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Selected lactobacilli strains inhibit inflammation in LPS-induced RAW264.7 macrophages by suppressing the TLR4-mediated NF-κB and MAPKs activation

Jialu SHI[a,b], Huizhen LI[a,b,c], Shengnan LIANG[a,b], Smith Etareri EVIVIE[a,b,d,e], Guicheng HUO[a,b], Bailiang LI[a,b], Fei LIU[a,b*]

Abstract

Probiotics are known to provide the host with immune-modulatory effects, which is widely used for the treatment of inflammatory bowel disease (IBD), diarrhea, and ulcers.*Lactobacillus acidophilus* KLDS 1.0901, *Lactobacillus helveticus* KLDS 1.8701, and *Lactobacillus plantarum* KLDS 1.0318 were isolated from Chinese fermented dairy food with antibacterial activity, antioxidant activity, and immunomodulatory activity, respectively. This study evaluated the anti-inflammatory potential of the tested strains in LPS (Lipopolysaccharide)-induced RAW264.7 cells. The results showed that all tested strains could inhibit the mRNA expression of iNOS and COX-2 and reduce the concentration of NO and PGE2 production. Furthermore, all tested strains markedly reduced proinflammatory cytokines' production (TNF-α, IL-1β, and IL-6). Moreover, these results may be associated with inhibiting TLR4-mediated NF-κB and MAPKs signaling pathway activation. These results indicated that *L. acidophilus* KLDS 1.0901, *L. helveticus* KLDS 1.8701, and *L. plantarum* KLDS 1.0318 possesses an anti-inflammatory potential and provide a molecular basis regarding the development of functional probiotic products.

Keywords: *Lactobacillus*; anti-inflammation; Toll-like receptor 4; nuclear factor-kappa B; mitogen-activated protein kinase.

Practical Application: The screening of anti-inflammatory lactobacilli provides a new idea for the further development of probiotics.

1 Introduction

Inflammation is a defensive immune response to protect the body from exogenous stimuli. However, excessive or persistent inflammation can lead to various inflammation-related diseases (Lee et al., 2013). Macrophages, derived from monocytes, play a crucial role in innate immunity by their unique phagocytosis function (Ryu et al., 2017). Lipopolysaccharide (LPS) comprises lipids and polysaccharides connected by a covalent bond and is the central part of gram-negative bacteria's outer membrane. When pathogenic bacteria invasion occurs, the host and intestinal mucosa barrier are compromised, allowing LPS release into the bloodstream (Guo & Chen, 2019). A combination of LPS, LPS binding protein (LBP), and CD14 forms the LPS-LBP-CD14 complex, which binds to the Toll-like receptor 4 (TLR4)/ myeloid differentiation protein 2 (MD-2) on the mononuclear-macrophage membrane (Lu et al., 2008). The TLR4 is stably expressed in cell membranes and binds to myeloid differentiation factor 88 (MyD88) through interactions with the Toll-interleukin-1 receptor (TIR) domains and activates the MyD88-dependent signaling pathway, responsible for downstream nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinase (MAPKs) pathways (Rauta et al., 2014; Yang et al.,2015). The activation of NF-κB and MAPKs could influence inflammation, immune regulation, cell proliferation, and apoptosis. RAW264.7 cells are an immortalized murine macrophage line, which are often used for the establishment of inflammation models (Lu et al., 2012). In the LPS-stimulated RAW 264.7 macrophage cells, the expression of proinflammatory mediators and cytokines, including nitric oxide (NO), prostaglandin E2 (PGE2), tumor necrosis factor (TNF) -α, interleukin (IL) -1β and IL-6, as well as other immune-related genes have been reported (Lawrence et al., 2002).

Probiotics are living microorganisms that can provide beneficial effects to the host when administered in adequate amounts, such as regulation of gut microbiota, strengthening the intestinal barrier, and inhibiting intestinal diseases (Gorbach, 2000; Rama et al., 2020). In recent years, lactobacilli strains have been a probiotic focus group for the presentment and treatment of inflammatory response (Fedorak et al., 2003; Xin et al., 2016). For instance, *L. acidophilus* SMC-S095 exerted protective effects against dextran sulfate sodium (DSS)-induced murine colitis. *L. acidophilus* SMC-S095 down-regulated the expression of IL-23 and transforming growth factor (TGF)-β1 and the phosphorylation of signal transducer and activator of transcription (STAT) 3, resulting in decreased the level of proinflammatory cytokine IL-17 mediated by T helper cell (Th) 17 cells (Chen et al., 2015). Furthermore, the administration of *L. helveticus* NS8 could increase the concentration of IL-10 in peripheral blood

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[a]*Key Laboratory of Dairy Science, Ministry of Education, Northeast Agricultural University, Harbin, China*
[b]*Food College, Northeast Agricultural University, Harbin, China*
[c]*School of Food Science and Technology, Jiangnan University, Wuxi, China*
[d]*Department of Food Science and Human Nutrition, Faculty of Agriculture, University of Benin, Benin City, Nigeria*
[e]*Department of Animal Science, Faculty of Agriculture, University of Benin, Benin City, Nigeria*
*Corresponding author: david.as@163.com

mononuclear cells (PBMCs) in vivo (Rong et al., 2015). Similarly, *L. plantarum* 21 significantly alleviated TNBS-induced colitis in rats by inhibiting the levels of TNF-α, IL-1β and NO in colon tissues, while increasing the contents of glutathione (GSH) and IL-10 (Satish Kumar et al., 2015).

