Morin inhibits *Listeria monocytogenes* virulence *in vivo* and *in vitro* by targeting listeriolysin O and inflammation

Gen Li
Jilin University
Guizhen Wang
Jilin University
Meng Li
Jilin University
Li Li
Jilin University
Hongtao Liu
Jilin University
Meiyang Sun
Jilin Provincial Cancer Hospital
Zhongmei Wen (wenzhongmei2019@163.com)
Jilin University First Hospital

Research article

Keywords: Listeria monocytogenes, listeriolysin O, morin, inflammation, anti-infection

Posted Date: April 17th, 2020

DOI: https://doi.org/10.21203/rs.2.21184/v2

Version of Record: A version of this preprint was published at BMC Microbiology on May 12th, 2020. See the published version at https://doi.org/10.1186/s12866-020-01807-6.

Abstract

Background: Listeria monocytogenes (L. monocytogenes) is a global opportunistic intracellular pathogen that can cause many diseases, including meningitis and abortion in humans and animals; thus, L. monocytogenes poses a great threat to public safety and the development of the aquaculture industry. The pore-forming toxin listeriolysin O (LLO) is one of the most important virulence factors of L. monocytogenes ; LLO can promote cytosolic bacterial proliferation and aid in evading the attacks of the host immune system. In addition, L. monocytogenes infection can trigger a series of severe inflammatory reactions.

Results: Here, on the basis of our previous studies, we further confirmed that morin lacking anti-Listeria activity could inhibit LLO oligomerization and found that morin could effectively alleviate the inflammation induced by Listeria in vivo and in vitro and has an obvious protective effect on infected cells and mice.

Conclusions: Thus, we further prove that morin is a valuable drug precursor to be developed for the treatment of Listeria infection.

Background

Listeria monocytogenes (*L. monocytogenes*) is a ubiquitous gram-positive intracellular pathogen that frequently grows under the conditions used for food preservation and is regarded as the etiologic agent of a series of severe diseases, including meningitis, sepsis, and even life-threatening infections, in both humans and animals. *L. monocytogenes* poses a particularly serious threat to immunocompromised individuals, such as the elderly and pregnant women [1, 2]. The ingestion of food contaminated with *L. monocytogenes* is the main method of infection, and thus, this pathogen poses a significant threat and severe challenge to the food chain and the food production industry [3]. A high mortality rate is an important manifestation of *L. monocytogenes* infections, and it has been reported that nearly 19% of annual deaths induced by foodborne infections are caused by *L. monocytogenes*. In fact, in an outbreak in 2011, hundreds of people were infected, and dozens died from the infection [4, 5], which suggests that *L. monocytogenes* is an important public health concern.

The successful establishment of infection is an important step in the process through which pathogenic bacteria evade the host immune system, and a series of complex virulence factors are crucial weapons carried by bacteria to exert virulence at each stage of the infection [6]. Multiple virulence factors are secreted during the intracellular lifecycle of *L. monocytogenes* to achieve colonization and infection in the host. Listeriolysin O (LLO), which is encoded by the *hly* gene, is one of the most important virulence factors of *L. monocytogenes*; this factor plays a central role in the process of bacterial escape from the phagosome [7] and belongs to the cholesterol-dependent cytolysin (CDC) family, which includes suilysin [8] and pneumolysin [9]. The pore-forming toxin is also a crucial factor involved in the cell-to-cell spread of *L. monocytogenes*; the virulence and pathogenicity of *L. monocytogenes* in which *hly* is mutated to

inhibit its hemolytic activity were almost lost in mouse infection models [10, 11]. In addition, LLO can induce a variety of apoptosis and cytotoxicity pathways [12-14], which suggests that LLO is an important and effective drug target for the treatment of *L. monocytogenes* infection.

L. monocytogenes infection can induce a variety of inflammatory reactions, including encephalitis, osteomyelitis, and peritonitis [15-17], and activates the assembly of AIM2, NLRC4, and NLRP3 inflammasomes, which are multiprotein complexes that induce activation of the proinflammatory cysteine protease caspase-1 during the disease course as well as the secretion of important pro-inflammatory cytokines, such as IL-1β, IL-6, and TNF-α [18-20]. These excessive inflammatory reactions cause great harm to the body, particularly immunocompromised populations [21, 22]. The severe inflammation induced by *L. monocytogenes* is also a crucial part of the disease process. Therefore, effective alleviation of the inflammatory response might be an important strategy for the treatment of *L. monocytogenes* infection.

The natural ingredient morin, which can be found in various fruits and vegetables, is a safe, edible flavonoid with multiple biological activities, including the induction of apoptosis, the killing of tumor cells to fight cancer, and antioxidant activities [23-25]. In addition, morin inhibits the LPS-induced inflammatory response [26], and molecular simulations in our previous study showed that morin can effectively inhibit the pore-forming activity of purified LLO by directly binding to the protein at very low doses [27], which suggesting that morin is a potential drug precursor, particularly in the fight against *L. monocytogenes* infection. However, previous studies have only remained at the level of molecular mimicry, and no in-depth studies have been performed. Thus, further *in vivo* and *in vitro* studies are still needed to confirm the effect of morin in the treatment of *L. monocytogenes* infection and to provide a preliminary theoretical basis for further drug development of the compound.

Results

Morin has no influence on *L. monocytogenes* growth

Morin (Figure 1A) is a kind of natural flavonoids and we found that the minimum inhibitory concentration of morin against L95 cells was more than 102.4 μg/mL. Furthermore, the addition of morin at various concentrations, including at the effective concentration (32 μg/mL) at which it functions, induced no obvious difference in the growth of L95 cells (Figure 1B). Moreover, morin (16 μg/mL) clearly suppressed the function of lysing red blood cells in the bacterial culture (Figure 1C), which is consistent with our previous finding that morin can directly bind to LLO *in vitro* [27]. However, in addition to direct binding, reducing the expression of LLO in the supernatant of the bacterial culture might also weaken the hemolytic activity. Therefore, we further tested the expression of LLO in the supernatant of bacterial cultures treated with different concentrations of morin and found that morin treatment did not interfere with the secretion of LLO (Figure 1D). In summary, morin does not affect the growth of *Listeria*, which suggests that the use of morin for the treatment of *Listeria* infection would not impose too much pressure on the survival of the bacteria.

Morin inhibits the oligomerization of LLO

Our previous studies have shown that morin can bind directly to LLO to inhibit its hemolytic activity [27]. Here, we further demonstrated that the binding of morin and LLO effectively inhibited the formation of LLO oligomers and did not induce changes in the total protein in the various groups (Figure 2A and B). The purified LLO protein was induced to oligomerize *in vitro*. The addition of morin into the reaction system caused the oligomeric bands to become narrow, significantly decreased the number of oligomers and reduced the hemolytic activity of LLO (Figure 2C).

Morin inhibits the secretion of inflammatory mediators in cells cultured with *L. monocytogenes*

All of the above results suggest that morin might play an inhibitory role in *Listeria* infection; therefore, we further studied the function of morin in *Listeria* infection of J774 cells. The secretion of TNF-α, IL-1β, and IL-6, which are considered important inflammatory mediators, was markedly downregulated, which indicates that morin exerts a significant anti-inflammatory effect *in vitro*. In addition, the use of morin ≤32 μg/mL≥alone exerted little effect on the expression of inflammatory factors compared with that observed with untreated cells (Figure 3A-C).

