Effect of honokiol on exotoxin proteins listeriolysin O and p60 secreted by *Listeria monocytogenes*

Rizeng Meng,1,2† Ziwen Zhao,1,3† Na Guo,1,3 Zonghui Liu,3 Xingchen Zhao,3 Wenli Li,3 Xiaoxu Li,3 Ce Shi,3 Dandan Nie,2 Weilin Wang,2 Tao Liu,4 Wenchen Ma,2 Lu Yu1,4 and Juan Li1

1Public Health College, Jilin University, 130062 Changchun, PR China
2Jilin Entry-exit Inspection and Quarantine Bureau, Changchun, 130062, PR China
3Department of Food Quality and Safety, Jilin University, 130062 Changchun, PR China
4Key Laboratory of Zoonosis Research, Ministry of Education, Institute of Zoonosis, College of Veterinary Medicine, Jilin University, 130062 Changchun, PR China

*Listeria monocytogenes* is considered one of the most important foodborne pathogens. The virulence-related proteins listeriolysin O (LLO) and p60 are critical factors involved in *Listeria* pathogenesis. In the present study, we investigated the effect of honokiol on LLO and p60 secreted from *L. monocytogenes*. A listeriolysin assay was used to investigate the haemolytic activities of *L. monocytogenes* exposed to honokiol, and the secretion of LLO and p60 was detected by immunoblot analysis. Additionally, the influence of honokiol on the transcription of LLO and p60 genes (*hly* and *iap*, respectively) was analysed by real-time reverse transcription PCR. TNF-α release assays were performed to elucidate the biological relevance of changes in LLO and p60 secretion induced by honokiol. According to the data, honokiol showed good anti-*L. monocytogenes* activity, with MICs of 8–16 μg ml⁻¹, and the secretion of LLO and p60 was decreased by honokiol. In addition, the transcription of *hly* and *iap* was inhibited by honokiol. Our results indicate that TNF-α production by RAW264.7 cells stimulated with *L. monocytogenes* supernatants was inhibited by honokiol. Based on these data, we propose that honokiol could be used as a promising natural compound against *L. monocytogenes* and its virulence factors.

INTRODUCTION

*Listeria monocytogenes* is a Gram-positive, facultative, intracellular pathogen of humans and several animal species. Ingestion of foods contaminated with *L. monocytogenes* can result in listeriosis, a severe infectious disease characterized by meningocellulitis, abortion, septicemia and a high fatality rate (30 %) in pregnant women, newborns, the elderly and immunocompromised individuals (Mateus et al., 2013). Several different virulence-associated exotoxin proteins have been characterized from *L. monocytogenes*. Among these, the proteins listeriolysin O (LLO) and p60 are considered highly important. LLO, a 58 kDa secretory protein, is the essential virulence factor that enables *L. monocytogenes* to escape from the phagosomal compartment of macrophages via its membrane-damaging activity (Jiang et al., 2006; Nishibori et al., 1996). LLO is encoded by the *hly* gene and has an important role in intracellular parasitism only in *L. monocytogenes* (Jiang et al., 2006). The protein p60 (invasion-associated protein), encoded by the *iap* gene, is a murein hydrolase enzyme that is essential during the final stage of *L. monocytogenes* cell division (Jiang et al., 2006). This protein is produced in relatively large amounts by all virulent *L. monocytogenes* strains, mainly as a secreted form, and a large amount of p60 accumulates in culture supernatants (Wuenscher et al., 1993) during the stationary growth phase. A p60 deletion mutant showed attenuated virulence in mice (Pilgrim et al., 2003).

Magnolia is one of a number of traditional medicinal plants used, particularly in China and Japan. Honokiol (C18H18O2), which is isolated from the phenolic extract from the bark of Magnoliaceae plants (Park et al., 2004), possesses antimicrobial activities against organisms such as Propionibacterium acne, Porphyromonas gingivalis and Prevotella intermedia (Chang et al., 1998; Clark et al., 1981; Ho et al., 2001; Hu et al., 2011; Park et al., 2004). However, to the best of our knowledge, the effects of honokiol on *L. monocytogenes* growth and virulence factors have not
been reported. The aim of the present study was to identify the effect of honokiol on the expression of two major virulence-associated exotoxin proteins, LLO and p60, secreted by *L. monocytogenes*.