L. acidophilus KLDS 1.0901, *L. helveticus* KLDS 1.8701, and *L. plantarum* KLDS 1.0318 were isolated from traditional fermented dairy food in Sinkiang (China) and identified using 16S rDNA sequence analysis. Our previous results showed that all tested strains exhibited high acid and bile salt-resistances and high cell adhesion. These characteristics implied that all tested strains could colonize the host intestine and confer probiotic properties. Furthermore, we reported the antibacterial, antioxidant, and immunomodulatory properties of three candidate strains-*L. acidophilus* KLDS 1.0901 (Du et al., 2017), *L. helveticus* KLDS 1.8701 (Li et al., 2019), and *L. plantarum* KLDS 1.0318 (Meng et al., 2019).

The current study aimed to assess the potentials of *L. acidophilus* KLDS 1.0901, *L. helveticus* KLDS 1.8701, and *L. plantarum* KLDS 1.0318 in suppressing proinflammatory gene expressions and the related-signaling pathways (NF-κB and MAPK) activation in LPS-stimulated RAW 264.7 murine cell lines. We hope that this study will provide a molecular basis regarding the development of functional probiotic products.

2 Materials and methods

2.1 Bacterial strain and culture conditions

The *L. acidophilus* KLDS 1.0901, *L. helveticus* KLDS 1.8701, and *L. plantarum* KLDS 1.0318 strains were isolated from traditional fermented dairy foods in Sinkiang Province (China) and preserved at the Northeast Agricultural University's (NEAU) Key Laboratory Dairy Science (KLDS) in Harbin, China. The tested strains were identified using the 16S rDNA sequence analysis. Both the bacterial strains were grown in MRS broth at 37 °C for 18 h (stationary phase), harvested (6,000 × g, 10 min, 4 °C), and washed twice with phosphate-buffered saline (PBS, pH 7.4). Then the bacterial cell pellets were resuspended with high Dulbecco's modified Eagle's medium (DMEM).

2.2 Cells culture

The murine macrophages cell line RAW264.7 was purchased from the Chinese Academy of Sciences Cell Bank (Shanghai, China) and cultured in high DMEM medium, including 10% fetal bovine serum (FBS) and 1% antibiotics (100 μg/mL streptomycin and 100 U/mL penicillin) at 37 °C (5% CO_2).

2.3 Cell viability

Murine RAW264.7 cells were cultivated in 96-well plates (1.0×10^5 cells/well) with *L. acidophilus* KLDS 1.0901, *L. helveticus* KLDS 1.8701, and *L. plantarum* KLDS 1.0318 suspensions at a multiplicity of infection (MOI) (ratio of bacteria number to epithelial cell number) of 10 (1.0×10^6CFU/well), 100 (1.0×10^7 CFU/well), and 1000 (1.0×10^8 CFU/well), respectively. After 24 h of incubation at 37 °C (5% CO2), cells were treated with 10% Cell Counting Kit-8 (CCK8) (APExBIO, USA) and incubated for another 2 h. The optical density (OD) was estimated at 450 nm using a microplate reader.

2.4 Nitric oxide (NO) assay

The concentration of nitric oxide (NO) was measured in LPS (Sigma, USA)-induced RAW264.7 cells based on the Griess reaction, as previously described (Park et al., 2015). RAW264.7 cells were plated in 96-well plates (1×10^5 cells/well) and pre-incubated with *L. acidophilus* KLDS1.0901, *L. helveticus* KLDS 1.8701, and *L. plantarum* KLDS1.0318 at an MOI of 10 (1.0×10^6 CFU/well) for 1 h, and then incubated with LPS (1 μg/mL) for 24 h. Cell culture media was centrifuged at 2000 × g for 10 min to remove debris and collected supernatants to assay immediately. Equal volumes of Griess reagent (Nanjing Jiancheng Bioengineering Institute, China) were mixed with the supernatants and static for 10 min at room temperature. The absorbance was measured at 550 nm using a microplate spectrophotometer.

2.5 Enzyme-Linked Immunosorbent Assay (ELISA)

RAW264.7 cells were incubated in 6-well plates (1×10^6 cells/well) and were treated with *L. acidophilus* KLDS 1.0901, *L. helveticus* KLDS 1.8701, and *L. plantarum* KLDS 1.0318 at an MOI of 10 (1.0×10^7 CFU/well) for 1 h prior to LPS (1 μg/mL) treatment incubator for 24 h. The supernatants were then collected and assayed immediately. According to the ELISA kits (Beijing Chenglin Bioengineering Institute, China) manufacturer's instructions, the production levels of PGE2, TNF-α, IL-1β, and IL-6 were determined, respectively.

2.6 Real-time quantitative polymerase chain reaction (RT-qPCR)

RAW264.7 cells were incubated in 6-well plates (1×10^6 cells/well) and were treated with the *L. acidophilus* KLDS 1.0901, *L. helveticus* KLDS 1.8701, and *L. plantarum* KLDS 1.0318 (1.0×10^7 CFU/well) for 1 h prior to LPS (1 μg/ml) treatment incubator for 24 h. The total RNA of RAW264.7 cells was extracted using Total RNA Kit (Tiangen Biotech Co., LTD., China). Reverse transcription was carried out using the a GoScript™ Reverse Transcription Mix (Promega, USA). Quantitative PCR (qPCR) was performed by GoTaq◯,R SYBR-Green qPCR Master Mix (Promega, USA). The relative expressions of target genes were analyzed using the $2^{-\Delta\Delta Ct}$ calculation method and β-actin was used as an endogenous housekeeping gene. The gene primers were obtained from the National Centre for Biotechnology Information (NCBI), then designed and synthesized by Sangon Biotech Co., Ltd (Shanghai, China) (Table 1).

2.7 Western blot analysis

RAW264.7 cells were planted in at a density of 1×10^6 cells/well and pretreated with *L. acidophilus* KLDS 1.0901, *L. helveticus* KLDS 1.8701, and *L. plantarum* KLDS 1.0318 (1.0×10^7CFU/well) or special inhibitors for 1 h prior to treatment with LPS (μg/ml) for 20 min (NF-κB and MAPKs signal pathway) or 24 h (TLR4,

Table 1. Primers designed for this study.