Morin can effectively alleviate the damage to cells infected with *L. monocytogenes*

To further explore the role of morin in *Listeria* infection *in vitro*, we detected the LDH levels in the cell culture supernatant of J774 cells cocultured with L95 cells and different concentrations of morin, and treatment with morin also effectively reduced the release of LDH by the infected cells. In addition, morin itself exerted no cytotoxic effects on cells (Figure 4A). In line with the results of bacterial infection, morin also significantly protected against LLO-induced cell damage (Figure 4B), which further suggested that morin can play an anti-infective role by inhibiting LLO activity. Consistent with the results of the above-described studies, morin reduced the survival of intracellular bacteria but hardly affected extracellular bacteria (Figure 4C and D). These results strongly suggest that morin exerts a significant protective effect during *Listeria* infection *in vitro*.

Morin protects mice infected with *L. monocytogenes* and reduces the bacterial burden and inflammation *in vivo*

To assess the therapeutic effect of morin in *Listeria* infections *in vivo*, we established a mouse *Listeri*a infection model. Mice were injected with 1×10^7 CFU of L95 cells in the peritoneal cavity to observe the survival rate and 2×10^6 CFU of L95 cells to determine the colonization of organs and inflammation of tissue. Mice died significantly after *Listeria* infection, but after subcutaneous administration of morin, the survival rate after infection with a high concentration of L95 cells was significantly increased compared with the control, indicating that morin has a protective effect on *Listeria* infection (Figure 5A).

The *in vivo* effect of morin was further assessed by analyzing the bacterial burden in and detecting the inflammation of tissue from mice infected with L95 cells. A mouse model of *Listeria* infection was

successfully established, and colonization in the tissues (liver and spleen) was significantly decreased by morin treatment (Figure 5E and F). In accordance with the above-described results, as shown in Figure 5B,5C,and 5D treatment with morin also significantly relieved inflammation in the infected mice. Taken together, these data suggest that morin treatment can protect mice during *in vivo Listeria* infections.

Discussion

L. monocytogenes is an important opportunistic foodborne bacterium of both humans and animals and is the etiological agent of *listeriosis* and a series of fatal infections [28]. At present, the treatment of diseases related to *Listeria* infection in clinical practice mainly involves the application of antibiotics for the control or killing of pathogenic microorganisms [29]. However, an increasing number of *Listeria* strains that can survive antibiotic treatment are being discovered clinically at higher frequencies, and the problem of *Listeria* resistance is becoming more serious [30]. Over the past few decades, antibiotics have been used as the major treatment for nearly all types of bacterial infectious diseases in both humans and animals. However, the abuse, abnormal use or overuse of antibiotics has led to a series of adverse effects, particularly the acceleration of multidrug resistance in bacteria, and have caused more serious and alarming infections, which have resulted in some bacterial infections for which there are no available drugs [31]. Even more concerning, the development of new types of bacteriostatic drugs is occurring at a markedly slower rate compared with the generation of drug resistance, which has resulted in the current embarrassing and dangerous situation characterized by higher rates of bacterial resistance and pathogenicity and the availability of fewer weapons that can be used to fight against bacterial infections [32]. In recent decades, the development of a new anti-virulence strategy for the treatment of anti-bacterial infection has provided an effective and promising method for coping with the declining efficacy of traditional antibiotics [33]. Anti-virulence strategies differ from traditional antibiotics in terms of their antibacterial effects: anti-virulence strategies could involve inhibiting core elements in the bacterial lifecycle, exerting anti-infective activity and reducing bacterial pathogenicity by acting on virulence factors, and these effects would apply a relatively low survival pressure in comparison with antibiotics and are not conducive to the induction of bacterial drug resistance [32, 33]. LLO is a crucial virulence factor in the pathogenesis of *Listeria*, and the deletion of LLO can significantly inhibit bacterial escape from the phagosome and reduce the pathogenicity of the bacteria [10, 11], which suggests that LLO deletion could be regarded as an important and effective target in the therapy of *Listeria* infections. In addition, the severe inflammatory response caused by *Listeria* infection also causes harm to the infected host and particularly induces a risk for abortion in pregnant women [34], which indicates that attenuating the inflammatory response could be an important strategy for the treatment of *Listeria* infection.

At present, morin is widely used in the treatment of diseases. Only a small amount of orally administered morin can cross the blood-brain barrier to the brain, whereas morin hydrate-loaded micellar nanocarriers show a markedly improved ability to cross the blood-brain barrier and play a therapeutic role in Alzheimer's disease [35, 36], which suggests that the appropriate modification of the form of morin administered can improve its therapeutic effect on meningitis induced by *Listeria* infection. In addition, some studies have shown that morin also exerts potential therapeutic effects in the treatment of

Staphylococcus aureus and *Streptococcus suis* infections [37, 38], which suggests that morin has potential as a broad-spectrum agent for the treatment of bacterial infections. Current studies have shown that LLO is associated with inflammation induced by *Listeria* infection, but the exact role remains unclear [39-41]. Therefore, whether there is a correlation between the anti-inflammatory activity of morin and its activity of inhibiting LLO is an interesting research direction in the future

In our previous research, we found that morin can clearly suppress the hemolytic function of LLO, and molecular simulations demonstrated that the inhibitory effect was achieved by direct binding between morin and LLO to form an LLO-morin complex. The predicted binding site is the region of residues 420–470 in LLO, which is important for the interaction of morin with LLO, and the C1-C2 double bond of morin (Figure 1A) is also essential for this process [27]. In this study, we further investigated the potential protective function of morin in the treatment of *Listeria* infection and found that morin did not interfere with bacterial growth or expression of LLO protein. These findings further confirmed that the morin-mediated suppression of LLO hemolytic function is achieved by its direct action on the protein itself, which indicates that morin could be regarded as an anti-virulence agent and therapeutic candidate for *Listeria* infection. In addition, we also discovered that morin can significantly alleviate the secretion of inflammatory mediators induced by *Listeria in vitro* and in tissue cultures from the liver and spleen *in vivo*. The expression of the important proinflammatory cytokines TNF-α, IL-6, and IL-1β was reduced, and morin also exhibited effective protection against *Listeria* at the cell level *in vitro* and decreased the bacterial burden *in vivo.* The inflammatory response is a key defensive response of the host to exogenous pathogens. However, excessive inflammatory reactions induced by bacterial infection can also cause damage to the host. And our research found that morin can effectively lower the up-regulation of inflammatory factors produced by *Listeria* infection, indicating that morin can inhibit the inflammatory response induced by *Listeria* infection. These also suggests that we can further investigate whether morin will plays a role in *Listeria* infection-induced inflammatory diseases such as meningitis. In addition, a recent study have found that morin could inhibit the formation of *Listeria* biofilms [42]. However, it is interesting that a cyclic dipeptide cyclo(L-leucyl- L-prolyl) can inhibit the hemolytic activity of *Listeria* and also reduce the formation of *Listeria* biofilms [43]. Whether the morin and cyclo(L-leucyl- L-prolyl) are similar in action is an important research direction in the future, and whether LLO is related to the biofilm formation process is also an interesting question worth thinking about.

