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: January 17th, 2020

DOI: <https://doi.org/10.21203/rs.2.21184/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

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 type of ubiquitous Gram-positive intracellular pathogen that frequently grows under the conditions of food preservation and is regarded as the etiologic agent of a series of severe diseases including meningitis, sepsis, even life-threatening infections in both humans and animals; *L. monocytogenes* poses a particularly serious threat to immunocompromised individuals such as the elderly or pregnant women[1, 2]. The ingestion of food contaminated with *L. monocytogenes* is the main way of infection, which poses a significant threat and severe challenge to the food chain and production process[3]. The 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 an outbreak in 2011, hundreds of people were infected, and dozens died from the infection[4, 5], suggesting that *L. monocytogenes* is an important public health concern.

Successful establishment of infection is an important step in the process of pathogenic bacteria evading the host immune system, and a series of complex virulence factors are crucial weapons carried by the bacteria to exert virulence at each stage of the infection[6]. Multiple virulence factors are secreted during the intracellular life cycle of *L. monocytogenes* to achieve the 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*; it 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 members such as 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* with *hly* 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], suggesting 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, peritonitis, etc[15-17] and activate the assembly of AIM2, NLRC4, and NLRP3 inflammasomes, which are multiprotein complexes that induce the activation of the proinflammatory cysteine protease caspase-1 in the course of disease and induce the secretion of important pro-inflammatory cytokines such as IL-1 β , IL-6, and TNF- α [18-20]. These excessive inflammatory reactions have great harm to the body, especially to the immunocompromised populations [21, 22]. The severe inflammation induced by *L. monocytogenes* is also a crucial part of the disease process. Therefore, effectively alleviating the inflammatory response may be an important way to treat *L. monocytogenes* infection.

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

Results

Morin has no influence on *L. monocytogenes* growth

The minimum inhibitory concentration of morin (Figure 1A) against L95 was greater than 1024 $\mu\text{g/mL}$. Furthermore, no obvious difference was observed in the growth of L95 when adding morin or not in the effective concentration (32 $\mu\text{g/mL}$) at which it functions (Figure 1B). Moreover, morin could clearly suppress the function of lysing red blood cells of the bacterial culture (Figure 1C), which is consistent with our previous study that morin could 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 LLO expression 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*, suggesting that the using morin to the treatment of *Listeria* infection does 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. Here, we further demonstrate that the binding of morin and LLO effectively inhibits the formation of LLO oligomers and the total protein of each group did not change (Figure 2A and B). The purified LLO protein was induced to oligomerize *in vitro*. After adding morin into the reaction system, the oligomeric bands became narrow, and the number of oligomers decreased significantly; the hemolytic activity of LLO was also reduced by treatment with morin (Figure 2C).

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

All above results suggest that morin may play an inhibitory role in *Listeria* infection; therefore, we further studied the function of morin in *Listeria* infection. The secretion of TNF- α , IL-1 β , and IL-6 that considered to be an important inflammatory mediators was remarkably downregulated, which indicates that morin has a significant anti-inflammatory effect *in vitro*. In addition, compared with untreated cells, the use of morin alone had little effect on the expression of inflammatory factors (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 LDH level in cell culture supernatant of J774 cocultured with L95 and different concentrations of morin, and treatment with morin also effectively reduced the LDH release of infected cells. In addition, morin itself had no cytotoxic effects on cells (Figure 4A). In line with the results of bacterial infection, morin also significantly protects LLO-induced cell damage (Figure 4B), further suggesting that morin can play an anti-infective role by inhibiting LLO activity. Consistent with the results of the above studies, morin reduces the survival of intracellular bacteria but hardly affects extracellular bacteria (Figure 4C and D). These results strongly suggest that morin has 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 *Listeria* infection model. Mice were injected with different concentrations of L95 in the abdominal cavity to observe the survival rate and determine the colonization of organs or tissue inflammation. After infection with a high concentration of L95, the survival rate increased significantly in comparison with the control. All mice in the control group died 96 h after infection; however, the survival rate rose to 42.46% (Figure 5A).