Genes	Primers
TNF-α	F:5'-GCC TCT TCT CAT TCC TGC TTG TGG-3'
	R:5'-GTG GTT TGT GAG TGT GAG GGT CTG-3'
IL-1β	F:5'-TCG CAG CAG CAC ATC AAC AAG AG-3'
	R:5'-AGG TCC ACG GGA AAG ACA CAG G-3'
IL-6	F:5'-AGA CAG CCA CCA CAC TGG AGA TAG-3'
	R:5'-CCT GCC TCC TGT TGA TGT GAA GTC-3'
iNOS	F:5'-ACT CAG CCA AGC CCT CAC CTA C-3'
	R:5'-TCC AAT CTC TGC CTA TCC GTC TCG-3'
COX-2	F:5'-GGT GCC TGG TCT GAT GAT GTA TGC-3'
	R:5'-GGA TGC TCC TGC TTG AGT ATG TCG-3'
TLR4	F:5'-CCG CTT TCA CCT CTG CCT TCA C-3'
	R:5'-ACC ACA ATA ACC TTC CGG CTC TTG-3'
β-actin	F:5'-GGT TGT CTC CTG CGA CTT CA-3'
	R: 5'-TGG TCC AGG GTT TCT TAC TCC-3'

COX-2, and iNOS). The cells were washed twice with ice-cold PBS and lysed with ice-cold radio-immunoprecipitation assay (RIPA) buffer (1 sodium dodecyl sulfate-containing 1% phosphatase inhibitor, 0.1% protease inhibitor, and 0.5% 100 mM PMSF). Total protein was extracted using the whole protein extraction kit (Invent Biotechnologies, USA), and concentrations required for analysis were quantified using the bicinchoninic acid (BCA, KeyGen, China) protein assay following the manufacturer's instructions. The total protein (40 μg) of each sample was separated by 10%-13% sodium decyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, KeyGen, China) and transferred onto nitrocellulose/ polyvinylidene difluoride (NC/PVDF) membranes. The NC/PVDF membrane was blocked in buffer containing 5% skimmed milk with shaking for 2 h, followed by incubation with primary antibodies that recognized p-38, *p*-p38, p65, JNK, TLR4, GAPDH (1:500; ImmunoWay, USA); ERK, *p*-ERK (1:1,000; Cell Signaling Technology, USA), IκB, *p*-IκB, *p*-JNK, β-actin (1:1,0000; Abcam, UK); COX2 (1:2000; Abcam, UK); iNOS (1:1000, Sangon Biotech, China) at 4 °C overnight. The membrane was washed with TBST four times and probed with secondary antibodies (1:5000) for 1 h at room temperature. After washing thrice in TBST, the chemiluminescence detection system was enhanced with the ECL kit to display antibody-specific proteins.

2.8 Statistical analysis

All data were assessed using SPSS 23.0 software (SPSS Inc., Chicago, IL, USA) and expressed as mean ± standard deviation (SD). The one-way analysis of variance (ANOVA) followed by Duncan's multiple range test was used to compare groups. In this study, *P*-values of 0.05, 0.01, and 0.001 were considered significant.

3 Results

3.1 The effect of the tested strains on cell viability

The effect of *L. acidophilus* KLDS 1.0901, *L. helveticus* KLDS 1.8701, and *L. plantarum* KLDS 1.0318 on cell viability were assessed by CCK8 assay. As shown in Figure 1, the tested strains significantly inhibited cell viability within MOI of 100 and 1000 compared with that of the control group. These may have been induced by the competition of nutrients between three strains and RAW264.7 cells. However, there was no significant difference between the control group and all tested strains-treated groups in the viability of RAW 264.7 cells when the MOI is 10. Given the above results, an MOI of 10 was applied during subsequent experiments.

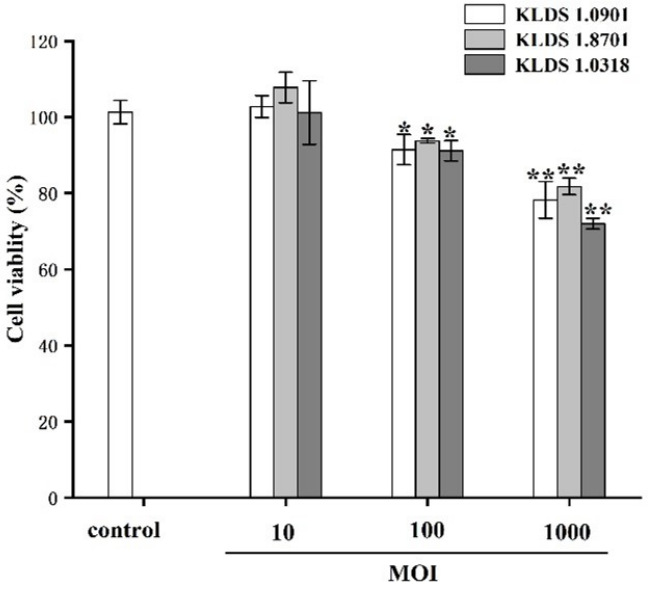

Figure 1. Effects of the tested strains at different MOI on the cell viability by the CCK8 assay. Data are expressed as means ± SD (n = 6). **p* < 0.05 and ***p* <0 .01 vs. the control group.