**METHODS**

**Bacterial strains, reagents and cell growth conditions.** Sixteen strains of *L. monocytogenes*: EGD (serovar 1/2a), ATCC 19115 (serovar 4b), ATCC 19111 (serovar 1), ATCC 15313 (serovar 1), ATCC 7644 (serovar 1/2c) and China Medical Culture Collection Center (CMCC) 54002 (serovar 1/2c), and 10 strains (serovar 1/2b, 1/2a and 1/2c) from dairy and meat, were obtained from the Jilin Entry–Exit Inspection and Quarantine Bureau. The bacteria were grown in brain–heart infusion (BHI) broth at 37 °C. Inspection and Quarantine Bureau. The bacteria were grown in DMEM (Dulbecco’s modified Eagle’s medium (Gibco) containing 10% fetal bovine serum (Gibco)) and 100 U penicillin ml⁻¹ and 100 μg streptomycin ml⁻¹ at 37 °C in a 5% CO₂ atmosphere. Honokiol was obtained from the National Institutes for Food and Drug control, dissolved in DMSO (Sigma-Aldrich) under sterile conditions and stored at 4 °C.

**Antimicrobial susceptibility testing.** The MICs of honokiol against the 10 *L. monocytogenes* isolates was determined using the microdilution method (Bertsch et al., 2013). Twofold serial dilutions of honokiol (100 μl) were prepared in 96-well microplates. An overnight culture of test bacteria was centrifuged, washed twice with PBS and diluted to achieve a cell density of 5 × 10⁴ number of colony forming units (c.f.u.) ml⁻¹. The cell suspension (100 μl) suspended in BHI broth was inoculated into each well of the microplate to give a total volume in each well of 200 μl. The microplates were incubated for 24 h at 37 °C, and turbidity was examined using a microtitre plate reader at a wavelength of 630 nm. All experiments were performed in triplicate, and the median MIC value was calculated. Meanwhile, the *in vitro* cytotoxicity of honokiol was assayed by a thiazolylblue tetrazolium bromide (MTT) assay live cell staining method as described by Liu et al. (2012).

**Growth curves.** Growth curve studies were performed in BHI broth with different concentrations of honokiol. *L. monocytogenes* strain EGD was grown at 37 °C to an OD₆0₀ of 0.3, and a 100 ml cell suspension was divided into five 100 ml Erlenmeyer flasks. Honokiol was added to four of the cultures to obtain final concentrations of 0.5, 1, 2 and 4 μg ml⁻¹. A sample containing 0.1 % DMSO was used as a control. The samples were further cultured at 37 °C with shaking. After 30, 60, 90 120, 150 and 180 min, 50 μl aliquots of each culture were collected and monitored spectrophotometrically at OD₆0₀ (Qiu et al., 2010). The experiment was repeated three times.

**Analysis of bacterial growth in macrophages.** To observe *L. monocytogenes* phagocytosis by the RAW264.7 macrophage-like cell line, RAW264.7 cells were infected with strain EGD for 2 h at an m.o.i. of 50. The infected cells were treated with honokiol at various concentrations in fresh DMEM containing 10 % FBS. The cells were then treated with Hoechst 33342 for 20 min to stain the nuclei and washed once with PBS. Non-treated, infected cells served as the control group. The experimental groups had triplicate samples. Images were captured using an Olympus FV1000 confocal laser scanning microscope (Olympus) with a ×60 objective lens. Image analyses and export were performed using a Fluoview version 1.7.3.0 (Olympus).