All of these findings lay theoretical foundations for further study of morin as a candidate for the challenge of *L. monocytogenes* infection. Morin can be easily obtained in large quantities from a variety of plants and is suitable for large-scale production, which provides a powerful advantage for potential drug development. However, our work has only completed some preliminary theoretical research, and a series of in-depth studies are still needed for the development of morin as a new drug that can be clinically applied for the treatment of *L. monocytogenes* infection. In particular, further studies should identify a suitable form for administration and perform effective structural modifications to further enhance the effect of the drug.

Conclusions

In this study, we demonstrated that morin exhibited no antibacterial activity against *L. monocytogenes* and did not influence the expression of LLO. However, morin significantly reduced the inflammation induced by *L. monocytogenes* infection *in vitro* and *in vivo*, alleviated cellular injury, protected infected mice and reduced the bacterial burden *in vivo*. These findings suggest that morin could be an effective and promising candidate to fight against *L. monocytogenes* infection.

Methods

Bacterial strains and reagents

L. monocytogenes strain ATCC 19115 (L95) was cultured in trypticase soy broth (TSB) with various concentrations of morin (purity ≥ 98%) that was purchased from Dalian Meilun Biotech Co., Ltd.

Anti-*L. monocytogenes* activity testing

The minimum inhibitory concentration (MIC) of morin for L95 cells was evaluated as described in a previous study [14]. In brief, L95 cells were cultured to the logarithmic growth stage in TSB, and the density was adjusted to 1×10^8 CFUs/mL. Morin was diluted continuously with TSB medium, and the bacterial culture was added to 5×10^5 CFUs/mL. The growth of bacteria in the presence of different concentrations of morin was observed every 12 h for 48 h. For the determination of bacterial growth, the absorbance at $OD_{600\ nm}$ of L95 cells cultured in TSB with the indicated concentrations of morin at 37°C was measured every 30 min for 5 h. Three independent trials were performed.

Hemolysis assay

L95 cells were cultured in TSB with various concentrations of morin (0, 2, 4, 8, and 16 μg/mL) at 37°C for 5 h. Following centrifugation (10,000 rpm, 2 min), 100 μL of the supernatant from each coculture sample was incubated with sheep erythrocytes (25 μL) and PBS (875μL) for 20 min. The system was centrifuged, and the absorption value at $OD_{543\ nm}$ of the supernatant was detected to determine the hemolytic activity; specifically, the activity of each sample was compared with that of the control sample (sheep erythrocytes treated with PBS supplemented with 2% Triton X-100), which was set as 100%. Three independent trials were performed.

The purified LLO protein was incubated with morin (0, 1, 2, 4, and 8 μg/mL), and the hemolytic activity in these samples was measured as described above. Three independent trials were performed.

Western blotting assay

L95 cells were cultured in TSB with morin at 37°C for 5 h. Following centrifugation, the supernatants of each sample were treated with Laemmli sample buffer, boiled for 10 min and separated on SDS-PAGE gels. After electrophoretic transfer, the LLO on the polyvinylidene fluoride membrane was blocked at room

temperature for more than 2 h, and the polyvinylidene fluoride membrane was incubated with a primary rabbit anti-LLO antibody (Abcam, 1:2000) (original concentration 0.12 mg/ml) for 2 h and then with a corresponding secondary antibody (Proteintech, 1:3000) (original concentration 0.5 mg/ml)for 1 h. ECL detection reagents were used to visualize the signals on the PVDF membranes with a Tanon-4200 imager. Three independent trials were performed.

The purification of LLO protein and the induction of its oligomerization were performed as previously described [44]. In brief, LLO was incubated with morin (64 μg/mL) for 20 min, and oligomerization was induced under high salt conditions *in vitro*. First, LLO protein was incubated with morin for 30 min under acidic PBS （pH 5.5）conditions, and saturated potassium chloride was then added to a concentration of 0.3 mg/mL. The mixture was incubated for another 10 min, and sheep erythrocytes were then added to the reaction system. After treatment on ice for 5 min, the reaction samples were boiled with SDS-PAGE loading buffer lacking 2-hydroxy-1-ethanethiol. Oligomerization was detected by Western blotting as described above. Three independent trials were performed.

Cell-line infections

L95 cells were cultured to the mid-logarithmic phase of growth ($OD_{600\ nm}$ of approximately 1.0), and the cells were washed with sterile PBS and then resuspended in complete DMEM (HyClone) containing no fetal bovine serum (FBS).

J774 macrophage-like cells were grown in high-glucose DMEM at 5% CO_2, and the cells were plated in Corning 96-well plates (approximately 2×10^4 cells per well) and cultured for 16 h. The J774 cells were then cocultured with L95 cells at 37°C for 5 h at an MOI (multiplicity of infection) of 8 or treated with LLO protein (12 ng per well) that was preincubated with morin (0, 2, 4, 8, and 16 μg/mL) for 30 min at 37°C for the detection of lactate dehydrogenase. The coculture supernatant was diluted continuously, and the extracellular bacteria were determined by colony counting. For the assessment of intracellular bacteria, the supernatant of the culture medium was removed and washed three times with PBS, and gentamicin (20 μg/mL) was added to kill the residual extracellular bacteria. The cells were then lysed with 0.2% saponins (sigma), and the colony count was determined by continuous dilution. Three independent trials were performed.

J774 cells grown in Corning six-well plates (1×10^6 cells per well) were infected with L95 cells in the presence of morin at an MOI of 10 at 37°C for 4 h for inflammation assays. Three independent trials were performed.

Inflammation assays

Following centrifugation (1,000 rpm, 10 min), the inflammatory mediators (IL-1β, IL-6, and TNF-α) contained in each coinfection sample were determined using IL-1β, IL-6, and TNF-α ELISA kit (BioLegend, San Diego, CA, USA). Three independent trials were performed.

Cytotoxicity assays

Following centrifugation (1,000 rpm, 10 min), the lactate dehydrogenase level in the infected cell samples was assessed using a cytotoxicity detection kit (Roche, Basel, Switzerland). Samples treated with Triton or DMEM were used as controls. Three independent trials were performed.

Animal experiments

Female BALB/c mice (6-8 weeks) were purchased from the Experimental Animal Center of Jilin University, and the animal experiments were approved by Jilin University (the First Hospital) institutional animal care committee and performed in accordance with their guidelines. All experimental animals were given sufficient food and water, provided sufficient ventilation, activity space and a good living environment. After the experiment, cervical dislocation was performed for euthanasia, and the experimental animals were not given pain during the whole process.