3.2 Inhibition effect of the tested strains on inflammatory mediator

To evaluate the potential anti-inflammatory property of the tested strains, the concentrations of NO and PGE2 and the mRNA expressions of iNOS and COX-2 were detected in LPS-stimulated RAW264.7 cells. Compared to the control group, the level of NO in the LPS group was increased (Figure 2A). However, the concentration of NO in *L. acidophilus* KLDS 1.0901 ($P < 0.001$), *L. helveticus* KLDS 1.8701 ($P < 0.05$), and *L. plantarum* KLDS 1.0318 ($P < 0.001$) group was significantly lower than that in the LPS group. This result was consistent with the changes in iNOS mRNA levels (Figure 2C). As shown in Figure 2B, the level of PGE2 in the LPS group was markedly higher in contrast to that of the control group. However, treatment with all tested strains decreased the production of PGE2. Furthermore, the mRNA expression of COX-2 that regulated PGE2 production showed a similar trend (Figure 2D). As aforementioned, all tested strains could decrease the concentration and mRNA expression of proinflammatory mediators. Furthermore, *L. plantarum* KLDS 1.0318 had a more substantial inhibition effect than *L. acidophilus* KLDS 1.0901, followed by *L. helveticus* KLDS 1.8701.

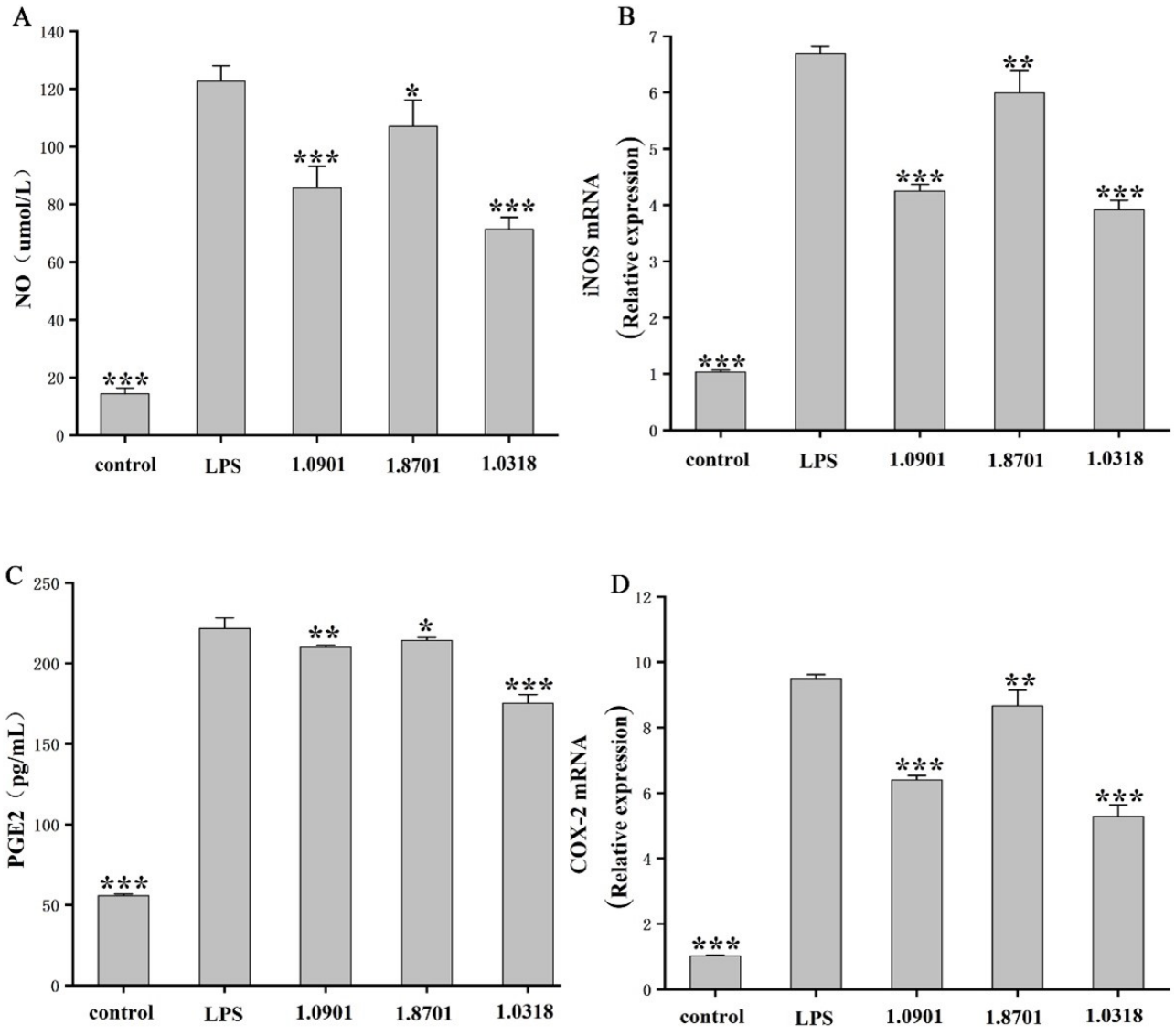

Figure 2. Effects of the tested strains on NO (A), PGE2 (B) production and iNOS (C), COX-2 (D) mRNA expression in LPS-induced RAW 264.7 cells. Cells were pretreated with the tested strains for 1h and then stimulated with LPS for 12 h. Data are expressed as means ± SD (n = 3). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ vs. the LPS group.

3.3 Inhibition effect of the tested strains on inflammatory cytokines

This study evaluated the effect of the tested strains against proinflammatory cytokines by measuring TNF-α, IL-1β, IL-6 production, and their mRNA expression. The TNF-α, IL-1β, and IL-6 concentrations in the LPS group were significantly higher than that in the control group, implying that LPS treatment could increase proinflammatory cytokines' secretion. While administering all the tested strains decreased those proinflammatory cytokines significantly (Figure 3A-3C), so did mRNA levels (Figure 3D-F). In summary, the above results showed that all the tested strains effectively inhibited the secretion of proinflammatory cytokines by down-regulating their gene transcription levels in LPS-induced RAW264.7 cells. Also, *L. plantarum* KLDS 1.0318 had the best anti-inflammatory effect.

