Immuno-Modulatory Effects of Bacteriocin-Producing *Pediococcus pentosaceus* JWS 939 in Mice

Hyun Jong Choi¹, Ji Ye Kim¹, Myeong Su Shin^{1,3}, Sang Myeong Lee², and Wan Kyu Lee^{1*}

¹College of Veterinary Medicine and Research Institute of Veterinary Medicine,
Chungbuk National University, Cheongju 361-763, Korea

²Division of Biotechnology, College of Environmental & Bioresource Sciences, Chonbuk National University,
Iksan 570-752, Korea

³Korea Bio Science Research Institute of Organic Bio Tech Co., Ltd., Jincheon 365-861, Korea

Abstract

Pediococcus pentosaceus JWS 939 (JWS 939) is a nonpathogenic bacteriocin-producing probiotic isolated from the duck intestine. This study assessed the immunomodulatory effects of JWS 939 and compared them with those of *Lactobacillus rhamnosus* GG (LGG), a well-known immune enhancer. The immune-enhancing effects of JWS 939 were measured by measuring the production of nitric oxide (NO) and cytokines in C57BL/6 mouse peritoneal macrophages. In addition, to assess the immune enhancement abilities of JWS 939, *in vivo*, a *Listeria monocytogenes* challenge mice model was used. The results showed that heat-killed JWS 939 induced more NO and interleukin (IL)-1 β production in mouse peritoneal macrophages than in LGG, and that oral administration of viable JWS 939 in mice increased more NO, IL-1 β , and tumor necrosis factor (TNF)- α level than in LGG in serum upon *L. monocytogenes* challenge. In addition, mice fed with JWS 939 had a longer survival time after lethal challenge with *L. monocytogenes*, and these effects were stronger than those induced by LGG. Collectively, *P. pentosaceus* JWS 939 is a remarkable strain that, by releasing bacteriocin and enhancing host immune responses, may have potential as a duck feed additive to suppress pathogens.

Key words: lactic acid bacteria, immune response, bacteriocin(s), *Listeria*, macrophage(s)

Introduction

Pediococcus pentosaceus is a Gram-positive probiotic that is commonly found in cheese and yogurt and is reported to be a beneficial microbe (Haakensen *et al.*, 2009). This probiotic produces various bacteriocins, called pediocins, and the bacteriocin produced by *P. pentosaceus* inhibits growth of pathogenic bacteria such as *Clostridium perfringens* and *Listeria monocytogenes* (Daeschel and Klaenhammer 1985; Wu *et al.*, 2004). *P. pentosaceus* also has immunomodulatory effects. Earlier studies reported that *P. pentosaceus* induces interferon- γ and interleukin (IL)-12, and suppresses IL-4 in mouse spleen cells or peritoneal macrophages (Igarashi, 2010; Jonganurakkun *et al.*, 2008).

Macrophages are phagocytes that have differentiated

from monocytes and exist within host tissues. These cells also play an important role in host immune responses (van Furth *et al.*, 1972). Various stimuli including bacteria, lipopolysaccharides (LPS), lymphokines, and interferons activate macrophages, and activated macrophages regulate host immunity (Lorsbach *et al.*, 1993). Nitric oxide (NO) and proinflammatory cytokines such as IL-1 β and tumor necrosis factor- α (TNF- α) are produced by macrophages (Marin *et al.*, 1997; Schreiber *et al.*, 1985). NO is a short-lived mediator that either kills or restrains the growth of bacteria and tumor cells (Snyder and Brecht, 1992). IL-1 β is a proinflammatory cytokine that triggers a cascade of immune responses to induce secretion of other cytokines and chemokines. It also induces a variety of cellular responses such as cell proliferation, differentiation, and apoptosis (Dinarello, 1996). TNF- α has a broad spectrum of systemic and cellular immunity activities and, also, suppresses intracellular pathogens and controls inflammatory processes to enhance resistance to infectious disease (Derouich-Guergour *et al.*, 2001).

Bacteriocins are substances from bacteria and produce

*Corresponding author: Wan-Kyu Lee, College of Veterinary Medicine and Research Institute of Veterinary Medicine, Chungbuk National University, Cheongju 361-763, Korea. Tel: 82-43-261-2960, Fax: 82-43-267-3150, E-mail: wklee@cnu.ac.kr

antimicrobial effects by suppressing the synthesis of the target bacteria's inner membrane. Bacteriocins produced by lactic acid bacteria have a particular ability to kill pathogenic bacteria such as *Listeria monocytogenes* or *Clostridium perfringens* (Chanos and Williams, 2011; Grilli *et al.*, 2009). Therefore, bacteriocin-producing lactic acid bacteria may be an effective substitute for antibiotics (Nes *et al.*, 2007).