L95 cells were cultured to the mid-logarithmic growth stage (OD_{600}=0.8-1.0), centrifuged, collected, washed three times with sterilized PBS and resuspended to a density of 1×10^8 CFU/mL. The mice that were intraperitoneally injected with 1×10^7 CFUs were administered subcutaneously 100 μg/g morin or DMSO (25 μL) 2 h after infection and then at 12-h intervals to assess the protective effect of morin. The mortality rate of the infected mice was observed for 96 h. In addition, mice intraperitoneally injected with the L95 cell suspension (2×10^6 CFUs/mouse) were used for detection of the bacterial burden and inflammation *in vivo*, and morin was administered subcutaneously (100 mg/kg) 2 h after infection and then at 12-h intervals. DMSO was administered every 12 h in control animals. The mice were sacrifice by euthanasia 48 h after infection. The liver and spleen were homogenate processing in sterile PBS and cultured on TSB agar medium by spreading plate, then calculate the number of bacteria. The pro-inflammatory cytokines in the tissue supernatant collected after centrifuge were determined by ELISA. Ten mice were arranged to each group for survival assays and three independent trials were performed (30 in total). For the inflammation and burden of the bacteria assays three mice were arranged to each group and three independent trials were performed (9 in total) (Table 1).

Statistical analysis

All the experimental data are presented as the means ± SDs and were obtained from experiments that were repeated independently at least three times. The statistical analysis was conducted using GraphPad Prism 5.0. Differences with P values < 0.05 and P values < 0.01 are marked in the figures (Student's t-test)

Abbreviations

Listeria monocytogenes *L. monocytogenes*

listeriolysin O LLO

Cholesterol-dependent cytolysin CDC

Trypticase soy broth TSB

Fetal bovine serum FBS

Interleukin-1 beta IL-1β

Interleukin-6 IL-6

Tumor Necrosis Factor-α TNF-α

Polyvinylidene fluoride membrane PVDF

Multiplicity of infection MOI

Declarations

Ethics approval and consent to participate

The animal experiments were approved by Jilin University (the First Hospital) institutional animal care committee and performed in accordance with their guidelines. All experimental animals were given sufficient food and water, provided sufficient ventilation, activity space and a good living environment. After the experiment, cervical dislocation was performed for euthanasia, and the experimental animals were not given pain during the whole process.

Consent for publication

Not applicable

Availability of data and materials

The datasets used in the current study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no conflicts of interest.

Funding

The animal assays were supported by the National Natural Science Foundation of China (grant 31602109), and the other experiments were supported by the Education Department of Jinlin Province Research Planning Project (JJKH20190173KJ).

Authors' contributions

Z.M.W., G.L. and G.Z.W. conceived the study and designed the experiments. G.L. and M.L. performed the experiments. M.Y.S., H.T.L. and L.L. contributed reagents/materials/analysis tools. G.L., G.Z.W. and Z.M.W. wrote the paper. All the authors read and approved the manuscript.

Acknowledgements

Not applicable

References

1. Pensinger DA, Aliota MT, Schaenzer AJ, Boldon KM, Ansari IU, Vincent WJB, Knight B, Reniere ML, Striker R, Sauer JD: **Selective Pharmacologic Inhibition of a PASTA Kinase Increases Listeria monocytogenes Susceptibility to beta-Lactam Antibiotics**. *Antimicrobial agents and chemotherapy* 2014, **58**(8):4486-4494.
2. Cossart P: **Illuminating the landscape of host-pathogen interactions with the bacterium Listeria monocytogenes**. *Proceedings of the National Academy of Sciences of the United States of America* 2011, **108**(49):19484-19491.
3. Jordan K, McAuliffe O: **Listeria monocytogenes in Foods**. *Advances in food and nutrition research* 2018, **86**:181-213.
4. Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson MA, Roy SL, Jones JL, Griffin PM: **Foodborne Illness Acquired in the United States-Major Pathogens**. *Emerging infectious diseases* 2011, **17**(1):7-15.
5. Williams MA, Schmidt RL, Lenz LL: **Early events regulating immunity and pathogenesis during Listeria monocytogenes infection**. *Trends Immunol* 2012, **33**(10):488-495.
6. Fittipaldi N, Segura M, Grenier D, Gottschalk M: **Virulence factors involved in the pathogenesis of the infection caused by the swine pathogen and zoonotic agent Streptococcus suis**. *Future microbiology* 2012, **7**(2):259-279.
7. Schnupf P, Portnoy DA: **Listeriolysin O: a phagosome-specific lysin**. *Microbes and infection* 2007, **9**(10):1176-1187.
8. Zhang S, Wang J, Chen S, Yin J, Pan Z, Liu K, Li L, Zheng Y, Yuan Y, Jiang Y: **Effects of Suilysin on Streptococcus suis-Induced Platelet Aggregation**. *Frontiers in cellular and infection microbiology* 2016, **6**:128.
9. Keller LE, Bradshaw JL, Pipkins H, McDaniel LS: **Surface Proteins and Pneumolysin of Encapsulated and Nonencapsulated Streptococcus pneumoniae Mediate Virulence in a Chinchilla Model of Otitis Media**. *Front Cell Infect Microbiol* 2016, **6**:55.
10. Osborne SE, Brumell JH: **Listeriolysin O: from bazooka to Swiss army knife**. *Philosophical transactions of the Royal Society of London Series B, Biological sciences* 2017, **372**(1726).
11. Lety MA, Frehel C, Berche P, Charbit A: **Critical role of the N-terminal residues of listeriolysin O in phagosomal escape and virulence of Listeria monocytogenes**. *Molecular microbiology* 2002,

46(2):367-379.

12. Valenti P, Greco R, Pitari G, Rossi P, Ajello M, Melino G, Antonini G: **Apoptosis of Caco-2 intestinal cells invaded by Listeria monocytogenes: Protective effect of lactoferrin**. *Exp Cell Res* 1999, **250**(1):197-202.
13. Rogers HW, Callery MP, Deck B, Unanue ER: **Listeria monocytogenes induces apoptosis of infected hepatocytes**. *J Immunol* 1996, **156**(2):679-684.
14. Zhou X, Zhang B, Cui YM, Chen SY, Teng ZH, Lu GJ, Wang JF, Deng XM: **Curcumin Promotes the Clearance of Listeria monocytogenes both In Vitro and In Vivo by Reducing Listeriolysin O Oligomers**. *Front Immunol* 2017, **8**.
15. Karlsson WK, Harboe ZB, Roed C, Monrad JB, Lindelof M, Larsen VA, Kondziella D: **Early trigeminal nerve involvement in Listeria monocytogenes rhombencephalitis: case series and systematic review**. *Journal of neurology* 2017, **264**(9):1875-1884.
16. Haneche F, Brocard A, Garioud A, Cadranel JF: **[Spontaneous bacterial peritonitis with listeria monocytogenes leading to a cirrhosis discovery]**. *Presse medicale* 2017, **46**(3):332-334.
17. Fernandez de Orueta L, Esteban Fernandez J, Aichner HF, Casillas Villamor A: **[Listeria monocytogenes osteomyelitis]**. *Medicina clinica* 2012, **139**(1):e1.
18. Qiu ZJ, Cervantes JL, Cicek BB, Mukherjee S, Venkatesh M, Maher LA, Salazar JC, Mani S, Khanna KM: **Pregnane X Receptor Regulates Pathogen-Induced Inflammation and Host Defense against an Intracellular Bacterial Infection through Toll-like Receptor 4**. *Scientific reports* 2016, **6**.
19. Wu JH, Fernandes-Alnemri T, Alnemri ES: **Involvement of the AIM2, NLRC4, and NLRP3 Inflammasomes in Caspase-1 Activation by Listeria monocytogenes**. *J Clin Immunol* 2010, **30**(5):693-702.
20. Meixenberger K, Pache F, Eitel J, Schmeck B, Hippenstiel S, Slevogt H, N'Guessan P, Witzenrath M, Netea MG, Chakraborty T *et al*: **Listeria monocytogenes-Infected Human Peripheral Blood Mononuclear Cells Produce IL-1 beta, Depending on Listeriolysin O and NLRP3**. *J Immunol* 2010, **184**(2):922-930.
21. Inoue T, Itani T, Inomata N, Hara K, Takimoto I, Iseki S, Hamada K, Adachi K, Okuyama S, Shimada Y *et al*: **Listeria Monocytogenes Septicemia and Meningitis Caused by Listeria Enteritis Complicating Ulcerative Colitis**. *Internal Med* 2017, **56**(19):2655-2659.
22. Koopmans MM, Bijlsma MW, Brouwer MC, van de Beek D, van der Ende A: **Listeria monocytogenes meningitis in the Netherlands, 1985-2014: A nationwide surveillance study**. *The Journal of infection* 2017, **75**(1):12-19.
23. Park C, Lee WS, Go SI, Nagappan A, Han MH, Hong SH, Kim GS, Kim GY, Kwon TK, Ryu CH *et al*: **Morin, a flavonoid from moraceae, induces apoptosis by induction of BAD protein in human leukemic cells**. *International journal of molecular sciences* 2014, **16**(1):645-659.
24. Dhanasekar C, Kalaiselvan S, Rasool M: **Morin, a Bioflavonoid Suppresses Monosodium Urate Crystal-Induced Inflammatory Immune Response in RAW 264.7 Macrophages through the Inhibition**

