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Original Article

Effect of Bacillus Subtilis on Immune Function of Hd11 Chicken Macrophages

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ABSTRACT

Poultry is frequently contaminated by *Salmonella*, a pathogen leading to human health concern worldwide. This study aimed to evaluate the effect of *Bacillus subtilis* (BS)strain 048 (BS048) on the activation, phagocytosis, sterilization, cytokine secretion, and nitrogen oxide synthesis of HD11 chicken macrophages subjected to *Salmonella* enteritidis challenge, using lipopolysaccharide treatment as a negative control. The results showed: (1) BS048 had no significant effect on extracellular lactate dehydrogenase activity ($p>0.05$), while lipopolysaccharide treatment significantly increased extracellular lactate dehydrogenase activity ($p<0.05$);(2)BS048 significantly upregulated the expression levels of pro-inflammatory cytokines (interleukin (IL)-1â and IL-6), anti-inflammatory cytokines(IL-10 and transforming growth factor-â1), and anti-viral cytokine, interferon-â ($p<0.01$); (3) BS048 significantly upregulated the mRNA expression level of the inducible nitric oxide synthase and its activity as well as extracellular nitrogen oxide level ($p<0.01$). In conclusion, BS048 could improve anti-inflammatory and immune functions of HD11 chicken macrophages, without cytotoxic effects on these cells.

INTRODUCTION

Bacillus subtilis (BS) is a kind of probiotic strain that can produce protective endospores. As a high-quality and safe probiotic, it has many biological functions, including improving animal growth, enhancing body immunity, displaying antibacterial effects, and improving intestinal flora and body antioxidant status. BS can secrete a variety of enzymes, such as cellulases, proteases, amylases, lipases, glucanases, phytases, pectinases, and xylanases. These enzymes can help animals effectively degrade non-starch polysaccharides and other complex compounds and can also reduce anti-nutritional factors in feed, thus improving feed digestibility.BS can produce many kinds of bioactive substances, including amino sugars, phospholipids, polypeptides, and lipopeptides, which can act as antibacterial or bacteriostatic agents; the organic acids produced by BS can help regulate the balance of intestinal microflora. Moreover, the addition of BS to broilers has been found to increase the number of T and B lymphocytes and to improve the level of humoral and cellular immunity (Hong *et al.*, 2005)(Inooka *et al.*, 1986), but its effect on the activity of splenic macrophages has not been investigated yet.

Macrophages, as phagocytes, have many functions, including phagocytosis, secretion, tissue repair, and antigen presentation. They play an important role in innate immunity (non-specific immunity) and acquired immunity (specific immunity), and are closely related to body defense, inflammation and homeostasis (Verschoor *et al.*, 2012)

(Mosser & Edwards, 2008). Therefore, we hypothesized that BS may affect the function of macrophages. The purpose of this work was to investigate the effect of BS strain 048 (BS048) on HD11 cell activation, phagocytosis and sterilization, cytokine secretion and nitrogen oxide (NO) synthesis, and to study the effect of BS048 on chicken macrophage function, in order to provide an experimental and theoretical basis for further determination of the immune regulation and the mechanism by which BS048 helps to prevent *Salmonella* infection in chickens.

MATERIALS AND METHODS

Test materials

Test strain: BS048 was obtained from Shandong Baolaililai Bioengineering Co., Ltd.; *Salmonella* enteritidis (SE) ATCC13076 was provided by Jiangsu Institute of Poultry Sciences, P.R.China.

Test cell: HD11 chicken macrophage cells were kindly provided by Professor Weifen Li, School of Animal Science, Zhejiang University, P.R. China.