Bacteriocin-producing *P. pentosaceus* JWS 939 (JWS 939) has been isolated from the intestines of ducks (Shin *et al.*, 2007). Bacteriocin produced by JWS 939 is effective in killing *L. monocytogenes* and *Streptococcus mutans* *in vitro* and this activity is not destroyed within a pH range of 2.0 to 9.0 or by heating (95°C for 30 min). It also retains this ability following exposure to enzymes such as catalase and amylase (Shin *et al.*, 2007).

The present study investigated the immune effects of bacteriocin-producing JWS 939. The JWS 939 was heat-killed and its immune effects *in vitro*, based on NO and inflammatory cytokines productions on C57BL/6 mouse peritoneal macrophages were examined. Thereafter, a *L. monocytogenes*-infected mice model was used to examine the protective effects of JWS 939 *in vivo*. The *in vitro* and *in vivo* effects were compared with those mediated by *Lactobacillus rhamnosus* GG (LGG), a well-known probiotic with immune enhancement effects.

Materials and Methods

Microorganism preparation

Probiotics (JWS 939 and LGG) were grown in MRS broth (BD, USA) at 37°C for 24 h, and viable probiotic cells were counted in an MRS plate. Then, the probiotic cells were collected by centrifugation at 12,000 rpm for 10 min at 4°C and the supernatant discarded. Cell pellets were washed twice and resuspended in 0.01 M sterile phosphate buffered saline (PBS; pH 7.2). For *in vitro* experiments, probiotic cells were killed by heating at 110°C for 15 min and stored at -20°C until use. All probiotic stocks were diluted to 5×10^8 CFU/mL for high concentration dosage or 1×10^8 CFU/mL for low concentration dosage. For the *in vivo* tests, the viable probiotic cell pellets were concentrated to 1×10^{10} CFU/mL in PBS containing 10% skim milk.

L. monocytogenes BA00092 (porcine origin; National Veterinary Research & Quarantine Service of Korea, Korea) were grown overnight in BHI broth (BD, USA) at 37°C and the number of viable bacterial cells was counted

in a BHI plate. Cell pellets were collected by centrifugation at 12,000 rpm for 5 min at 4°C; subsequently, the cells were washed twice and diluted to 2×10^6 CFU/mL in PBS.

Isolation of murine peritoneal macrophages

Mouse peritoneal macrophages were isolated according to the method of Zhang *et al.* (2008). Briefly, Brewer thioglycollate medium (Sigma, USA) was injected via an intra-peritoneal (*i.p.*) route to C57BL/6 mouse. After 4-5 d, peritoneal macrophages were collected from the peritoneal cavities of the treated mice. Mice were euthanized with CO₂ and 5 mL PBS were injected into the peritoneal cavity. Then, the cavity fluid was aspirated and centrifuged at 1,500 rpm for 8 min at 4°C. The cell pellet was washed twice with PBS and counted in a hemacytometer, cell viability was checked before they were diluted to 1×10^6 cells/mL in a RPMI 1640 medium (Sigma, USA) supplemented with 10% (v/v) FBS (Invitrogen, USA) along with, 100 mg/mL streptomycin and 100 U/mL penicillin (Invitrogen, USA).

Activation of peritoneal macrophages

Peritoneal macrophages (5×10^5 cells/well) were cultured in triplicate and heat-killed probiotics (100 μ L containing 5×10^7 CFU/mL or 1×10^7 CFU/mL of LGG or JWS 939) were added to the wells. The probiotic concentrations were such that the macrophages were exposed to either 20 or 100 probiotic cells per macrophage, at 37°C with 5 % CO₂. Instead of probiotics, PBS was only added to wells as a negative control, and positive control was added LPS (100 or 500 ng/mL; Sigma, USA) in PBS-containing wells. After 24 h, the culture supernatants were collected and the concentrations of NO and cytokines (IL-1 β and TNF- α) in the supernatants were determined.