The bacterial burden analysis and detection of inflammation in tissue from mice infected with L95 were used to further assess the effect of morin *in vivo*. A mouse model of *Listeria* infection was successfully established, and the colony colonization in the tissues (liver and spleen) was significantly decreased by morin treatment (Figure 5E and 5F). In accordance with the above results, as shown in Figure 5B, 5C, and 5D treatment with morin also significantly relieved inflammation in 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]. Currently, the treatment of diseases related to *Listeria* infection in clinical practice mainly involves applying antibiotics to control or kill the pathogenic microorganism [29]. However, an increasing number of *Listeria* strains that can survive antibiotic treatment are more frequently being discovered clinically, and the problem of *Listeria* resistance is becoming much more serious [30]. In 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 have led to a series of adverse effects, especially the acceleration of multidrug resistance in bacteria and have caused more serious infections in alarming situations, which have resulted in some bacterial infections with no available drugs[31]. Even more concerning the situation that developing new types of bacteriostatic drugs is much slower than the rate of the generation of drug resistance, which has resulted in this embarrassing and dangerous situation of increasing bacterial resistance and pathogenicity with fewer weapons being available 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 to cope with the declining efficacy of traditional antibiotics [33]. The anti-virulence strategies differ from traditional antibiotics in terms of antibacterial effects by inhibiting core elements in bacterial life, exerting anti-infective activity and reducing bacterial pathogenicity by acting on virulence factors, which would apply a relatively small survival pressure in comparison with antibiotics and not be conducive to inducing bacterial drug resistance[32, 33]. Listeriolysin O (LLO) is a crucial virulence factor in the pathogenesis of *Listeria*, and the deletion of LLO can significantly inhibit bacteria escaping from the phagosome and reduce the pathogenicity of the bacteria[10, 11], which would 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, which is a particular risk for abortion in pregnant women[34], indicating that attenuating the inflammatory response is an important part of the treatment of *Listeria* infection.

At present, morin are widely used in the treatment of diseases, and only a small amount of oral morin can cross the blood-brain barrier to the brain, while morin hydrate-loaded micellar nanocarriers can greatly improve their ability to cross the blood-brain barrier and play a therapeutic role in Alzheimer's disease. This suggests that applying appropriate modification of morin dosage form can improve its therapeutic effect on meningitis induced by *Listeria* infection[35, 36]. There are few studies on the stability of morin, but the effect of oral morin is very poor, indicating that the state of oral morin is not stable [37], so intravenous injection may become an important direction for its research and application. Although the oral effect of morin can be greatly improved after some modification, whether it is convenient for large-scale production and development remains to be studied[38]. In addition, some studies have shown that morin also has potential therapeutic effects in the treatment of *Staphylococcus aureus* and *Streptococcus suis* infections [39, 40], suggesting 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 is still unclear[41-43]. Therefore, whether the anti-inflammatory effect of morin on *Listeria* infection depends on its inhibition of LLO is an interesting direction for further research.

In our previous research, we found that morin could clearly suppress the hemolytic function of LLO and demonstrated by molecular simulation that the inhibitory effect was achieved by direct binding between morin and LLO to form an LLO-morin complex. The predicted binding sites is the residues 420–470 region of LLO, which is important to morin interacting with LLO and the C1-C2 double bond of morin (Figure 1A) are also essential for this process[27]. In this paper, we further studied 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, further confirming that the suppression of morin to LLO hemolytic function is achieved by direct action on the protein itself, which could be regarded as an anti-virulence agent and therapeutic candidate in *Listeria* infection. In addition, we also discovered that morin could 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 important pro-inflammatory cytokines TNF- α , IL-6, and IL-1 β was reduced, and morin also exhibited effective protection against *Listeria* at cell level *in vitro* and decreased bacterial burden *in vivo*. All of these findings lay the theoretical foundations for the further study of morin as a candidate for the challenge of *L. monocytogenes* infection. Morin is easy to obtain 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 morin to become a new drug for clinical application in the treatment of *L. monocytogenes* infection. In particular, selecting a suitable dosage form and performing effective structural modifications to further enhance the effect of the drug is necessary.

Conclusions

In this study, we further demonstrated that morin had 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.

Abbreviations

Listeria monocytogenes *L. monocytogenes*

listeriolysin O LLO

Cholesterol-dependent cytolysin CDC

Trypticase soy broth TSB

Fetal bovine serum FBS

Interleukin-1 beta IL-1 β

Interleukin-6 IL-6

Tumor Necrosis Factor- α TNF- α

Methods

Bacterial strains and reagents

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

Anti-*L. monocytogenes* activity testing

The minimum inhibitory concentration (MIC) of morin for L95 was evaluated as described in previous work [14]. In brief, L95 was cultured to the stage of logarithmic growth in TSB and adjust the density to 1×10^8 CFUs/ml. The morin was diluted continuously with TSB medium and then adding bacterial culture to 5×10^5 CFUs/ml, respectively. The growth of bacteria under different concentration of morin was observed every 12 hours for 48 hours. For the determination of bacterial growth, the absorbance at OD600 nm of L95 cultured in TSB with the indicated concentrations of morin at 37°C was measured every 30 min for 5 h.