Reagents and instruments

DMEM/F12 medium, fetal calf serum (FBS), 0.25% pancreatin (containing 0.02% EDTA solution), and penicillin streptomycin solution (100×, containing penicillin10000U/mL, streptomycin 10000 μg/mL) were purchased from GIBCO company. Lipopolysaccharide (LPS) was obtained from Sigma Aldrich company. The acid phosphatase (ACP), lactate dehydrogenase (LDH), inducible nitric oxide synthase (iNOS, a014-1), and NO assay kits (nitrate reductase method) were purchased from Nanjing Jiancheng Bioengineering Research Institute (Nanjing, P.R. China). The extraction reagent RNA iso plus, M-MLV reverse transcriptase (RTase), RNase H^-, fluorescence quantitative reagent SYBR, and Premix Ex TaqGammaII (TLI RNaseH plus), were purchased from Takara Bao Bioengineering (Dalian) Co., Ltd. Other conventional reagents and analytical reagents were purchased from Bio engineering (Shanghai) Co., Ltd.

Spectramax M5 enzyme labeling instrument was purchased from Molecular Devices. The 7500 real time PCR system was obtained from Applied Biosystems, USA. Nanodrop 2000 Ultra micro nucleic acid and protein analyzer was purchased from Thermo Fisher, USA. The 5804r desktop high-speed large capacity refrigerated centrifuge was purchased from Eppendorf (Germany); The MCO-20AIC carbon dioxide incubator was purchased from Sanyo Motor Company, Japan. The Cx41-12c02 inverted microscope was purchased from Olympus, Japan.

TEST METHODS

Cell culture

HD11 cells were cultured in a complete medium (DMEM/F12 medium containing 10% FBS and two antibiotics (100U/mL penicillin and 100μg/mL streptomycin) at 41 ºC and under 5%CO2. The cells were passaged for 3-5 generations before being used in the experiments.

Culture of BS048

BS048 was cultured on LB agar plates at 37 ºC, and a single colony was selected for isolation and purification. The growth curve of BS048 was drawn using the plate counting method. The purified bacteria were cultured in a shaker at 37 ºC, and the test bacteria were prepared according to the optical density growth curve.

Cytotoxic evaluation of BS048

HD11 cells were seeded into a 12-well cell culture plates, and divided into a control group (treated with complete culture medium without BS048 or LPS as a negative control), a BS048 group (treated with culture medium containing 108CFU/mL BS048) and an LPS group (treated with culture medium containing 100ng/mL LPS, as a negative control). Each group consisted of 3 replicates. The cells were incubated at 41 ºC and 5%CO2 for 12 hours, and then the culture supernatant was transferred to a sterile centrifuge tube and centrifuged at 400×g for 5 min. Next, the supernatant was removed, and the cell pellet was stored at - 80 ºC for subsequent use. The activity of LDH (extracellular LDH) in the culture supernatant was determined using an LDH assay kit. This index was positively correlated with the cytotoxicity of BS.

Detection of HD11 cell activation

HD11 cells were seeded into 12-well cell culture plates and divided into three test groups as described above. Each group consisted of three replicates, which were incubated at 41 ºC and 5% CO2 for 12 hours. Then, the cells were lysed with 0.1% Triton X-100 in phosphate buffer solution (PBS) and the lysate was collected into an aseptic centrifuge tube. After centrifugation, the lysates were transferred to new tubes and stored at - 80 ºC until analysis. The activities of ACP and LDH in HD11 cell lysates were determined using an ACP and a LDH assay kit, respectively.

Detection of phagocytosis and bactericidal ability of HD11 cells

HD11 cells were seeded into 12-well cell culture plates at various concentrations, including the control group and the BS048 group (10^8CFU/mL). Each group consisted of three replicates, which were incubated at 41 ºC and 5% $CO_2$for 12 hours. Then complete medium containing 10^8CFU/mL of SE was added, and the incubation was continued for 6 hours. Next, the SE-containing medium was discarded, the cells were washed three times with PBS, complete medium was added, and the incubation was continued. Cells were analyzed for internalized SE at 0, 1, 6, and 12 hours following the removal of the SE-containing medium, as follows. Cells were lysed with Triton X-100 solution and the lysates were collected into sterile centrifuge tubes. The typical SE colonies were counted using the plate method. There was a positive correlation between the initial amount of intracellular SE bacteria (0 hour) and phagocytic capacity of macrophages, and a negative correlation between the residual percentage of intracellular SE (the amount of intracellular SE remaining 1, 6, and 12 hours following the removal of non-phagocytosed SE divided by the initial amount of intracellular SE bacteria x 100%) and the bactericidal activity of macrophages.