of Inflammatory Mediators, Intracellular ROS Levels and NF-kappa B Activation. *PloS one* 2015, **10**(12).

25. Chung SS, Oliva B, Dwabe S, Vadgama JV: **Combination treatment with flavonoid morin and telomerase inhibitor MST-312 reduces cancer stem cell traits by targeting STAT3 and telomerase**. *Int J Oncol* 2016, **49**(2):487-498.
26. Qureshi AA, Guan XQ, Reis JC, Papasian CJ, Jabre S, Morrison DC, Qureshi N: **Inhibition of nitric oxide and inflammatory cytokines in LPS-stimulated murine macrophages by resveratrol, a potent proteasome inhibitor**. *Lipids Health Dis* 2012, **11**.
27. Wang J, Zhou X, Liu S, Li G, Zhang B, Deng X, Niu X: **Novel inhibitor discovery and the conformational analysis of inhibitors of listeriolysin O via protein-ligand modeling**. *Scientific reports* 2015, **5**:8864.
28. Lomonaco S, Nucera D, Filipello V: **The evolution and epidemiology of Listeria monocytogenes in Europe and the United States**. *Infection, genetics and evolution : journal of molecular epidemiology and evolutionary genetics in infectious diseases* 2015, **35**:172-183.
29. Morgand M, Leclercq A, Maury MM, Bracq-Dieye H, Thouvenot P, Vales G, Lecuit M, Charlier C: **Listeria monocytogenes-associated respiratory infections: a study of 38 consecutive cases**. *Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases* 2018.
30. Camargo AC, de Castilho NP, da Silva DA, Vallim DC, Hofer E, Nero LA: **Antibiotic Resistance of Listeria monocytogenes Isolated from Meat-Processing Environments, Beef Products, and Clinical Cases in Brazil**. *Microbial drug resistance* 2015, **21**(4):458-462.
31. Page SW, Gautier P: **Use of antimicrobial agents in Livestock**. *Rev Sci Tech Oie* 2012, **31**(1):145-188.
32. Escaich S: **Antivirulence as a new antibacterial approach for chemotherapy**. *Curr Opin Chem Biol* 2008, **12**(4):400-408.
33. Rasko DA, Sperandio V: **Anti-virulence strategies to combat bacteria-mediated disease**. *Nature reviews Drug discovery* 2010, **9**(2):117-128.
34. Roulo RM, Fishburn JD, Amosu M, Etchison AR, Smith MA: **Dose response of Listeria monocytogenes invasion, fetal morbidity, and fetal mortality after oral challenge in pregnant and nonpregnant Mongolian gerbils**. *Infection and immunity* 2014, **82**(11):4834-4841.
35. Singh M, Thakur V, Deshmukh R, Sharma A, Rathore MS, Kumar A, Mishra N: **Development and characterization of morin hydrate-loaded micellar nanocarriers for the effective management of Alzheimer's disease**. *Journal of microencapsulation* 2018, **35**(2):137-148.
36. Sharma D, Singh M, Kumar P, Vikram V, Mishra N: **Development and characterization of morin hydrate loaded microemulsion for the management of Alzheimer's disease**. *Artificial cells, nanomedicine, and biotechnology* 2017, **45**(8):1620-1630.
37. Chemmugil P, Lakshmi PTV, Annamalai A: **Exploring Morin as an anti-quorum sensing agent (anti-QSA) against resistant strains of Staphylococcus aureus**. *Microb Pathog* 2019, **127**:304-315.

38. Li G, Lu G, Qi Z, Li H, Wang L, Wang Y, Liu B, Niu X, Deng X, Wang J: **Morin Attenuates Streptococcus suis Pathogenicity in Mice by Neutralizing Suilysin Activity**. *Frontiers in microbiology* 2017, **8**:460.
39. Walls ZF, Gong H, Wilson RJ: **Liposomal Coencapsulation of Doxorubicin with Listeriolysin O Increases Potency via Subcellular Targeting**. *Molecular pharmaceutics* 2016, **13**(3):1185-1190.
40. Hernandez-Flores KG, Calderon-Garciduenas AL, Mellado-Sanchez G, Ruiz-Ramos R, Sanchez-Vargas LA, Thomas-Dupont P, Izaguirre-Hernandez IY, Tellez-Sosa J, Martinez-Barnetche J, Wood L *et al*: **Evaluation of the safety and adjuvant effect of a detoxified listeriolysin O mutant on the humoral response to dengue virus antigens**. *Clin Exp Immunol* 2017, **188**(1):109-126.
41. Noor S, Goldfine H, Tucker DE, Suram S, Lenz LL, Akira S, Uematsu S, Girotti M, Bonventre JV, Breuel K *et al*: **Activation of cytosolic phospholipase A2alpha in resident peritoneal macrophages by Listeria monocytogenes involves listeriolysin O and TLR2**. *The Journal of biological chemistry* 2008, **283**(8):4744-4755.
42. Sivaranjani M, Gowrishankar S, Kamaladevi A, Pandian SK, Balamurugan K, Ravi AV: **Morin inhibits biofilm production and reduces the virulence of Listeria monocytogenes - An in vitro and in vivo approach**. *International journal of food microbiology* 2016, **237**:73-82.
43. Gowrishankar S, Sivaranjani M, Kamaladevi A, Ravi AV, Balamurugan K, Karutha Pandian S: **Cyclic dipeptide cyclo(l-leucyl-l-prolyl) from marine Bacillus amyloliquefaciens mitigates biofilm formation and virulence in Listeria monocytogenes**. *Pathogens and disease* 2016, **74**(4):ftw017.
44. Wang J, Qiu J, Tan W, Zhang Y, Wang H, Zhou X, Liu S, Feng H, Li W, Niu X *et al*: **Fisetin inhibits Listeria monocytogenes virulence by interfering with the oligomerization of listeriolysin O**. *The Journal of infectious diseases* 2015, **211**(9):1376-1387.

