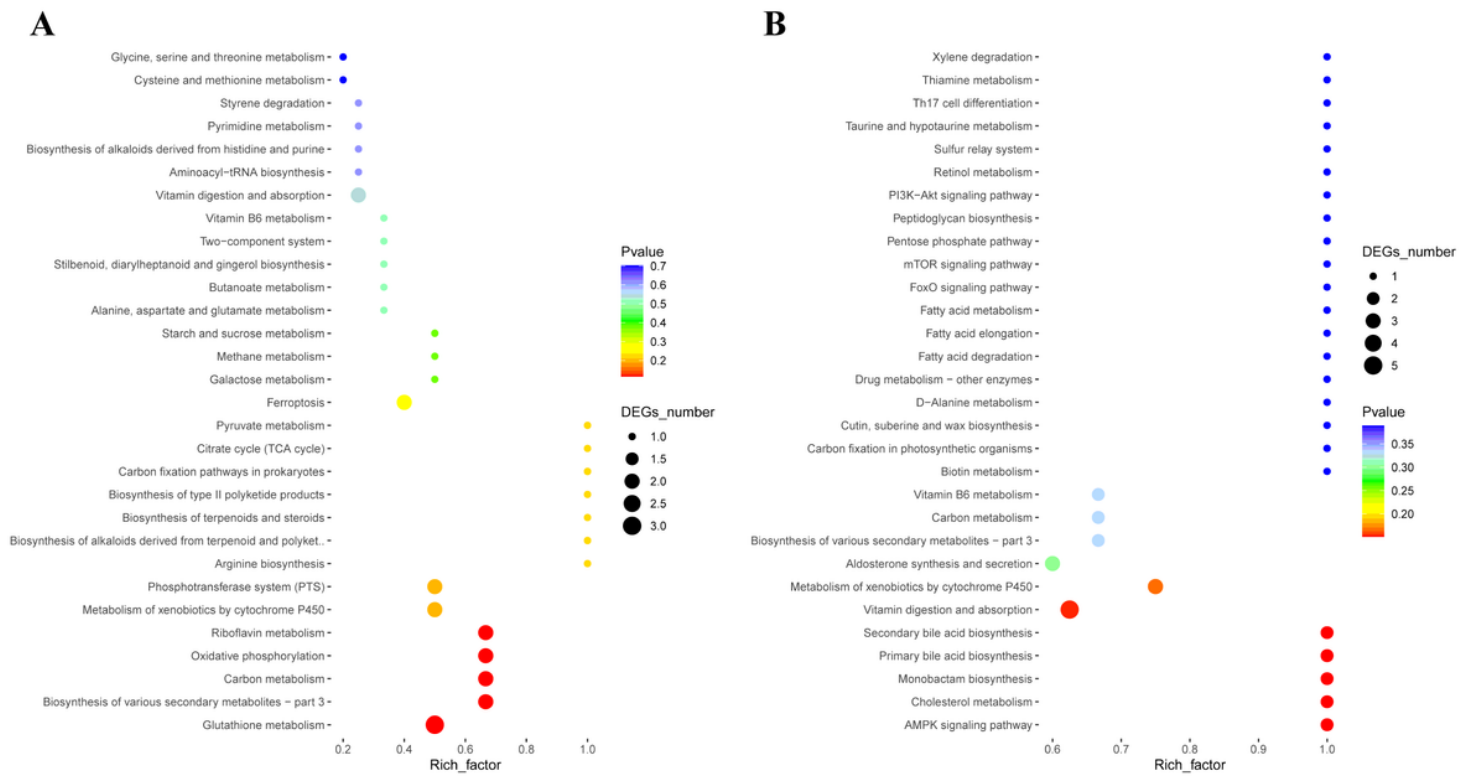


Figure 4

KEGG annotation based on metabolome analysis. (A) Baicalein versus polymyxin B + baicalein. (B) Polymyxin B versus polymyxin B + baicalein.