To quantify the number of viable *L. monocytogenes* in RAW264.7 cells, a classical gentamicin protection assay based on the quantification of c.f.u. in infected cells was used (Kühbacher et al., 2014). *L. monocytogenes* EGD was added at an m.o.i. of 50 to RAW264.7 cells grown in DMEM containing 10 % FBS at 24-well plates at 2.5 × 10⁴ cells per well. The 24-well plate was centrifuged at 2000 g for 10 min and incubated for 2 h at 37 °C in a humidified incubator containing 5 % CO₂. Two hours later, the infected cells were exposed to 100 μg gentamicin ml⁻¹ in culture medium for 1 h, followed by three thorough washes with PBS to remove killed extracellular bacteria. The infected RAW264.7 cells were then treated with honokiol at various concentrations in fresh DMEM containing 10 % FBS for 3 h. The surviving intracellular bacteria were harvested by lysis with 500 μl 0.1 % Triton X-100 in PBS at 37 °C for 10 min. The cell lysates were immediately serially diluted with PBS and spread onto agar plates. The number of c.f.u. was determined after incubation for 24 h at 37 °C. The experiment was repeated three times.

**LLO activity assay.** LLO activity was assayed using sheep red blood cells, as described by McKellar (1992). *L. monocytogenes* EGD was cultured in BHI broth containing graded subinhibitory concentrations of honokiol to the early stationary growth phase (OD₅₅₀=3.6). A bacterial suspension without the test compound served as a control. The *L. monocytogenes* cultures were harvested and centrifuged at 5500 g (3 min, 4 °C). A 0.1 ml sample of supernatant was made up to 1 ml with haemolysin buffer (0.145 M NaCl, 0.02 M CaCl₂) prior to the addition of 25 μl defibrinated sheep red blood cells. A volume of 0.1 ml culture medium served as a negative control. The mixture was incubated for 30 min at 37 °C and centrifuged at 5500 g (3 min, 4 °C). The inhibition of haemolysis by honokiol was observed visually, and the absorbance values at 543 nm (A₅₄₃) of the supernatants were measured. The percentage of haemolysis was determined by comparison to a control sample without honokiol.

**Immunoblot analysis of LLO and p60.** The bacterial culture supernatants described above were also used for immunoblot analysis of the virulence factors LLO and p60 secreted by *L. monocytogenes* EGD. The proteins were size separated by discontinuous SDS-PAGE (12.5 % acrylamide) and transferred to PVDF membranes using a semi-dry transfer apparatus (Bio-Rad). After blocking for 2 h, the membranes were then incubated with the primary antibody, a mouse anti-*L. monocytogenes* LLO antibody (Abcam) or a mouse anti-*L. monocytogenes* p60 (Millipore), at 4 °C overnight. The membranes were washed three times with TBS and 0.05 % Tween 20 before incubating with HRP-conjugated anti-mouse antibodies used as the secondary antibody (Cell Signalling) at 25 °C for 2 h. The protein bands were detected using an ECL Plus kit (Beyotime). The protein images were scanned using a CanoScan LiDE 100 scanner (Canon) and analysed using ImageJ software.

**Challenge test to detect the hly and iap genes in pasteurized milk samples using real-time reverse transcription PCR.** Samples (24 ml) of pasteurized milk in the presence or absence of graded subinhibitory concentrations of honokiol (0, 1, 2 and 4 μg ml⁻¹) were inoculated with 1 ml serially diluted overnight culture of *L. monocytogenes* EGD to obtain a final concentration of 1 × 10⁴ c.f.u. in 25 ml, and were cultured to the post-exponential growth phase. The cells were then collected by centrifugation at 10 000 g for 5 min at 4 °C, and total RNA was extracted with TRIzol reagent (Invitrogen). The cell pellets were resuspended in 1 ml TRIzol, and cDNA was synthesized using a PrimeScript RT Reagent kit (Takara Bio). Target gene mRNA expression was quantified using SYBR Premix Ex Taq II (Takara Bio). The following primers were used: *hly* gene: forward primer 5'-AAGAATTCCTGTAGAGGAGGATGAAAC-3' and reverse primer 5'-GGCGGATCCTTTTTATTACTTT-3' (fang et al., 2006); *iap* gene: forward primer 5'-TAGGCACATTGGTAAACG-3' and reverse primer 5'-TGTCACGCTTTATTTAAG-3' (Jiang et al., 2012); 16S rRNA gene, forward primer 5'-GAGTGATGATGTTGATTACC-3' (Oba et al., 2005).
5'-GATGCATAGCGACCTGAGA-3' and forward primer 5'-TGC-TCCGTACACTTTGCAGT-3' (van der Veen & Abee, 2010). The reactions were performed using an ABI Prism 7000 sequence detection system with an initial step of 10 min at 95 °C and 40 cycles of 15 s at 95 °C and 1 min at 60 °C. All samples were analysed in triplicate and normalized against 16S rRNA gene expression. The threshold cycle (Ct) values were determined and the mean value determined to detect the fold change in expression (García et al., 2010).