3.4 Effect of the tested strains on TLR4 mRNA and protein expression

To estimate the strains' capacity to inhibit LPS receptor expression, TLR4 mRNA and protein expression levels were detected using RT-qPCR and western blot analysis, respectively. Compared with the control group, the mRNA expression of TLR4 in the LPS group increased from 1.35 ± 0.27 to 9.65 ± 0.28 (Figure 4A). However, compared to the LPS group, the TLR4 mRNA expression level was reduced to 5.79 ± 0.21 (*L. acidophilus* KLDS 1.0901), 7.17 ± 0.40 (*L. helveticus* KLDS 1.8701), and 4.89±0.34 (*L. plantarum* KLDS 1.0318) following the treatment of the tested strains, respectively. Because of LPS intervention, the protein expression of TLR4 increased significantly (Figure 4B). Compared to the LPS group, the TLR4 relative expressions with the tested strains were significantly down-regulated by 37.75%

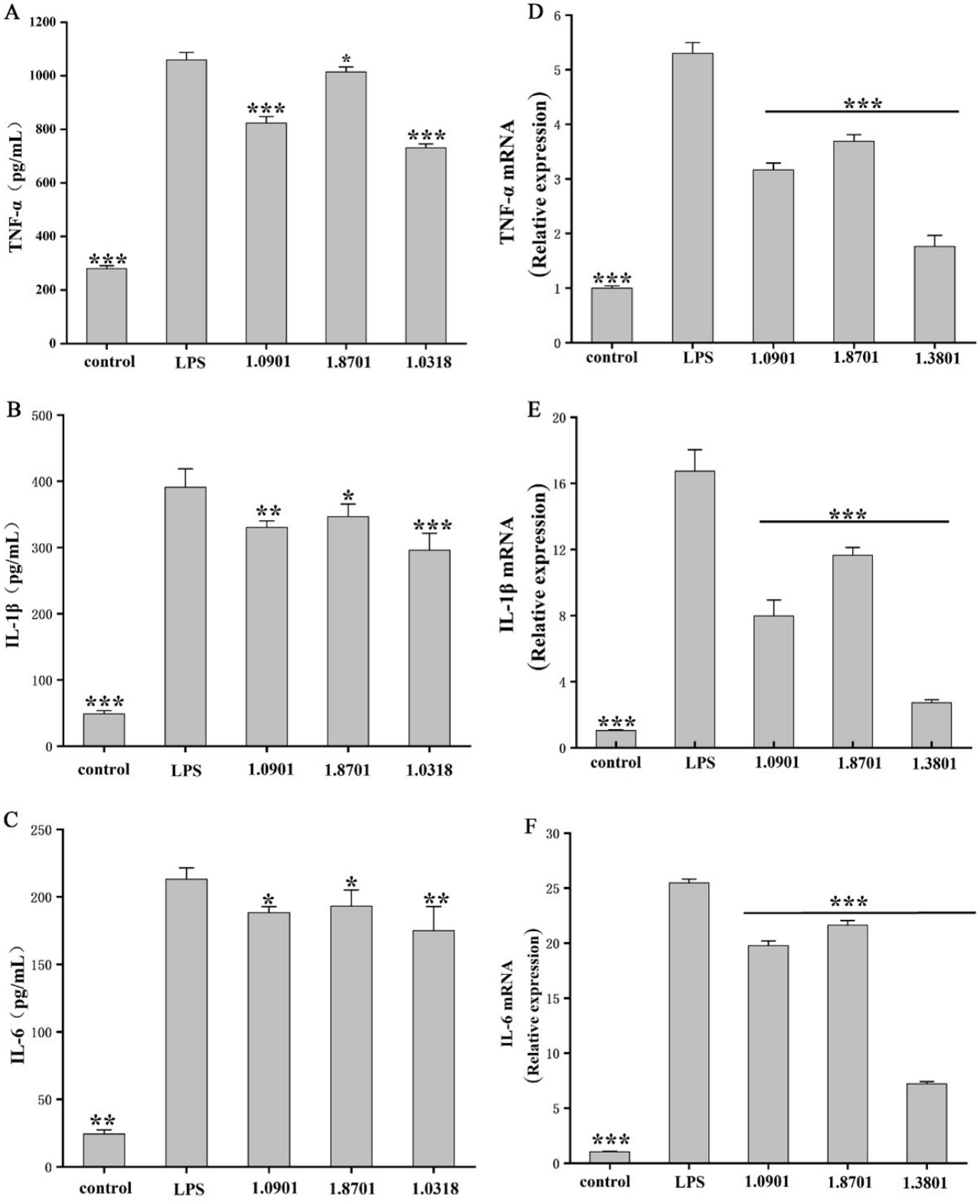

Figure 3. Effects of the tested strains on production and gene expression of proinflammatory cytokines. Cells were with *L. acidophilus* KLDS1.0901, *L. helveticus* KLDS1.8701 and *L. plantarum* KLDS1.0318 for 1 h. TNF-α (A), IL-1β (B) and IL-6 (C) production and TNF-α (D), IL-1β (E) and IL-6 (F) mRNA expression were detected by ELISA and RT-qPCR after LPS stimulated for 12 h. Data are expressed as means ± SD (n = 3). $^{*}p < 0.05$,$^{**}p < 0.01$ and $^{***}p < 0.001$ vs. the LPS group.