Measurement of NO

NO levels of macrophages were measured using Griess reagent (Promega, USA). Briefly, 50 μ L of macrophage culture supernatant were mixed (in triplicate) with an equal volume of 1% sulfanilamide in 5% phosphoric acid and 0.1% *N*-1-naphylethylenediamine dihydrochloride at room temperature for 10 min in the dark. Absorbance was then measured at 540 nm by using a microplate reader (Molecular Devices, USA). The production levels of NO were calculated from a standard curve for a nitrite standard (0-100 μ M sodium nitrite).

Measurement of cytokines

Cytokines (IL-1 β and TNF- α) were measured using ELISA kits (BD, USA) in accordance with the manufacturer's instructions. Samples were assayed in triplicate and absorbance read at 450 nm in a microplate reader. Cytokine levels were then calculated from a standard curve of the cytokine standards (0-2,000 pg/mL for IL-1 β ; 0-1,000 pg/mL for TNF- α).

Animals and *L. monocytogenes* challenge methods

Female 7-9 wk old BALB/c mice were purchased from Nara Biotech (Korea) and were housed under 12 h light/dark cycles with a commercial rodent diet and water *ad libitum*. Mice were given a daily dose of 1×10^9 CFU of viable LGG or viable JWS 939 in 100 μ L PBS containing 10 % skim milk via a gastric tube for 2 wk. The negative control group (NC) and positive control group (PC) was given 100 μ L PBS containing 10 % skim milk without probiotics. After 2 wk, both the probiotics-fed groups and the PC were injected with *L. monocytogenes* (1.2×10^5 CFU in 100 μ L PBS) via the tail vein. The NC was injected with 100 μ L PBS without *L. monocytogenes*.

Collection of serum and tissue samples for immunological and bacteriological analyses

Three days after infection with *L. monocytogenes*, 7 mice per group were euthanized and their body weight determined. In addition, serum was collected and their livers and spleens were weighed. Mice sera were analyzed for levels of NO and cytokines (IL-1 β and TNF- α). Liver and spleen weights were expressed relative to body weight. The liver was aseptically homogenized by passage through a 70 μ m nylon mesh (Fisher Scientific, USA); subsequently, the number of viable *L. monocytogenes* cells was counted in a BHI plate. The results were expressed as log-based data.

Survival analysis

Ten mice per group were used for survival time analysis. Mice were fed probiotics or PBS containing 10% skim milk for 2 wk and then challenged with *L. monocytogenes* as described above. Survival time was monitored every 8 h until death.

Statistical analysis

SPSS for Windows version 12.0 (SPSS Inc., USA) was used for statistical analysis of data. Significant differences between groups were tested by ANOVA and compared using Tukey's and Duncan's multiple range tests (p

< 0.05), except for survival time analysis. Survival time was analyzed by using Student's t-test ($p < 0.05$).

Results

NO and cytokines production of peritoneal macrophages *in vitro*

To examine the immunomodulatory effects of JWS 939, the production of NO and cytokines (IL-1 β and TNF- α) triggered by heat-killed JWS 939 was measured *in vitro* using peritoneal macrophages. Although NO production was induced by both JWS 939 and LGG heat-killed probiotics, the NO production from JWS 939 treatment was greater than that from LGG (Fig. 1A). Productions of NO in the low concentration (1×10^7 CFU/mL) of JWS 939 and in both the low and high concentration of LGG were not statistically different from that in the PBS control. However, the high concentration (5×10^7 CFU/mL) of JWS 939 induced NO production of 3.78 ± 0.07 μ M/mL, which was significantly higher than the production in the 100 ng/mL LPS-treated positive control.

JWS 939 and LGG increased IL-1 β production in a dose-dependent manner (Fig. 1B) with production from JWS 939 treatment higher than that from LGG treatment. IL-1 β was barely detectable in low dose (1×10^7 CFU/mL) LGG-treated macrophages. LGG at the high dosage (5×10^7 CFU/mL) induced 222.32 ± 4.87 pg/mL of IL-1 β production, which was significantly lower than that induced by JWS 939 at 1×10^7 CFU/mL (1683.64 ± 35.00 pg/mL). Moreover, JWS 939 at the high dosage (5×10^7 CFU/mL) induced IL-1 β production at a level significantly higher than that induced by 500 ng/mL LPS (2845.45 ± 97.71 pg/mL vs. 2097.97 ± 15.44 pg/mL, respectively).