**Determination of TNF-α in RAW 264.7 macrophages stimulated by L. monocytogenes supernatants.** The production of TNF-α secreted by RAW264.7 macrophages was determined by immunoblot assay. Briefly, L. monocytogenes EGD was grown overnight in fresh BHI broth to an OD600 of >2.5, and the supernatant was collected by centrifugation at 5500 g (3 min, 4 °C). The L. monocytogenes supernatants were then diluted 30-fold into 40 ml DMEM. After incubation at 37 °C for 30 min with constant shaking, 10 ml each was placed into four triangular flasks. The culture samples were combined with graded subinhibitory concentrations of honokiol (0, 1, 2 and 4 μg ml⁻¹) and further incubated at 37 °C for 4 h with constant shaking. Finally, the L. monocytogenes supernatants containing secreted proteins were collected and filtered through a 0.2 mm pore-sized filter. RAW264.7 cells were plated at 1 × 10⁵ cells per well in a 48-well tissue culture plate for adherence. After 24 h, the adherent cells were cultured in medium containing 150 μl DMEM and 50 μl L. monocytogenes supernatant at 37 °C for 16 h. The supernatants were then collected and centrifuged at 1000 g for 5 min. The concentration of TNF-α in the supernatants was measured using a mouse TNF-α platinum ELISA kit (eBioscience).

**RESULTS**

**Antimicrobial susceptibility and growth inhibition of honokiol against L. monocytogenes**

The MICs of honokiol against 16 L. monocytogenes strains ranged from 8 to 16 μg ml⁻¹. From the growth curves (Fig. 1), we found that 0.0625 × MIC (0.5 μg ml⁻¹), 0.125 × MIC (1 μg ml⁻¹) and 0.25 × MIC (2 μg ml⁻¹) of honokiol had no obvious effects on the growth of L. monocytogenes EGD compared with the control. However, the growth velocity was attenuated to 64 % after 30 min in the presence of 0.5 × MIC (4 μg ml⁻¹) compared with the untreated culture. At the same time, the cytotoxicity of honokiol in RAW264.7 cells was evaluated using an MTT assay. The concentration inducing 50 % apoptosis (IC50) of honokiol was 29 μg ml⁻¹. Our results indicated that honokiol inhibits L. monocytogenes growth and the secretion of LLO and P60 in a dose-dependent manner far below the IC50 value.

**Analysis of L. monocytogenes growth in macrophages**

The number of intracellular L. monocytogenes in RAW264.7 cells was assessed by confocal microscopy, which showed that the number of intracellular bacteria in the groups treated with honokiol was much lower than that of the control group (Fig. 2a). The number of viable bacteria in the RAW264.7 cells was also determined by c.f.u. quantification. The results indicated that the number of intracellular bacteria in the groups treated with 8 and 16 μg ml⁻¹ honokiol was much lower than that of the untreated group (Fig. 2b; P < 0.05 and P < 0.01, respectively). Thus our results indicated that honokiol can improve L. monocytogenes clearance in macrophages.

**Honokiol attenuates LLO activity and the expression of LLO and p60 proteins**

Compared with the control group, the LLO activity of L. monocytogenes EGD supernatants was attenuated when pre-incubated with subinhibitory concentrations of honokiol (Fig. 3). The LLO activity was reduced to 71, 61 and 43 % when the selected strain EGD was cultured with 1, 2 and 4 μg honokiol ml⁻¹, respectively. As shown in Fig. 4, subinhibitory concentrations of honokiol reduced the secretion of exotoxin proteins LLO and P60 by L. monocytogenes in a dose-dependent manner by immunoblot assay.