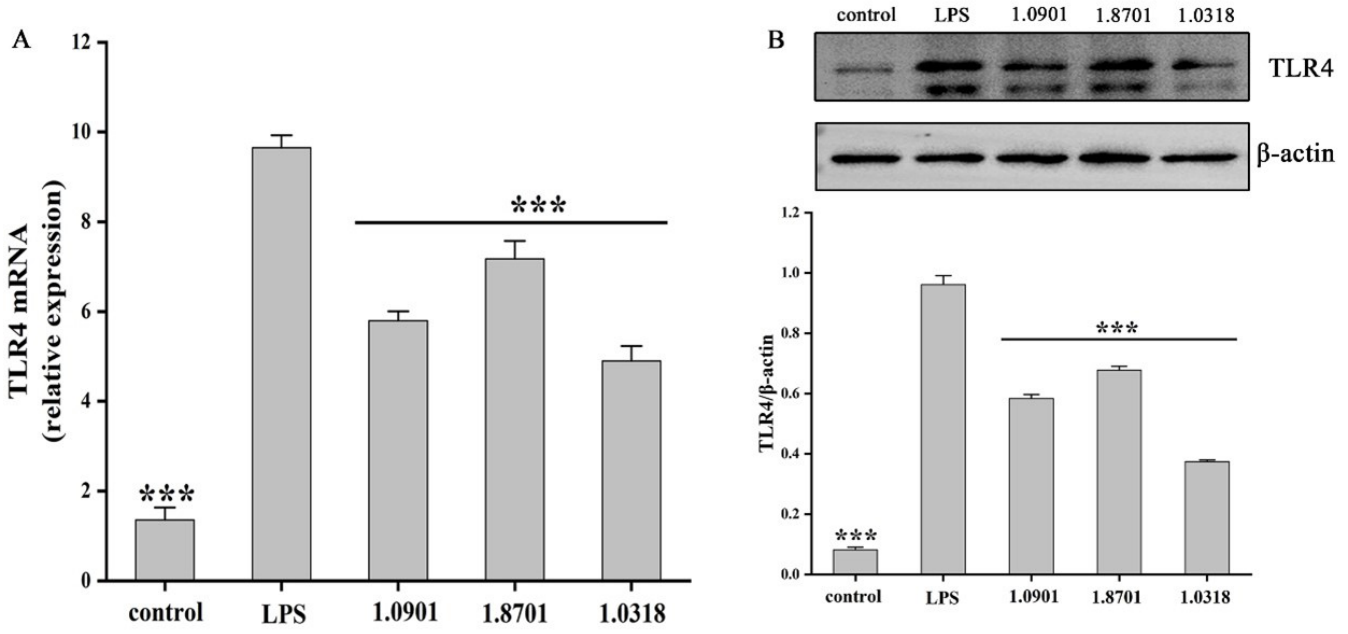

Figure 4. Effects of the tested strains on mRNA and protein relative expression of TLR4. Cells were with L. acidophilus KLDS1.0901, L. helveticus KLDS1.8701 and L. plantarum KLDS1.0318 for 1 h. TLR4 mRNA (A) and protein (B) were determined by RT-qPCR and western blot analysis after LPS stimulated for 12 h. Data are expressed as means ± SD (n = 3). ***p <0.001 vs. the LPS group.

(*L. acidophilus* KLDS 1.0901), 28.32% (*L. helveticus* KLDS 1.8701), and 58.70% (*L. plantarum* KLDS 1.0318), respectively. These findings implied that all tested strains could significantly inhibit TLR4 mRNA and protein expression in the following order: *L. plantarum* KLDS 1.0318 > *L. acidophilus* KLDS 1.0901 > *L. helveticus* KLDS 1.8701.

3.5 Effect of the tested strains on NF-κB activation

We analyzed the inhibition effect of the tested strains on the NF-κB signaling pathway activation. Compared with the control group, the expression level of the *p*-IκB in the LPS group increased significantly ($P < 0.05$), while the expression level of NF-κB p65 (cytoplasm) in the LPS group reduced observably, with a corresponding increase in the expression level of NF-κB p65 in the nucleus (Figure 5). However, compared to the LPS group, *p*-IκB expression with the tested strains was considerably down-regulated by 4.05% (*L. acidophilus* KLDS 1.0901), 8.20% (*L. helveticus* KLDS 1.8701), and 22.43% (*L. plantarum* KLDS 1.0318), respectively. And the expression of NF-κB p65 in the nucleus decreased by 18.19% (*L. acidophilus* KLDS 1.0901), 16.88% (*L. helveticus* KLDS 1.8701), and 39.16% (*L. plantarum* KLDS 1.0318) after the tested strains pretreatment compared with that of the LPS group, respectively. The above data implied that all tested strains could inhibit the NF-κB signaling pathway activation by blocking the phosphorylation of IκB, thus reducing the transport of NF-κB p65 into the nucleus.

3.6 Effect of the tested strains on MAPKs activation

To determine whether the MAPKs signaling pathway has a dominant position of the tested strains' anti-inflammatory potential against the inflammation response induced by LPS, the phosphorylation level of MAP kinases (p38, JNK, and ERK) was measured. As shown in Figure 6, the LPS group showed a significantly increase in the phosphorylation level of p38, JNK, and ERK compared with that of the control group ($P < 0.001$). However, all tested strains pretreatment restored the changes in MAP kinase. The *p*-p38/p38 expression with the tested strains pretreated was significantly reduced by 12.97% (*L. acidophilus* KLDS 1.0901), 11.06% (*L. helveticus* KLDS 1.8701), and 27.14% (*L. plantarum* KLDS 1.0318), respectively. Similarly, *p*-ERK/ERK protein level significantly down-regulated by 20.87% (*L. acidophilus* KLDS 1.0901), 23.05% (*L. helveticus* KLDS 1.8701), and 45.02% (*L. plantarum* KLDS 1.0318), respectively. Furthermore, the reduction of *p*-JNK/JNK protein expression (24.39%, 7.03%, and 16.86%) was found in *L. acidophilus* KLDS 1.0901, *L. helveticus* KLDS 1.8701, and *L. plantarum* KLDS 1.0318 group, respectively. The above results indicated that all tested strains could reduce p38, JNK, and ERK phosphorylation to suppress MAPKs signaling pathway activation.