The level of TNF- α production showed a different pattern (Fig. 1C). LGG induced TNF- α production in dose-dependent manner and triggered significantly greater TNF- α synthesis than 500 ng/mL LPS. On the other hand, the low concentration (1×10^7 CFU/mL) JWS 939 dosage induced higher levels of TNF- α than the same concentration of LGG or 500 ng/mL LPS, but the JWS 939 and LGG difference was not statistically significant. Moreover, JWS 939 did not show a dose-dependent effect on TNF- α production.

Immune effects of JWS 939 in *L. monocytogenes* challenge model

To assess whether JWS 939 stimulates macrophages *in vivo* and protects the host from acute lethal bacterial infection, a well-established *L. monocytogenes* infection

Fig. 1. Nitric oxide (NO) (A), IL-1 β (B), and TNF- α (C) production by murine peritoneal macrophages induced by two concentrations of heat-killed *L. rhamnosus* GG (LGG) and *P. pentosaceus* JWS 939 (JWS 939). Murine peritoneal macrophages (5×10^5 cells/mL) were stimulated with probiotics or PBS for 24 h. LGG and JWS 939 concentrations were 1×10^7 CFU/mL. LGG $\times 5$ and JWS 939 $\times 5$ concentrations were 5×10^7 CFU/mL. L100 or L500 means LPS at 100 ng/mL or 500 ng/mL, respectively. Data are mean $\pm$ SD values of triplicates. Different superscript letters (a, b, c and d) indicate statistical differences as determined by ANOVA ($p < 0.05$).

model was used. Viable JWS 939 or LGG cells were orally administrated to female BALB/c mice daily for 2 wk prior to *L. monocytogenes* challenge.

All three *L. monocytogenes* challenge groups (PC, LGG-fed, and JWS 939-fed mice) lost body weight compared to that in the NC (no *L. monocytogenes* infection); however, no differences in body weights were observed between the three *L. monocytogenes* challenge groups (Table 1). The three *L. monocytogenes* challenge groups had significantly higher relative liver weights than that in the NC. In addition, LGG-fed and JWS 939-fed groups had significantly heavier relative liver weight than that in the PC. With regard to relative spleen weights, it was not significantly higher in the JWS 939-fed group than in the NC, but the relative spleen weights in the PC and LGG-fed groups were significantly higher than that in the NC.

Both of the probiotics-fed groups had significantly lower numbers of viable *L. monocytogenes* in their livers compared to the PC (Fig. 2). The JWS 939-fed mice had fewer bacteria in the liver than the LGG-fed mice, but the difference was not significant. Livers from the PC contained an average of 4.3×10^8 CFU/g *L. monocytogenes* cells; those of the JWS 939-fed and LGG-fed groups contained 89% and 87% fewer bacteria, respectively.

The level of NO in the sera of JWS 939-fed mice was significantly higher than that in the NC (8.45 ± 0.73 μ M vs. 4.70 ± 0.64 μ M, respectively), but the NO levels in the PC (7.08 ± 1.37 μ M) and LGG-fed mice (6.96 ± 0.67 μ M) were not significantly different from that in the NC. The NO level in the JWS 939-fed group was higher than that in any of the other groups, but it was only statistically different from that in the NC (Fig. 3A).

Table 1. Effect of *Pediococcus pentosaceus* JWS 939 on the body weight and relative liver and spleen weights of mice 3 d after *Listeria monocytogenes* challenge

Group	Body weight (g)	Liver/BW (g/kg)	Spleen/BW (g/kg)
NC	18.38 $\pm$ 0.27 ^a	46.99 $\pm$ 1.53 ^a	5.47 $\pm$ 0.08 ^a
PC	15.31 $\pm$ 0.26 ^b	69.56 $\pm$ 2.01 ^b	7.35 $\pm$ 0.49 ^b
LGG	14.67 $\pm$ 0.40 ^b	70.45 $\pm$ 0.71 ^c	6.81 $\pm$ 0.22 ^b
JWS939	14.30 $\pm$ 0.21 ^b	71.79 $\pm$ 1.30 ^{cd}	5.21 $\pm$ 0.21 ^a

Data represent the mean $\pm$ SE of seven mice in each group. Different superscript letters (^{a,b,c} and ^d) indicate the statistical differences determined by ANOVA ($p < 0.05$). NC, normal control group (no probiotic-feeding and no *L. monocytogenes* infection); PC, positive control group (no probiotic-feeding and infected with *L. monocytogenes*); LGG, *Lactobacillus rhamnosus* GG-fed and infected with *L. monocytogenes* group; JWS 939, *Pediococcus pentosaceus* JWS 939-fed and infected with *L. monocytogenes* group.