**Honokiol represses the transcription of genes encoding LLO and p60 in L. monocytogenes**

As shown in Fig. 5, a significant reduction in hly and iap transcription was observed in L. monocytogenes EGD when incubated with honokiol. Compared with the control, the transcript levels of hly were decreased to 80 %, 60 % (P < 0.01) and 40 % (P < 0.01) when L. monocytogenes EGD was exposed to 1, 2 and 4 μg ml⁻¹ honokiol, respectively. Similarly, the transcript levels of iap were also reduced to 70 % (P < 0.05), 62 % (P < 0.05) and 30 % (P < 0.01). Therefore, both genes were notably inhibited at the transcriptional level by honokiol in a dose-dependent manner. This result is consistent with the results of the immunoblot analysis of LLO and P60 (Fig. 4). Previous studies (Hein et al., 2001; Nogva et al., 2000; Norton and Batt, 1999) indicate that the LLO gene (hly) and the protein p60 gene (iap)
are the most commonly used PCR amplification targets for detection of *L. monocytogenes*. Thus, detection of these two genes at the transcript level has important practical significance in food samples. Our data indicated that honokiol acts as a potential inhibitor of transcription of the exotoxin *hly* and *iap* genes in food samples such as milk.

**TNF-α production by RAW264.7 cells stimulated with supernatants of *L. monocytogenes***

It has been shown that the ability to produce LLO is unique to virulent *L. monocytogenes*, with only LLO-producing strains having the ability to induce T-cell-mediated immunity, which plays a key role in the acquired resistance of the host (Nishibori *et al.*, 1996). This prompted us to examine the influence of *L. monocytogenes* supernatants on the levels of TNF-α produced by RAW264.7 cells. As shown in Fig. 6, compared with the control group (291 pg ml⁻¹), the production of TNF-α was reduced to 122, 57 and 11 pg ml⁻¹ when RAW264.7 cells were cultured with the supernatants of *L. monocytogenes* EGD treated with 1, 2 and 4 µg honokiol ml⁻¹, respectively. Our experimental results showed that honokiol did not influence cytokine production by RAW264.7 cells (data not shown).

---

**Fig. 2.** Honokiol improves *L. monocytogenes* clearance in macrophages. (a) RAW264.7 cells infected by *L. monocytogenes* EGD were treated with honokiol at 8 and 16 µg ml⁻¹. The nuclei were stained with Hoechst 33342 and the cells observed by confocal microscopy. The number of intracellular bacteria in each group were counted and expressed as c.f.u. DIC, differential interference contrast. (b). The experiments were repeated three times and the mean ± SD value was calculated (*P*<0.05, **P**<0.01.)
DISCUSSION

*L. monocytogenes*, a human pathogen, is associated with foodborne disease. The anti-listerial properties of many components, such as essential oils of eugenol (Filgueiras & Vanetti, 2006), cinnamon, clove, oregano, pimento, thyme (Aureli *et al.*, 1992) and rosemary (Pandit & Shelef, 1994), have been described. In the present study, we evaluated the activity of honokiol against 16 *L. monocytogenes* strains. As a natural plant product, honokiol showed a marked anti-*L. monocytogenes* effect, with MIC values of 8–16 µg ml⁻¹. This finding is consistent with previous studies demonstrating that honokiol possesses good antimicrobial activity (Ho *et al.*, 2001; Hu *et al.*, 2011). The anti-*L. monocytogenes* activity of honokiol, with a lower MIC value, was stronger than other naturally occurring compounds, such as the essential oils eugenol (Filgueiras & Vanetti, 2006), bay, cinnamon, clove, thyme (Fazlara *et al.*, 2008; Smith-Palmer *et al.*, 1998) and rosemary (Pandit & Shelef, 1994).

The results from the present study indicate that LLO produced by *L. monocytogenes* is sensitive to subinhibitory concentrations of honokiol. Values represent means ± SD for three independent experiments (*P*<0.05, **P**<0.01, compared to honokiol-untreated cells).