3.7 Contrast KLDS 1.0318 and NF-κB /MAPKs inhibitors on inflammatory pathways

Based on the above results, NF-κB and MAPKs inhibitors were used to assess further the anti-inflammatory effect of *L. plantarum* KLDS 1.0318, which was most effective. As shown in Figure 7, compared with the control group, the increase of iNOS and COX-2 protein expressions was found in the LPS group ($P < 0.001$). However, the iNOS and COX-2 expression levels in *L. plantarum* KLDS 1.0318 group were noticeably decreased ($P < 0.001$), which showed the same trend with the

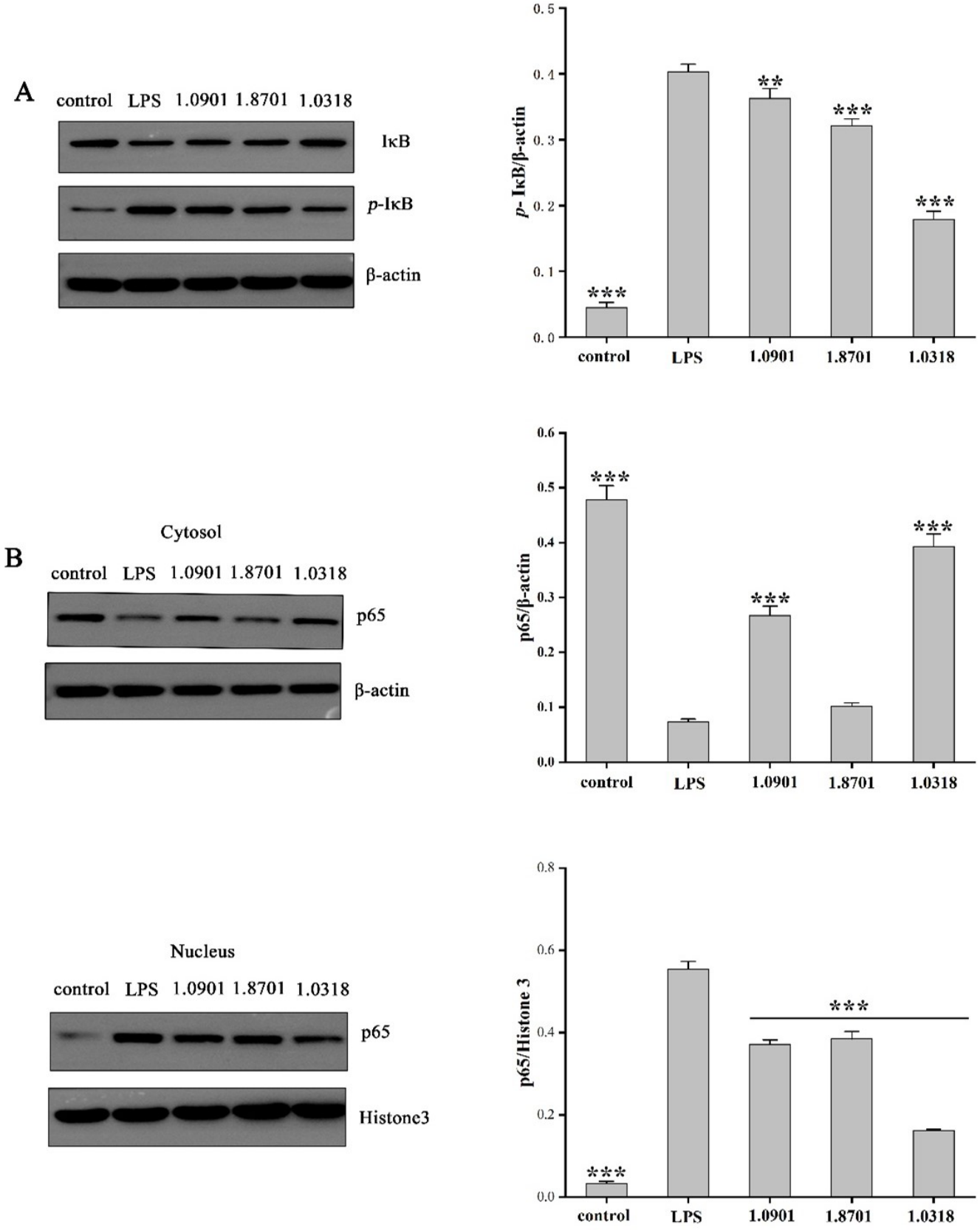

Figure 5. Effects of the tested strains on LPS-induced NF-κB signaling pathway. Cells were pretreated with *L. acidophilus* KLDS1.0901, *L. helveticus* KLDS1.8701 and *L. plantarum* KLDS1.0318 for 1 h. The IκB, phosphorylation of IκB(A)and nuclear p65, cytoplasm p65 (B) were determined by western blot analysis. Data are expressed as means ± SD (n = 3). $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. the LPS group.