Detection of gene expression of HD11 cytokines and iNOS

HD11 cells were inoculated into 12 well cell culture plates, and the experimental groups was the same as mentioned previously. Each group consisted of three replicates, which were cultured at 41 ºC and 5% CO_2 for 3 hours and 6 hours respectively. The cells were taken for total RNA extraction, reverse transcription and real-time PCR, and the mRNA expression levels of cytokine genes (interleukin (IL)-1β, IL-6, IL-12α, interferon (IFN)-γ, IL-10, transforming growth factor (TGF)-β1, and IFN-β) and iNOS were detected. The real-time PCR primers corresponding to these genes are shown in Table 1.

Detection of the NO synthesis ability of HD11 cells

HD11 cells were seeded into 12 well cell culture plates. Each group consisted of 3 replicates. Cells were incubated under 5%CO_2 at 41 ºC for 12 hours. Next, the supernatant of cell culture was transferred to sterile centrifuge tubes. Cells were lysed with Triton X-100 solution and lysates were transferred into new sterile centrifuge tubes. The iNOS activity of cell lysates and the NO content of culture supernatants were measured using the corresponding kits to evaluate the NO synthesis ability of the HD11 cells.

Table 1 – List of real-time PCR primers.

Gene	Genebank	Primer (5′-prime-3′-prime)	Product length
IL-1β	NM_204524.1	F:CGACATCAACCAGAAGTGCTT R: GTCCAGGCGGTAGAAGATGA	298
IL-6	NM_204628.1	F: CTCCTCGCCAATCTGAAGTC R:CCTCACGGTCTTCTCCATAAAC	99
IFN-γ	NM_205149.1	F: ACAAGTCAAAGCCGCACATC R: CACCTTCTTCACGCCATCAG	83
IL-10	NM_001004414.2	F: ACCAGTCATCAGCAGAGCAT R: CCTCCTCATCAGCAGGTACTC	222
TGF-β1	NM_001318456.1	F: GGATGGACCCGATGAGTATTG R:TTGAACACGAAGAAGATGCTGT	117
IFN-β	NM_001024836.1	F: TCAGAATACGGCTCCACCTC R: ATGGCTGCTTGCTTCTTGTC	105
iNOS	NM_204961.1	F:CCACCAGGAGATGTTGAACTATG R: CAGGAGTAATGACGCCAAGAG	160
GAPDH	NM_204305.1	F: GGTGAAAGTCGGAGTCAACGG R: CGATGAAGGGATCATTGATGGC	107

F, forward; R, reverse.

Statistics Analysis

Statistical software SPSS (version 22.0) was used to analyze the data, and independent t-test was used to analyze the significance of the data between the control group and the test group (BS048 group or LPS group). $p<0.05$ was considered a significant difference, $p\geq0.05$ was considered not significant, and $0.05<p<0.1$ indicated a trend of change. The test data are expressed as mean and standard deviation.

RESULTS

Cytotoxicity of BS048

Compared with the control group (Table 2), the extracellular LDH activity of BS048-administratedgroup did not change significantly ($p>0.05$), while its activity in the LPS-treated group showed a significant increase (about 3-fold; $p<0.01$), indicating that BS048 had no visible cytotoxicity on HD11 cells, in contrast to the cytotoxic effect of 100ng/mL LPS.

Table 2 – Effect of BS048 on LDH toxicity of HD11 cells.