Fig. 2. Viable cells of *L. monocytogenes* in liver of BALB/c mice in the *L. monocytogenes* challenge model. Data represent log data of mean $\pm$ SE of seven mice in each group. PC, positive control group (no probiotic-feeding and infected with *L. monocytogenes*). LGG, *L. rhamnosus* GG-fed and infected with *L. monocytogenes*; JWS 939, *P. pentosaceus* JWS 939-fed and infected with *L. monocytogenes*. Different superscript letters (^a and ^b) indicate statistical differences as determined by ANOVA ($p < 0.05$)

The levels of IL-1 β and TNF- α in the serum showed a similar tendency. Only the JWS 939-fed mice showed significant differences from the other groups (Fig. 3B and C). Levels of IL-1 β and TNF- α in the LGG-fed group were not significantly different from those in the NC or PC. On the other hand, JWS 939-fed mice induced 1082.57 $\pm$ 298.12 pg/mL of IL-1 β and 895.67 $\pm$ 280.54 pg/mL of TNF- α production, showing significant differences from all other groups.

JWS 939-fed mice survived longer than those in the PC and LGG-fed groups (Table 2). Mice in the NC did not die during the experimental period, but all mice in the PC and probiotics-fed groups died within 150 h after *L. monocytogenes* challenge. Mean survival time of the LGG-fed group was not significantly different from that in the PC, but that in the JWS 939-fed group was significantly different from survival in the PC.

Discussion

Probiotics are widely used as food supplements or therapeutic agents because they enhance host immune responses by increasing the production of NO and cytokines in macrophages (Saarela *et al.*, 2000; Tejada-Simon and Peska, 1999). Here, JWS 939, isolated from duck intestines, was examined to assess its immune enhancing effects. To that end, levels of NO and inflammatory cytok-

Fig. 3. Nitric oxide (A), IL-1 β (B), and TNF- α (C) production in BALB/c mice sera in the *L. monocytogenes* challenge model. Data represent mean $\pm$ SE of seven mice in each group. NC, normal control group (no probiotic-feeding and no *L. monocytogenes* infection); PC, positive control group (no probiotic-feeding and infected with *L. monocytogenes*). LGG, *L. rhamnosus* GG-fed and infected with *L. monocytogenes*; JWS 939, *P. pentosaceus* JWS 939-fed and infected with *L. monocytogenes*. Different superscript letters (^a, ^b and ^c) indicate statistical differences as determined by ANOVA ($p < 0.05$).

Table 2. Mean survival time of mice fed LGG or JWS 939 and infection to *L. monocytogenes*

Group	Survival time (h)
NC (no probiotic-feeding and no <i>L. monocytogenes</i> infection)	-
PC (no probiotic-feeding and infected with <i>L. monocytogenes</i>)	102.00±3.86
LGG (<i>Lactobacillus rhamnosus</i> GG-fed and infected with <i>L. monocytogenes</i>)	112.95±5.42
JWS 939 (<i>Pediococcus pentosaceus</i> JWS 939- fed and infected to <i>L. monocytogenes</i>)	116.14±5.32*

Data are mean±SE of 10 mice in each group. All mice of NC did not die in 160 h. *indicates the statistical different with PC as determined by student's *t*-test ($p<0.05$).

ines were measured in mouse peritoneal macrophages and in a *L. monocytogenes* infection model. The mediation of immune effects by JWS 939 was compared with that by LGG, a probiotic strain known to have immunomodulatory effects. LGG has been shown to induce immune cells to produce NO and inflammatory cytokines. For example, dendritic cells stimulated by LGG have shown increased production of IL-12, IL-17, and TNF- α (Mileti *et al.*, 2009). Another study reported that macrophages released both NO and IFN- γ when co-cultured with LGG and a pathogenic antigen (Ghadimi *et al.*, 2010).

Here, heat-killed JWS 939 stimulated mouse peritoneal macrophages to increase the production of NO, IL-1 β , and TNF- α *in vitro* experiment. Moreover, these immune effects were stronger than that induced by LGG, except for TNF- α production. For the *in vitro* experiments, JWS 939 and LGG were killed by heating in order to investigate the effect on macrophages of killed probiotics, and to prevent macrophage cell death caused by overgrowth of the probiotic cells and the resulting acidic pH. Heat-killed probiotics have been shown to activate macrophages and induce cytokine production (Lin *et al.*, 2007; Nonaka *et al.*, 2008); in addition, earlier study has shown that heat-killed LGG was as effective as viable LGG (Korhonen *et al.*, 2001).