Table

Table 1 The details of animal assays arrangement

Test category	Survival			Colony counting			Inflammatory factors		
group	infection	treatment	blank	infection	treatment	blank	infection	treatment	blank
Number	10/10/10	10/10/10	10/10/10	3/3/3	3/3/3	3/3/3	3/3/3	3/3/3	3/3/3
Pepetition	3								
Infection routine	intraperitoneal injection								
Treatment	subcutaneous injection								
Time bigin	2 h after infection								
Tiem stop	96 h			48 h			48 h		
Interval	12 h								
Details	6-8 weeks, weight around of 20 g, female, get diet and water freely, euthanasia								

Figures

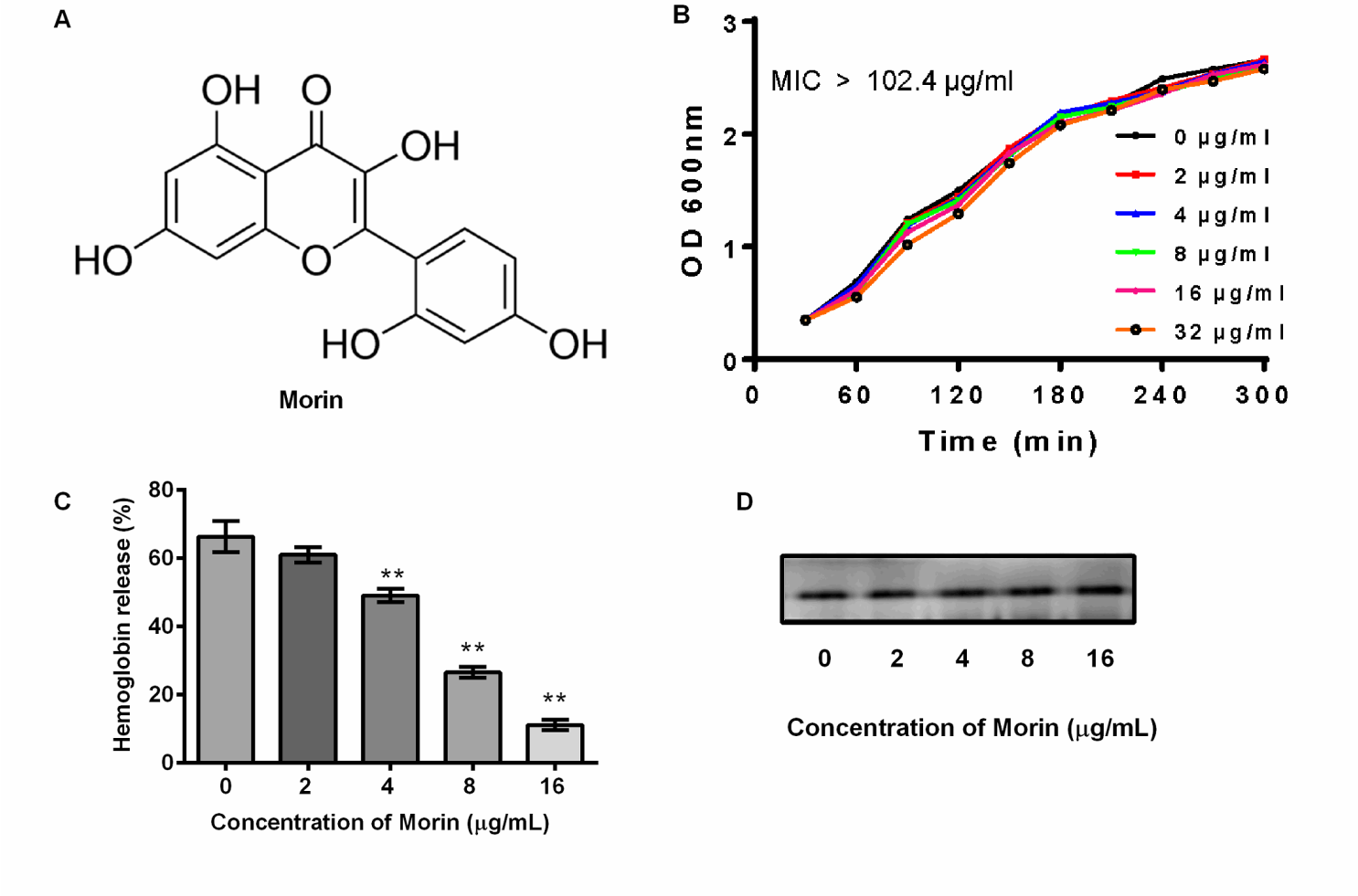

Figure 1

Inhibition of L. monocytogenes culture by morin. (A) Chemical structure of morin. (B) Growth kinetics curve of L95 cells treated with different concentrations of morin. L95 cells were incubated in TSB in the presence of various dosages of morin, and the growth of each sample was monitored every 30 min by measuring the value of OD600 nm at each indicated time point. (C) Suppression of the hemolytic activity of L95 culture supernatants by morin. L95 cells were cocultured with morin, and the cocultured supernatants were harvested by centrifugation. The hemolytic activity was assessed by a hemolysis assay. The hemolysis percent of each cocultured supernatant was compared with that of the positive control group (treatment with Triton X-100). (D) The expression of LLO in L. monocytogenes cultures treated with different concentrations of morin was assessed by Western blotting using a specific antibody against LLO. Three independent trials were performed for each index.* indicates $P < 0.05$, and ** indicates $P < 0.01$. (Student's t-test).