Group	Extracellular LDH activity (U / L)
Control	4.88 ± 0.84
BS048	5.44 ± 1.65
LPS	15.71 ± 2.50**

** $p<0.01$ LPS group compared with the control group.

Effect of BS048 on HD11 cell activation

As shown in Table 3, the activities of ACP and LDH in cells of the BS048 group increased by 26.62% ($p<0.01$) and 20.81% ($p<0.05$), respectively, when compared with the control group, while in the LPS group these activities increased by 43.27% ($p<0.05$) and 59.63% ($p<0.05$), respectively, suggesting that addition of both BS048 and LPS resulted in the activation of HD11 cells.

Table 3 – Effect of BS048 on ACP and LDH activities in HD11 cells.

Group	Intracellular ACP activity (U/100mL)	Intracellular LDH activity (U/L)
Control	3.07 ± 0.25	56.78 ± 9.29
BS048	3.89 ± 0.15**	81.34±10.20*
LPS	3.71 ± 0.19*	90.63±15.90*

*$p<0.05$, **$p<0.01$ test group compared with control group.

Effect of BS048 on phagocytosis and bactericidal ability of HD11 cells

We next subjected HD11 cells to treatment with SE, in the absence or presence of BS048. Table 4 showed that, compared with the control group, the number of intracellular SE in the BS048 group at 0 hour significantly increased (by 33.98%; $p<0.01$), indicating that the presence of BS048 led to a significant increase in phagocytotic activity of HD11 cells; Meanwhile, the percentage of surviving intracellular SE was greatly reduced in the BS048 group compared with the control group, at 71.83% vs. 87% after 1 hour ($p<0.05$), 1.07% vs. 2.81% after 6 hours ($p<0.01$), and 0.12% vs. 0.41% after 12 hours ($p<0.01$), respectively, which, in turn, indicated that BS048 treatment significantly reduced the number of residual SE inside HD11 cells.

Table 4 – Effect of BS048 on phagocytic function and intracellular bactericidal activity of HD11 cells.

Group	Sampling time(h)							
	0		1		6		12	
	Intracellular bacteria x10^7	Percentage	Intracellular bacteria x10^7	Percentage	Intracellular bacteria x10^5	Percentage	Intracellular bacteria x10^4	Percentage
Control	2.22±0.11	100.00	1.93±0.08	87.00±3.80	6.23±0.40	2.81±0.10	8.97±0.42	0.41±0.04
BS048	2.97±0.22**	100.00	2.13±0.10	71.83±2.56*	3.13±0.35**	1.07±0.20**	3.37±0.61**	0.12±0.03**

In the text group. *$p<0.05$, ** $p<0.01$.

Taken together, these results indicated that BS048 significantly enhanced the phagocytic function of HD11 cells as well as their ability of killing intracellular SE.