It has been reported that probiotics prevent infectious disease in mice. Mice infected with *Salmonella* Typhimurium or *L. monocytogenes* were protected by *L. rhamnosus* or *Lactobacillus casei* (Gill *et al.*, 2001; Nomoto *et al.*, 1985). Also, *P. pentosaceus* protected against the invasion of *Salmonella* in mice (Chiu *et al.*, 2008). Here, to evaluate the protective effects of JWS 939 against infection by pathogens, a *L. monocytogenes* challenge model was used. *L. monocytogenes* is a food-borne pathogen that causes listeriosis and is widely used in experimental model studies of host immune responses to bacterial infection. The coordinated action of various immune cells and cytokines of a host's immune system is required to protect against *L. monocytogenes* infection (Edelson and Unanue, 2000; Milon, 1997).

Here, we found that relative liver and spleen weights of mice increased following *L. monocytogenes* infection. Relative spleen weights were increased in both the PC and LGG-fed groups, but not in JWS 939-fed group. A decrease in relative spleen weight is indicative of increased host resistance; for example, *Lactobacillus plantarum* reduced the spleen weight in *L. monocytogenes*-infected mice and increased the survival rate of the mice (Puertollano *et al.*, 2008). Here, the LGG-fed and JWS 939-fed groups showed relatively heavier livers than the PC, a result similar to that in an earlier study in which oral administration of probiotics had increased liver weight in female rats (Tsai *et al.*, 2004).

JWS 939 increased NO and inflammatory cytokines (IL-1 β and TNF- α) levels in serum more than did LGG after *L. monocytogenes* infection. NO and cytokines produce beneficial effects by maintaining a balance within the immune system and by protecting against infections (Park *et al.*, 1999). Moreover, JWS 939 decreases numbers of viable *L. monocytogenes* in mice liver more than does LGG. JWS 939 activated macrophages of mice such as *in vitro* experiment, as a result of that activation; the numbers of viable *L. monocytogenes* was lower in the JWS 939-fed group than in the LGG-fed group. Consequently, we suggest that JWS 939 increases host resistance to *L. monocytogenes*.

JWS 939-fed mice had a significantly longer survival time that of the PC; regardless, none of the mice infected with *L. monocytogenes*, including the JWS 939-fed group, survived the infection. We expected survival time to be higher in the JWS 939-fed group, but the observed difference was not as dramatic as expected. Perhaps, if a lower dose of *L. monocytogenes* or a less pathogenic strain is used, the survival rate and survival time difference may be more dramatic. Moreover, mice were infected with *L. monocytogenes* via an intra-venous (*i.v.*) route to evaluate the immune effect of JWS 939 in the absence of an antimicrobial effect of the bacteriocin produced by JWS 939, and most recent studies used the *i.v.* route. If *L. monocytogenes* infection was via the oral route, JWS 939 may

show a much stronger protective effect, because JWS 939 has the ability to suppress *L. monocytogenes* via a direct bacteriocin-based bactericidal effect and can indirectly and simultaneously stimulate the host's immune system.

When probiotics are used for the same animal species from which they were isolated, they have intestinal adherence, settlement, and safety advantages (Kosin and Rakshit, 2006). Ehrmann *et al.* (2002) reported that two probiotic strains isolated from duck have potential as effective feed additives in duck. JWS 939 is a non-pathogenic member of duck microflora that has the ability to produce bacteriocin and has immuno-stimulatory effects, both *in vitro* and *in vivo*. Feeding a bacteriocin to an animal can enhance its immune responses. For example, broiler chickens have been protected from *C. perfringens* infection by feeding the bacteriocin of *P. pentosaceus* (Grilli *et al.*, 2009). Therefore, JWS 939 may have great potential as a feed supplement that can enhance the immune response in duck; more so, since JWS 939 produces bacteriocin and activates macrophages.

In conclusion, JWS 939 has the ability to induce immune responses both *in vitro* and *in vivo* and prolongs mouse survival time after lethal *L. monocytogenes* infection. Moreover, these effects are stronger than those from LGG. Further studies are needed to determine whether JWS 939 enhances immunity and provides protection against bacterial or viral infections in duck.

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