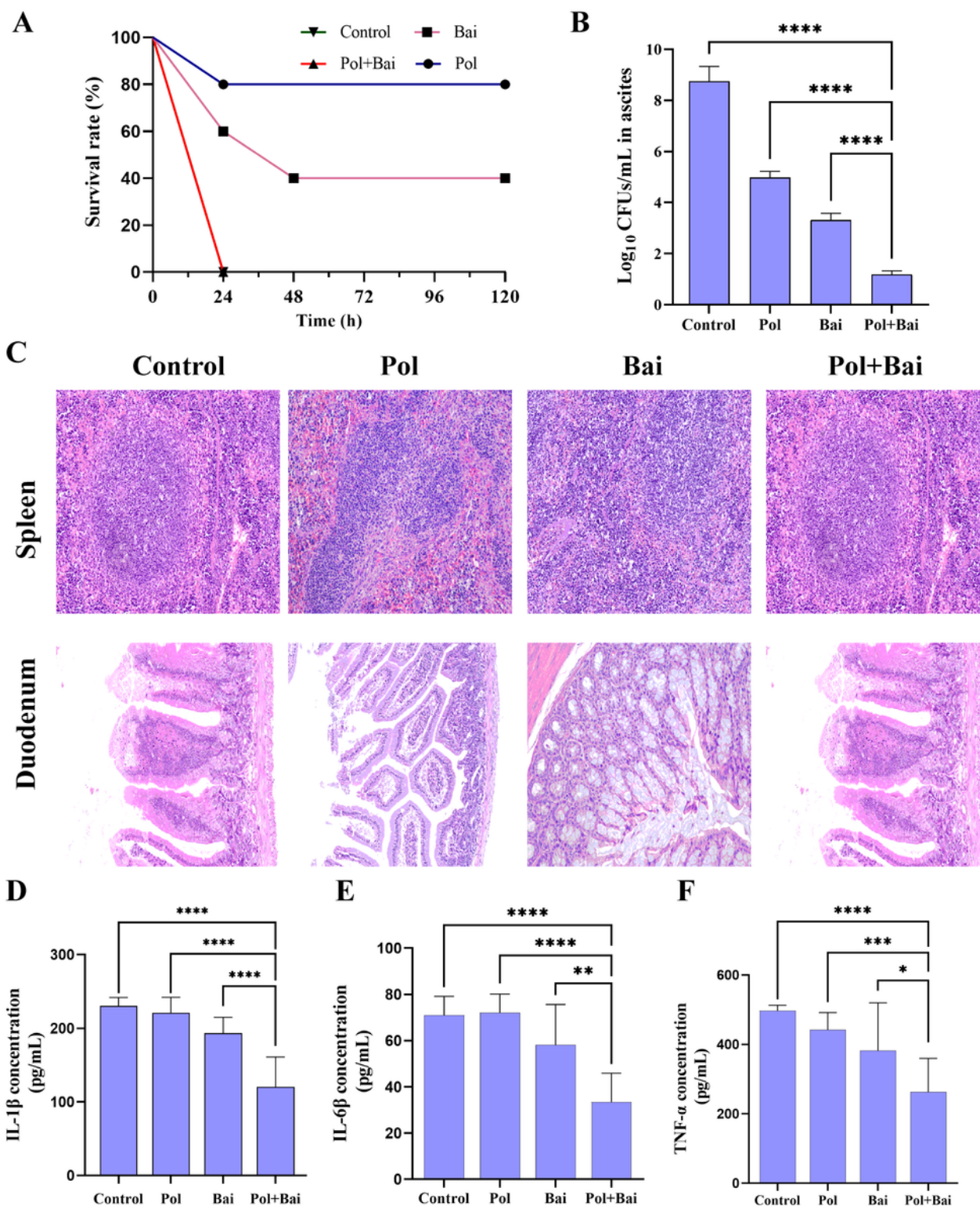


Figure 5

Baicalein reverses antibacterial activity of polymyxin B in the mice infection model. (A) The co-treatment of polymyxin B and baicalein significantly increased the survival rate of infected mice. (B) The combined drug significantly reduced bacterial loads in the ascites of infected mice. (C) Pathological slices of spleen and duodenum from infected mice under different therapeutic regimes. (D), (E) and (F) The co-treatment of polymyxin B and baicalein obviously alleviated the inflammatory reactions in infected mice