Effect of BS048 on the expression of cytokines and iNOS in HD11 cells

As shown in Table 5, the relative expression level of IL-1β mRNA in the BS048 group increased about 820-fold ($p<0.01$) and 222-fold ($p<0.01$) after 3 hours and 6 hours, respectively, when compared with the control group. Moreover, the relative expression level of IL-6 mRNA increased about 2.35-fold ($p<0.01$) and 1.81-fold ($p<0.05$) after 3 hours and 6 hours, respectively, while the relative expression level of IFN-γ mRNA had decreased by about 65% ($p<0.01$) after 3 hours and was unchanged after 6 hours, respectively. The relative expression level of IL-10 mRNA had increased about 21.64-fold ($p<0.01$) and 33.45-fold ($p<0.01$) at 3 hours and 6 hours, respectively. The relative expression level of TGF-β1 mRNA had increased about 7.03 times ($p<0.01$) and 10.18-fold ($p<0.01$) after 3 hours and 6 hours, respectively. The relative expression level of iNOS mRNA had increased about 84.32-fold ($p<0.01$) and 11.01-fold ($p<0.01$) after 3 hours and 6 hours, respectively. In the LPS-administrated group, the relative expression level of IL-1β mRNA had increased about 26822-fold ($p<0.01$) and 830-fold ($p<0.01$) after 3 hours and 6 hours, respectively, when compared with the control group. The relative expression level of IFN-γ mRNA decreased by 73.01% ($p<0.01$) and 70.09% ($p<0.01$) after 3 hours and 6 hours of treatment, respectively. The relative expression level of IL-10 mRNA increased about 34.51-fold ($p<0.01$) and 47.01-fold ($p<0.01$) after 3 hours and 6 hours of treatment, respectively, while the relative expression level of TGF-β1 mRNA increased about 2-fold ($p<0.01$) and 6-fold after 3 hours and 6 hours of treatment, respectively. Interestingly, compared with the control group, the relative expression level of IFN-β mRNA had increased about two-fold ($p<0.01$) after 3 hours in the BS048 group and about 1.6-fold ($p<0.01$) after 3 hours in the LPS group, respectively. These results together indicated that BS048 treatment resulted in a significantly upregulated gene expression of pro-inflammatory factors IL-1β, IL-6, anti-inflammatory factors IL-10, TGF-β1, anti-viral factor IFN-β and iNOS in HD11 cells. LPS treatment also resulted in an upregulation of the gene expression of these cytokines and iNOS in HD11 cells.

Table 5 – Effect of BS048 on cytokine and iNOS gene expression in HD11 cells.

Group		Control	BS048	LPS
IL-1β	3 h	1.03 ± 0.24	844.48±189.57**	27580.87±3472.53**
	6 h	1.01 ± 0.14	226.17±28.89**	839.19±107.53**
IL-6	3 h	1 ± 0.09	2.35 ± 0.17**	3.49 ± 0.75**
	6 h	1.02 ± 0.22	1.85 ± 0.36*	0.88 ± 0.12
IFN-γ	3 h	1.02 ± 0.17	0.36 ± 0.04**	0.27 ± 0.03**
	6 h	1.01 ± 0.14	0.92 ± 0.28	0.30 ± 0.04**
IL-10	3 h	1.01 ± 0.15	22.86 ±2.73**	35.90±8.52**
	6 h	1.01 ± 0.13	34.75 ±6.41**	48.43±6.47**
TGF-β1	3 h	1.03 ± 0.23	8.25 ± 1.26**	2.02 ± 0.53*
	6 h	1.01 ± 0.15	11.31±2.38**	6.69 ± 1.41**
IFN-β	3 h	1.01 ± 0.12	1.95 ± 0.29**	1.63 ± 0.12**
	6 h	1.02 ± 0.19	1.29 ± 0.28	0.94 ± 0.22
iNOS	3 h	1.01 ± 0.16	86.35 ± 13.73**	138.76 ± 37.38**
	6 h	1.02 ± 0.20	12.26 ± 2.80**	18.48 ± 2.56**

In the test group No "*", $p>0.05$;*$p<0.05$, **$p<0.01$.

Effect of BS048 on NO synthesis by HD11 cells

As shown in Table 6, compared with the control group, the iNOS activity and NO level in cells of BS048 group increased significantly by 51.10% ($p<0.01$) and 148.57% ($p<0.01$), respectively, while those of LPS group increased significantly by 35.55% ($p<0.05$) and 81.898% ($p<0.01$), respectively, indicating that BS048 and LPS can enhance the NO synthesis function of HD11 cells.

Table 6 – Effects of BS048 on iNOS activity and NO production in HD11 cells.

Group	iNOS activity(U / 100mL)	Extracellular NO level (μmol/L)
Contrast	14.26 ± 0.44	3.51 ± 0.19
BS048	21.55 ± 1.28**	8.73 ± 0.85**
LPS	19.33 ± 2.21*	6.39 ± 0.47**

*$p<0.05$, **$p<0.01$ test groups compared to the control.