Hemolysis assay

L95 was cultured in TSB with various concentrations of morin (0, 2, 4, 8, 16 μ g/mL) at 37°C for 5 h. Following centrifugation (10,000 rpm, 2 min), 100 μ L of the supernatant of each co-culture sample was incubated with sheep erythrocytes and PBS in a system of 1 mL for 20 min. Then, the system was centrifuged and the absorption value at OD_{543nm} of supernatant was detected to determine the hemolytic activity by comparing each sample with the control sample (sheep erythrocytes treated with PBS supplemented with 2% Triton X-100), which was set as 100% hemolysis activity.

The purified protein was incubated with morin (0, 1, 2, 4, 8 μ g/mL) and the detection of hemolytic activity in these samples were measured as described above.

Western blotting assay

L95 was 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 gel. After electrophoretic transfer, the interest protein on the polyvinylidene fluoride membrane was blocked at room temperature for more than 2 h, and then the polyvinylidene fluoride membrane was in a incubation with a primary rabbit anti-LLO antibody (Abcam, 1:2000) for 2 h and a corresponding secondary antibody

(Proteintech, 1:3000) for 1h. The ECL detection reagents were used to visualize the signals on the PVDF membranes with a Tanon-4200 imager.

Purification and the induction of oligomerization of LLO protein were performed as previously described[44]. In brief, the LLO incubated with morin (64 µg/mL) for 20 min and the oligomerization was induced under high salt conditions *in vitro*. Firstly LLO protein was incubated with morin for 30 minutes under acidic PBS conditions, then saturated potassium chlorid was added to the concentration of 0.3 mg/mL and incubated for another 10 min. Next, sheep erythrocytes were added to the reaction system and treated on ice for 5 minutes. The reaction samples were boiled with SDS-PAGE loading buffer lacking 2-hydroxy-1-ethanethiol. And the oligomerization was detected by western blotting described above.

Cell-line infections

L95 was cultured to mid-logarithmic growth at OD₆₀₀ about 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 grew in high-glucose DMEM at 5% CO₂ and we plated the cells in corning 96-well plates (about 2×10⁴ cells per well) to culture for 16 h and then the cells co-cultured with L95 at 37°C for 5 h at a MOI of 8 or treated with LLO protein (12 ng per well) that pre-incubated with morin (0, 2, 4, 8, 16 µg/mL) for 30 min at 37°C for the detection of lactate dehydrogenase. And the co-culture supernatant was diluted continuously and the extracellular bacteria determined by colony counting. In order to assess the intracellular bacteria, the supernatant of the culture medium was removed, washed with PBS for three times, and gentamicin was added to kill the residual extracellular bacteria. After that, the cells were lysed with saponins, and the colony count was carried out by continuous dilution.

Cells grew in corning 6-well plates (1×10⁶ cells per well) were infected with L95 in the presence of morin treatment at an MOI of 10 at 37°C for 4 h for inflammation assays.

Inflammation assays

Following centrifugation (1,000 rpm, 10 min), inflammatory mediator (IL-1β, IL-6, and TNF-α) contained in each co-infection sample were determined using the corresponding ELISA kit (Biolegend ,CA, USA).

Cytotoxicity assays

Following centrifugation (1,000 rpm, 10 min), the lactate dehydrogenase level in samples of cell infection was assessed by a Cytotoxicity Detection Kit. The samples treated with Triton or DMEM were set as controls.

Animal experiments

Female BALB/c mice (6-8 weeks) were bought from the Experimental Animal Center of Jilin University, and the animal experiments were in accordance with the Jilin University institutional animal care

committee guidelines. All of the animals were euthanized by cervical dislocation at the end of the experiment.