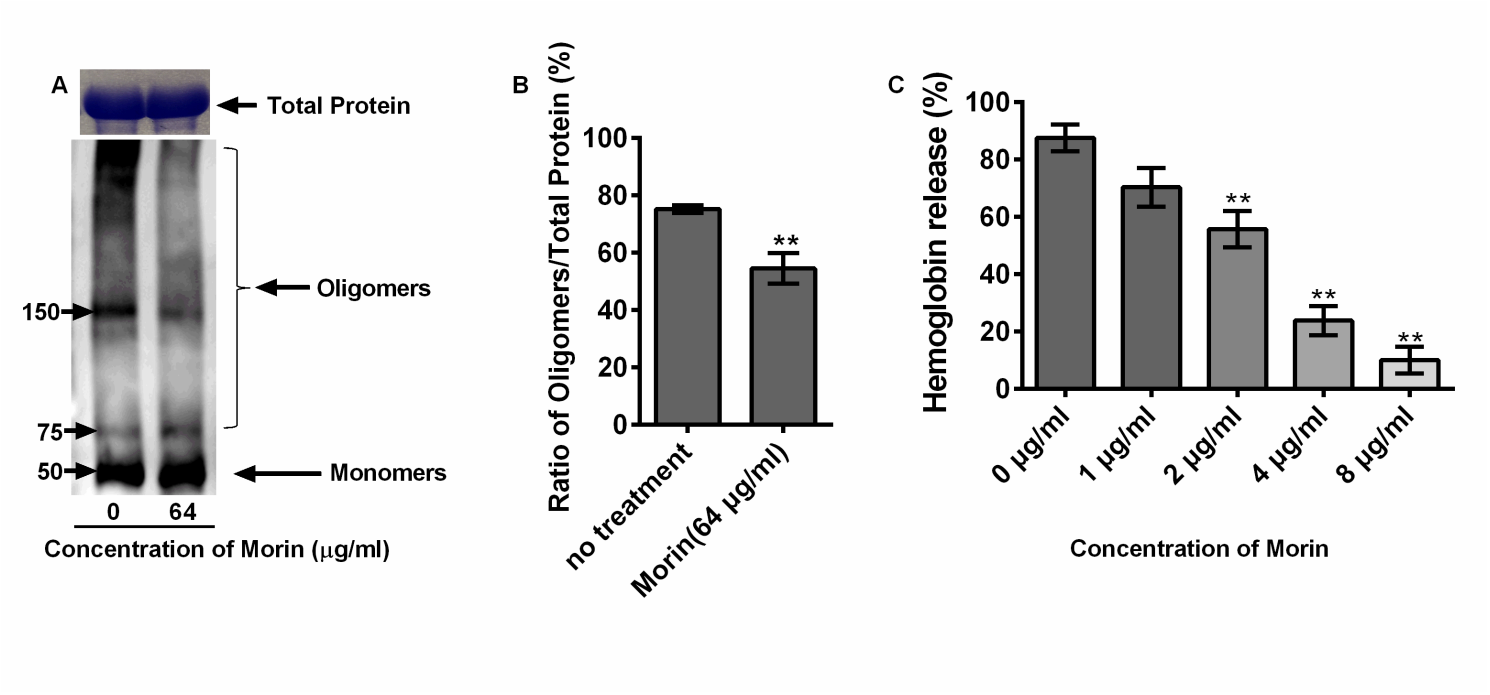

Figure 2

Inhibitory effects of morin on oligomer formation and the hemolytic activity of LLO. (A and B) Purified LLO proteins were treated with morin, and the inhibition of LLO oligomerization by morin was assessed by Western blotting. The total protein level in the groups without oligomerization induction was determined by Coomassie blue staining. (C) Purified LLO protein was coincubated with morin in PBS, and the cocultured systems were harvested by centrifugation. The hemolytic activity was assessed by a hemolysis assay. Three independent trials were performed for each index.* indicates $P < 0.05$, and ** indicates $P < 0.01$. (Student's t-test).

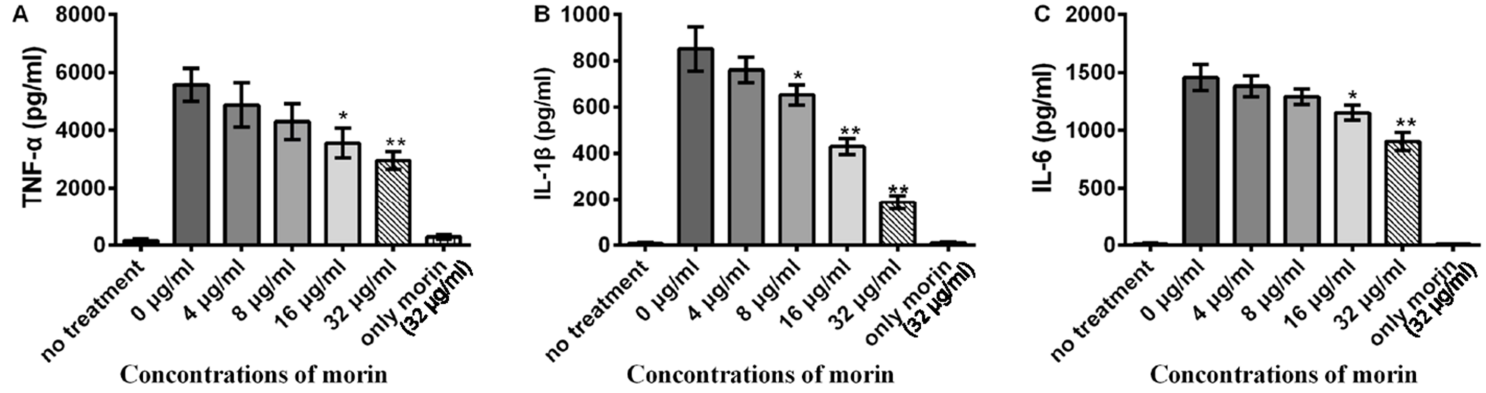

Figure 3

Inhibition of the production of inflammatory cytokines by morin in cells infected with L. monocytogenes. J774 macrophage-like cells were cocultured with L. monocytogenes in the presence or absence of morin (0, 8, 16, and 32 μg/mL) at an MOI of 10 for 5 h. The coculture supernatants were harvested by centrifugation, and the levels of TNF-α (A), IL-1β (B) and IL-6 (C) were detected by ELISA. Cells treated

with morin in the absence of L. monocytogenes were used as a control. Three independent trials were performed for each index.* indicates P < 0.05, and ** indicates P < 0.01. (Student's t-test).