DISCUSSION

Safety of the use of BS048 on HD11 cells

Safety is the primary premise of probiotics and the only standard to judge whether a probiotic is qualified (Saarela *et al.*, 2000). LDH is a cytoplasmic enzyme that is stably expressed in all cells (Weyermann *et al.*, 2005). Only when the cell membrane permeability increases due to cell death or damage, LDH will leak out of the cell (Korzeniewski & Callewaert, 1983; Patel & Markx, 2008). Therefore, the activity of extracellular LDH can be used as an index to measure the cytotoxicity of test substance (AFRC, 1989; Hove *et al.*, 1999; Gusils *et al.*, 1999). In this study, BS048 did not lead to significant changes in extracellular LDH activity, indicating that the plasma membrane of the HD11 cells was not damaged and that the use of BS048 was safe and displayed no visible cytotoxicity. Our results are consistent with previous studies, which have shown that BS stain was safe and exhibited no pathogenicity (Hong *et al.*, 2008; Sorokulova *et al.*, 2008; Tompkins *et al.*, 2008).

The Effect of BS048 on the Activation of HD11 Cells

ACP and LDH are a lysozyme inhibitor and an intracellular enzyme, respectively, which are closely related to phagocytic activity (Sugiura *et al.*, 2000a), and they can be regarded as marker enzymes of macrophages (Chen *et al.*, 2008). Previous studies have shown that macrophage activation can significantly increase the activity of ACP and LDH (Ryan *et al.*, 1979; Sugiura *et al.*, 2000b; Xu *et al.*, 2012; Huang *et al.*, 2013), and therefore they can be used as indicators of macrophage activation. In this study, we showed that BS048 significantly improved the activity of ACP and LDH in HD11 cells, indicating that the presence of BS048 induces the activation of HD11 macrophage cells. Macrophages are initially in a resting state with only basic cellular functions. They will be activated by exogenous stimuli (such as pathogens and foreign bodies) or endogenous signals (such as cytokines and hormones) to provide their immune function (such as phagocytosis, secretion, and antigen presentation) (Martinez *et al.*, 2008). In conclusion, BS048 may improve the immune function of chickens by activating macrophages.

Effect of BS048 on phagocytosis and bactericidal ability of HD11 cells

Macrophages are scavengers of cellular debris and wastes in human and animal bodies. Using their powerful phagocytic function, they can remove pathogens (eg. viruses, bacteria), foreign matters (eg. dust, pollen), cell fragments, abnormal cells (infected, apoptotic, and tumor cells) and other substances in the body (Zhang & Mosser, 2008), so as to maintain the homeostasis of the body. Phagocytic vesicles formed during phagocytosis of the above targets by macrophages will fuse with lysosomes, thus releasing a variety of acid hydrolases (eg. proteases, lipases, ribonucleases, phosphatases) aiming to kill and degrade the targets. In this study, we showed that BS048 significantly enhanced the phagocytic function of HD11 cells and their ability to internalize and kill SE. The results showed that BS048 enhanced the phagocytic function of chicken macrophages and their intracellular bactericidal activity, thereby potentially reducing *Salmonella* infections in chickens.

There was a clear difference between the control group and the BS048 group in the number of intracellular SE at the beginning of the intracellular bactericidal test (i.e. after removing the media with the non-phagocytosed SE and replacing it with fresh culture medium). If the number of intracellular SE retained (absolute comparison method) was used to measure the intracellular bactericidal ability of HD cells, these results were objective and not accurate. Therefore, in this study, the survival percentage was selected to exclude the effect of different initial intracellular SE quantity in each group on the test results. For example, one hour after the start of the intracellular bactericidal activity test, the intracellular SE survival percentage was significantly lower in the BS048 group than that of the control group, but there was no significant difference in the number of intracellular SE between the two groups.