---

**Fig. 3.** (a) Haemolysis assays performed with *L. monocytogenes* EGD culture supernatants in the absence or presence of graded concentrations of honokiol added to sheep red blood cells in haemolysin buffer. (b) Haemolysis was monitored by measuring 843. Values represent means ± SD derived from three experiments. Student’s paired t-test was used to compare each culture with honokiol to the untreated culture (*P*<0.05, **P**<0.01).

**Fig. 4.** Immunoblot analysis of LLO (a) and P60 (b) production by *L. monocytogenes* EGD exposed to different subinhibitory concentrations of honokiol.

**Fig. 5.** Relative expression levels of the *hly* and *iap* genes in *L. monocytogenes* EGD with different subinhibitory concentrations of honokiol. Values represent means ± SD from three independent experiments (*P*<0.05, **P**<0.01, compared to honokiol-untreated cells).

**Fig. 6.** TNF-α production by RAW264.7 cells stimulated with supernatants of *L. monocytogenes* EGD treated with different subinhibitory concentrations of honokiol. Values represent means ± SD for three independent experiments. Student’s paired t-test was used to compare each culture with honokiol with the untreated culture (**P**<0.01).
concentrations of honokiol. Compared with previous studies, our results indicate that the honokiol was more effective in inhibiting LLO secretion than eugenol (Filgueiras & Vanetti, 2006), sorbate and NaCl (Mckellar, 1993). The major extracellular protein (p60) produced by L. monocytogenes, which acts as a murein hydrolase required in the last step of cell division, was also analysed. The results of the immunoblot assay revealed that honokiol at subinhibitory concentrations was effective in inhibiting p60 secretion in a dose-dependent manner. Our results indicate that both L. monocytogenes growth and LLO and p60 production are sensitive to honokiol. This study presents alternative solutions for reducing the virulence of L. monocytogenes.

Previous studies (Hein et al., 2001; Nogva et al., 2000; Norton & Batt, 1999) have indicated that the LLO gene (hly) and p60 gene (iap) are the most commonly used PCR amplification targets for the detection of L. monocytogenes. Thus, the detection of these two genes at the transcript level has important practical significance in food samples. The results of transcription of virulence-associated genes result are consistent with the results of the immunoblot analysis of LLO and p60. Our data indicated that honokiol acts as a potential inhibitor of exotoxin hly and iap gene transcription in food samples, such as milk.

A previous study indicated that virulent L. monocytogenes strains with the ability to produce LLO are known to induce the expression of various cytokines in the infected host and in the macrophage-like cell line P388D1. Among the inflammatory cytokines induced by LLO, pro-inflammatory TNF-α appears to be produced mainly by macrophages (Nishibori et al., 1996). Sashinami et al. (2010) reported that mouse macrophage RAW264.7 cells produce TNF-α in response to stimulation with recombinant p60. These findings indicate that LLO and p60 modulate innate immune responses against L. monocytogenes infection. Therefore, TNF-α release assays were performed to elucidate the biological relevance of changes in LLO and p60 secretion induced by honokiol. Our results indicated that the reduction in the amount of TNF-α produced by RAW264.7 cells stimulated with L. monocytogenes supernatants might be related to a decrease in LLO and p60 secreted by L. monocytogenes after treatment with subinhibitory concentrations of honokiol.

In conclusion, honokiol clearly exhibits inhibitory action on L. monocytogenes growth and the secretion of the exotoxin proteins LLO and p60. In view of the broad spectrum of antimicrobial activities of honokiol, as reported previously, as well as the findings reported in this study, we propose that honokiol could be used as an anti-L. monocytogenes compound and virulence inhibitor in the food and pharmaceutical industries. Although honokiol inhibits L. monocytogenes growth and the secretion of LLO and p60, there is little information regarding the actual mechanism. Therefore, further experiments, such as animal and clinical studies, should be carried out.

ACKNOWLEDGEMENTS

Financial support for this work came from the following sources: the National Nature Science Foundation of China (nos 31271951 and 31172364), China Postdoctoral Science Foundation (2013M530142), the Program for New Century Excellent Talents in University (NCET-13-024 and NCET-09-0434) and the Important National Science and Technology Specific Projects (2012ZX10003002).

REFERENCES