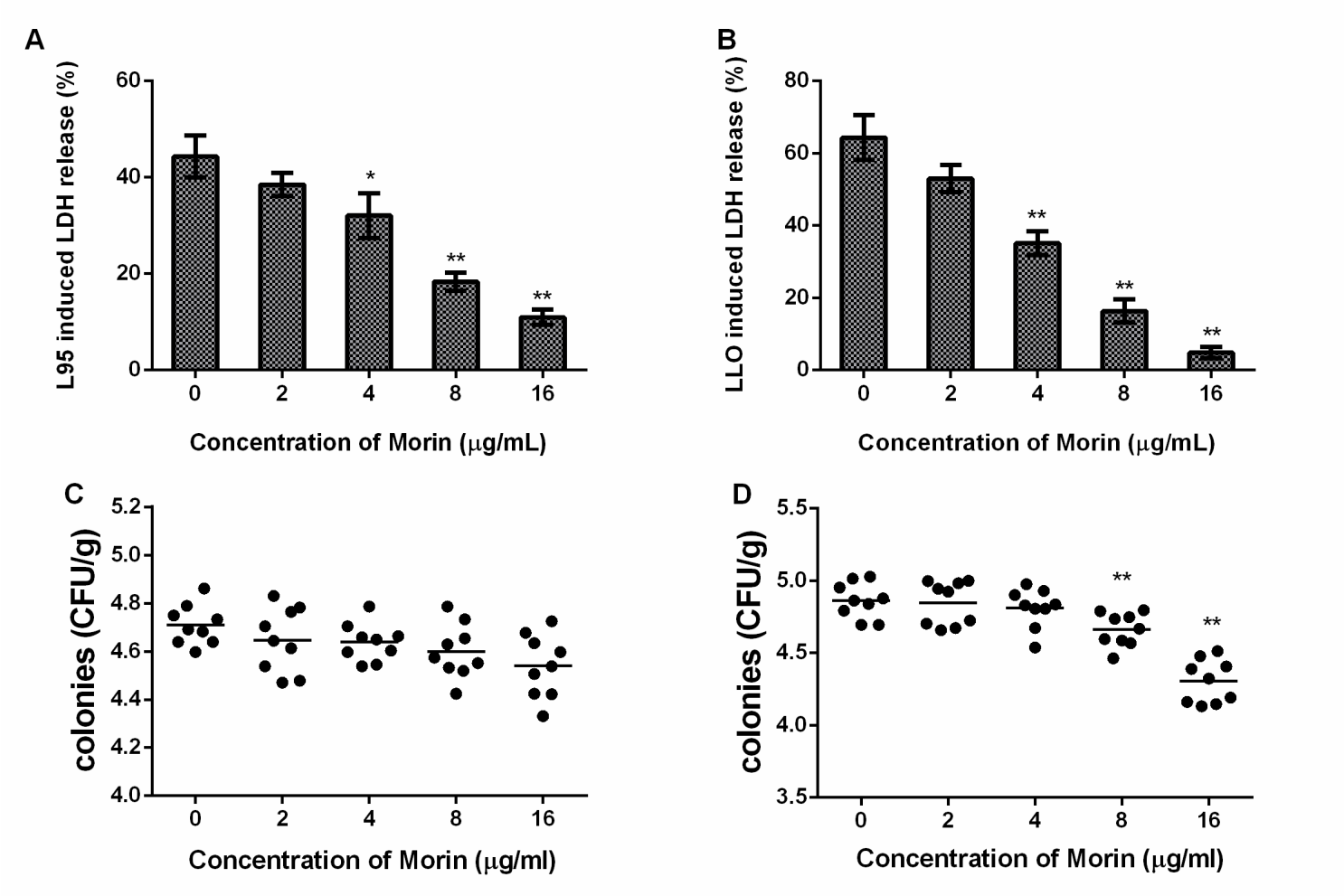

Figure 4

Morin-mediated attenuation of cell damage after infection with L95 cells or treatment with LLO. The level of LDH released into the supernatants of cells infected with L95 cells (A) or treated with LLO (B) in the presence or absence of various concentrations of morin was detected using a cytotoxicity detection kit. The extracellular (C) and intracellular (D) colonies were calculated by colonization. Three independent trials were performed for each index.* indicates P < 0.05, and ** indicates P < 0.01. (Student's t-test).

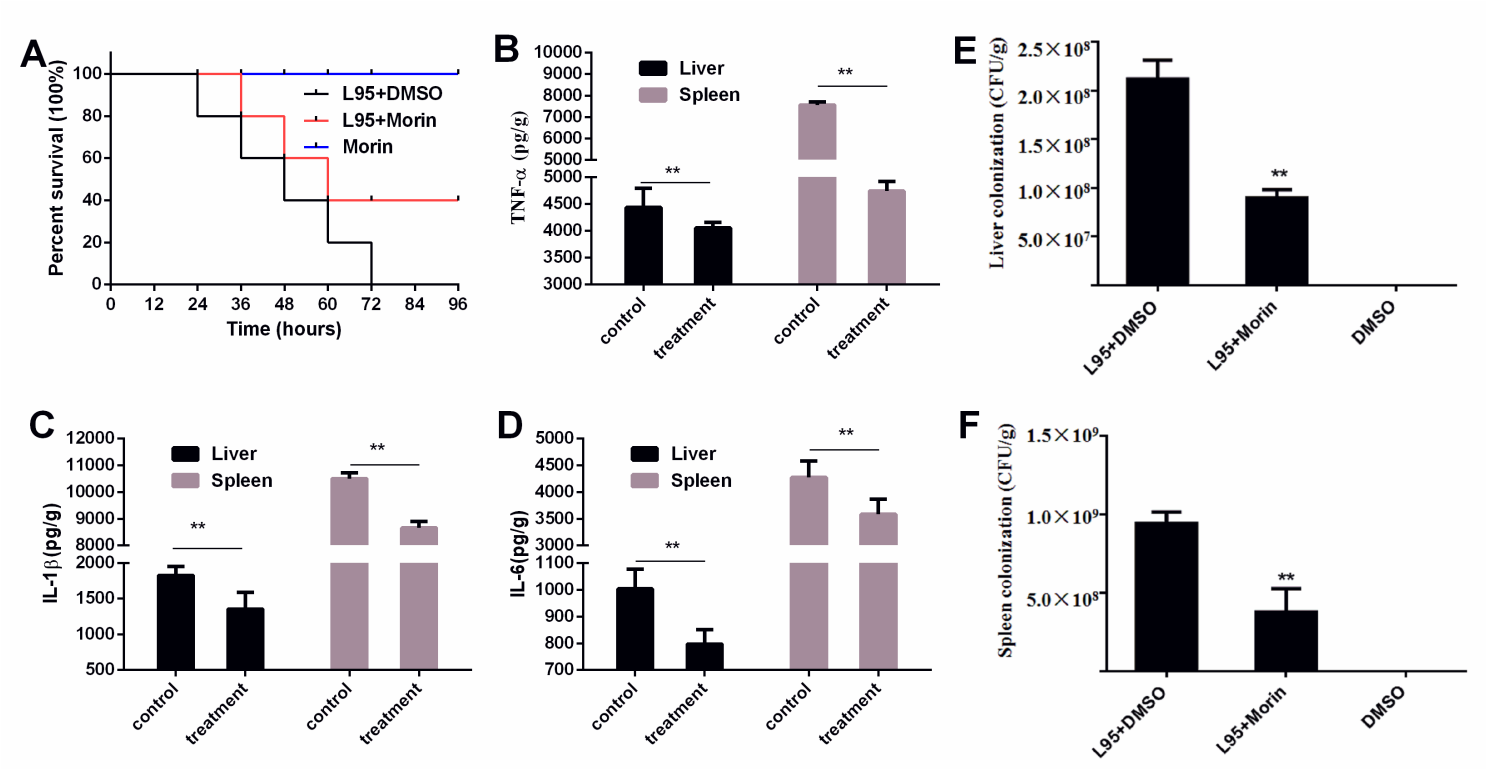

Figure 5

Protective effect of morin against L. monocytogenes infection in vivo. (A) Mice were intraperitoneally injected with 1×107 CFUs of L95 cells and treated with 100 mg/kg morin or DMSO 2 h after infection and at 8-h intervals. The survival rate of the infected mice was observed for 96 h. The mice were intraperitoneally injected with 2×106 CFUs of L95 cells and treated with 100 mg/kg morin or DMSO 2 h after infection and again at 8-h intervals. Forty-eight hours after infection, all of the mice were euthanized, the tissues of the liver and spleen were homogenized, and the number of colonies in ground tissue was calculated via serial dilution (E-F). The levels of IL-6, IL-1β and TNF-α were detected by ELISA (B-D). Ten mice were arranged to each group for survival assays and three independent trials were performed (30 in total). For the inflammation and burden of the bacteria assays three mice were arranged to each group and three independent trials were performed (9 in total).* indicates $P < 0.05$, and ** indicates $P < 0.01$. (Student's t-test).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- ARRIVEChecklist.docx