Effect of BS048 on cytokine secretion of HD11 Cells

Macrophages can secrete a variety of cytokines (such as IL family members, TNF family members, and IFN family members) (Cavaillon, 1994), that can regulate immune and inflammatory responses (Arai *et al.*, 1990). Inflammatory factors refer to cytokines that can promote or inhibit the process of inflammation. They are divided into pro-inflammatory factors (including IL-1β, IL-6, IL-8, IL-12, TNF-α, IFN-γ) and anti-inflammatory factors (including IL-4, IL-10, IL-13, TGF-β1)(Zhang & An, 2007). At present, our laboratory is not equipped to produce relevant ELISA kits. However, due to the fact that the gene expression level of cytokines is usually positively correlated with their secretion level, we measured the gene expression levels of IL-1β, IL-6, IFN-γ, IL-10, TGF- β1 and IFN-β in HD11 cells by real-time PCR in order to indirectly measure the secretion level of these cytokines. In this study, BS048 significantly induced the expression of IL-1β and IL-6 in HD11 cells. Pro-inflammatory factors can activate or enhance the function of macrophages, dendritic cells, lymphocytes and other immune cells, thereby initiating non-specific and specific immune defenses against pathogens and infected cells, leading to an enhanced immune function and anti-infection ability of the body. BS048 enhanced the immune function and bactericidal ability of macrophages of chickens by stimulating macrophages to produce pro-inflammatory factors such as IL-1β and IL-6. Anti-inflammatory factors can downregulate the production of pro-inflammatory factors, avoid excessive inflammatory reaction to damage cells and tissues, and promote the elimination of inflammation and tissue repair (Ariel & Timor, 2013). In this study, we showed that the addition of BS048 led to a significant induction of the expression of anti-inflammatory factors IL-10 and TGF-β1 in HD11 cells, which indicated that there was a self-regulation mechanism in HD11 cells to maintain the dynamic balance of pro-inflammatory response and anti-inflammatory response, in order to protect cells and tissues. We also found that BS048 could significantly induce IFN-β gene expression in HD11 macrophage cells. IFN-β belongs to type I IFN, which displays a strong antiviral activity, and its induction quickly starts the antiviral immune response of the body (Goodbourn *et al.*, 2000). In addition, IFN-β has many other important biological functions, including immune regulation and anti-tumor activity (Theofilopoulos *et al.*, 2005). We observed, that BS048 can induce macrophages to produce IFN-β which may enhance the antiviral, immunomodulatory and antitumor abilities of chicken macrophage cells.

Effect of BS048 on NO synthesis of HD11 Cells

Synthesis of NO is mainly dependent on iNOS with L-arginine as a substrate, and NO is an important mediator involved in many physiological and pathological processes (Soszynski & Chelminiak, 2007), such as immune regulation (Gomez-Flores & Weber, 1998), cytotoxicity and apoptosis (Albina & Reichner, 1998). However, NO produced by macrophages is not only indispensable for the defense of pathogenic microorganisms and tumor cells, but also essential for the inhibition of T cell proliferation and local inflammatory response (Chang *et al.*, 2001, Friedl *et al.*, 2001). In this study, combined with the level of iNOS gene expression in real-time PCR test and the test results of NO synthesis activity, we found that BS048 enhanced the iNOS activity and NO synthesis ability by inducing HD11 cells to produce iNOS. Thus, BS048 can enhance the immune function and anti-infection ability of chicken macrophages by enhancing the NO synthesis function.

CONCLUSIONS

Our *in vitro* experiments showed that the use of BS048 was safe for chicken macrophages. Addition of BS048 caused the activation of chicken macrophages and regulated their functions, including: (1) enhancing their phagocytic and intracellular bactericidal ability; (2) enhancing their NO synthesis ability; (3) enhancing their cytokine production ability. Our results revealed

one of the mechanisms by which BS048 enhanced the immunity and anti-*Salmonella* bactericidal activity of chicken macrophages.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest. All authors have read and approved this research article.

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