L95 cultured to mid-logarithmic growth stage ($OD_{600}=0.8-1.0$), followed by centrifugation and collection of the bacteria, which were then washed 3 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 CFU received 100 μ g/g of morin or DMSO (25 μ L) at 2 h after infection and then at 12-h intervals to assess the protective effect of morin. The mortality rate of infected mice (30 mice per group) was observed for 96 h. In addition, the mice intraperitoneally injected with the L95 suspension (2×10^6 CFUs/mouse) were used to detect the bacterial burden and inflammation *in vivo*, morin was administered subcutaneously (100 mg/kg) 2 h after infection, then at 12-h intervals. DMSO was administered as the control. The liver and spleen were dissected and ground in PBS after sacrificing by euthanasia 48 h postinfection. The ground tissue supernatant was collected by centrifugation and then diluted onto a solid TSB medium to calculate the number of bacteria. The pro-inflammatory cytokines in the ground tissue supernatant were determined by ELISA.

Statistical analysis

All experimental data presented as the mean $\pm$ SD and repeated independently at least for three times. The statistical analysis was conducted by GraphPad Prism 5.0. P value < 0.05 and P value < 0.01 are marked in the figures (Student's t-test).

Declarations

Ethics approval and consent to participate

The animal experiments were in accordance with the Jilin University institutional animal care committee guidelines.

Consent for publication

Not applicable

Availability of data and materials

The datasets used during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no conflict of interest.

Funding

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

Authors' contributions

Z.M.W., G.L. and G.Z.W. conceived 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 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 Sullysin 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, Witzentrath 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. Li J, Yang Y, Ning E, Peng Y, Zhang J: **Mechanisms of poor oral bioavailability of flavonoid Morin in rats: From physicochemical to biopharmaceutical evaluations.** *European journal of pharmaceutical sciences : official journal of the European Federation for Pharmaceutical Sciences* 2019, **128**:290-298.
38. Jangid AK, Agrawal H, Gupta N, Yadav UCS, Sistla R, Pooja D, Kulhari H: **Designing of fatty acid-surfactant conjugate based nanomicelles of morin hydrate for simultaneously enhancing anticancer activity and oral bioavailability.** *Colloids and surfaces B, Biointerfaces* 2019, **175**:202-211.
39. 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.
40. 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.
41. Walls ZF, Gong H, Wilson RJ: **Liposomal Coencapsulation of Doxorubicin with Listeriolysin O Increases Potency via Subcellular Targeting.** *Molecular pharmaceuticals* 2016, **13**(3):1185-1190.
42. 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.
43. 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.
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.

Figures

Figure 1

The inhibition of morin to *L. monocytogenes* culture (A) The chemical structure of Morin. (B) The growth kinetics curve of L95 treated with different concentrations of morin. L95 was 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 at OD600 at each indicated time point. (C) The suppression of the hemolytic activity of L95 culture supernatants by morin. L95 was co-cultured with morin and the cocultured supernatants were harvested by centrifugation. The hemolytic activity was assessed by a hemolysis assay. The hemolysis percent of each co-cultured supernatant was compared with the positive control group. (D) The expression of LLO in *L. monocytogenes* cultures treated with different concentrations of morin was assessed by Western blot using a specific antibody against LLO. *indicates $P < 0.05$, and ** indicates $P < 0.01$.

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 blot. Total protein in groups without inducing oligomerization was determined by Coomassie blue staining (C) Purified LLO protein was co-incubated with morin in PBS, and the co-cultured systems were harvested by centrifugation. The hemolytic activity was assessed by a hemolysis assay. *indicates $P < 0.05$, and ** indicates $P < 0.01$.

Figure 3

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

cells treated with morin in the absence of *L. monocytogenes* were used as a control.*indicates $P < 0.05$, and ** indicates $P < 0.01$.

Figure 4

Attenuation of cell damage by morin in cells infected with L95 or treated with LLO. The level of LDH released in supernatants of cells infected with L95 (A) or treated with LLO (B) in the presence or absence of various concentrations of morin was detected by a cytotoxicity detection kit. Extracellular (C) and intracellular (D) colonies are calculated by colonization *indicates $P < 0.05$, and ** indicates $P < 0.01$.

Figure 5

The protective effect of morin against *L. monocytogenes* infection in vivo (A) Mice were intraperitoneally injected with 1×10^7 CFU of L95 and treated with morin or DMSO at 100 mg/kg after infection for 2 h and then again at an 8-h interval. The survival rate of infected mice was observed for 96 h. Mice were intraperitoneally injected with 2×10^6 CFU of L95 and treated with morin or DMSO at 100 mg/kg after infection for 2 h and then again at an 8-h interval. After 48 h, 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 after serial dilution (E-F). The levels of IL-6, IL-1 β and TNF- α were detected by ELISA (B-D). *indicates $P < 0.05$, and ** indicates $P < 0.01$.