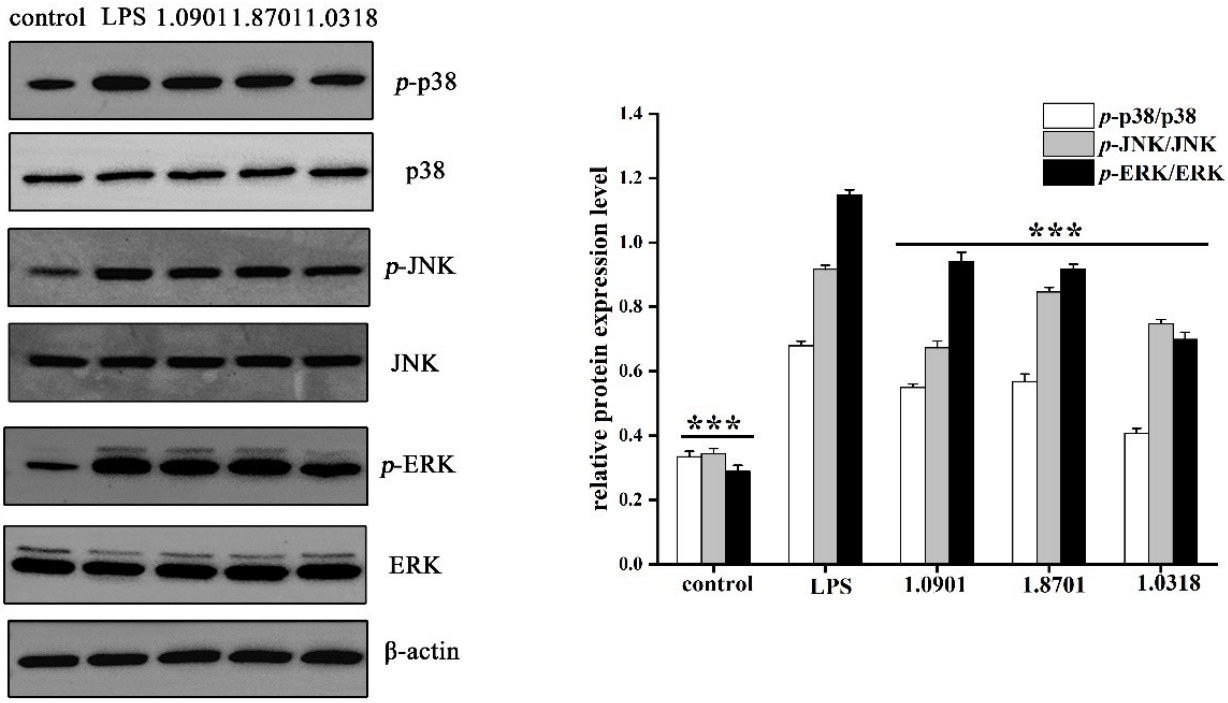

Figure 6. Effects of the tested strains on LPS-induced MAPKs signaling pathway. Cells were pretreated with *L. acidophilus* KLDS1.0901, *L. helveticus* KLDS1.8701 and *L. plantarum* KLDS1.0318 for 1 h. The phosphorylation of p38, ERK and JNK were detected by western blot analysis after LPS stimulated for 20 min. Data are expressed as means ± SD (n = 3). ***p <0.001 vs. the LPS group.

BAY11-7082 (NF-kB inhibitor, 2.5 μM), SB203580 (p38 inhibitor, 10 μM), SP600125 (JNK inhibitor, 10 μM), and PD98059 (ERK inhibitor, 10 μM) inhibitors groups. In particular, the combination *L. plantarum* KLDS 1.0318 with specific inhibitors groups showed more effective suppression, compared with *L. plantarum* KLDS 1.0318 or specific inhibitors alone. Furthermore, the iNOS level was more effectively suppressed by the combined treatment of *L. plantarum* KLDS 1.0318 with BAY11-708 or SB203580, compared with *L. plantarum* KLDS 1.0318 alone. Moreover, *L. plantarum* KLDS 1.0318 with PD98059 or BAY 11-708 showed a higher inhibitory effect than the *L. plantarum* KLDS 1.0318 group in terms of COX-2 expression. These findings indicated that *L. plantarum* KLDS 1.0318 exhibits synergistic inhibition when combined with the specific inhibitors.

4 Discussion

Inflammation responses can eliminate harmful external invasion or heal tissue injury. However, excessive inflammatory responses are closely related to the development of mild or chronic disease. Therefore, reducing inflammation is a critical preventive and therapeutic strategy for controlling various diseases. Furthermore, due to the limitations and side effects of traditional anti-inflammatory drugs, it is essential to develop alternative measures that are effective and without side effects. There are currently no suitable food-grade products to treat inflammation for long-term oral administration and minimizing drug toxicity. Many research works have shown that probiotics can improve infectious and inflammatory diseases, such as IBD, allergy and diabetes (Bock et al., 2021; Castagliuolo et al., 2010; Vanderhoof & Mitmesser, 2010). Therefore, in our present study, the anti-inflammatory potential of *L. acidophilus* KLDS 1.0901, *L. helveticus* KLDS 1.8701, and *L. plantarum* KLDS 1.0318 were evaluated, and the associated cellular signaling pathways were investigated.

Drug cytotoxicity requires evaluation before development towards therapeutic applications. Therefore, we assessed the effect of *L. acidophilus* KLDS 1.0901, *L. helveticus* KLDS 1.8701, and *L. plantarum* KLDS 1.0318 on the viability of RAW 264.7 macrophages. All tested strains did not show cytotoxicity at the MOI of 10. Therefore, the tested strains (MOI = 10) were further studied to illustrate the anti-inflammatory potential. NO and PGE2 are crucial biomarkers in the inflammatory response, modulated by iNOS and COX-2, respectively (Sautebin, 2000). iNOS and COX-2 are undetectable in the basal state, while LPS stimulated RAW264.7 cells to induce iNOS and COX-2 expression by activating NF-κB and MAPKs signal pathways to the secretion of excessive NO and PGE2. NO acts on vascular smooth muscle cells at the inflammatory site, increasing vascular permeability and accelerating the infiltration of inflammatory, pain mediators, and monocytes into the inflammatory site. Besides, PGE2 is associated with fever, swelling, and pain perception (Degagné et al., 2009). Therefore, inhibiting the overexpression of iNOS and COX-2 is an essential strategy for preventing and treating inflammatory diseases. In the current study, all tested strains significantly reduced NO and PGE2 production by inhibiting iNOS and COX-2 mRNA levels. Many experimental pieces of evidence support these results to validate claims that probiotics can ameliorate inflammation.

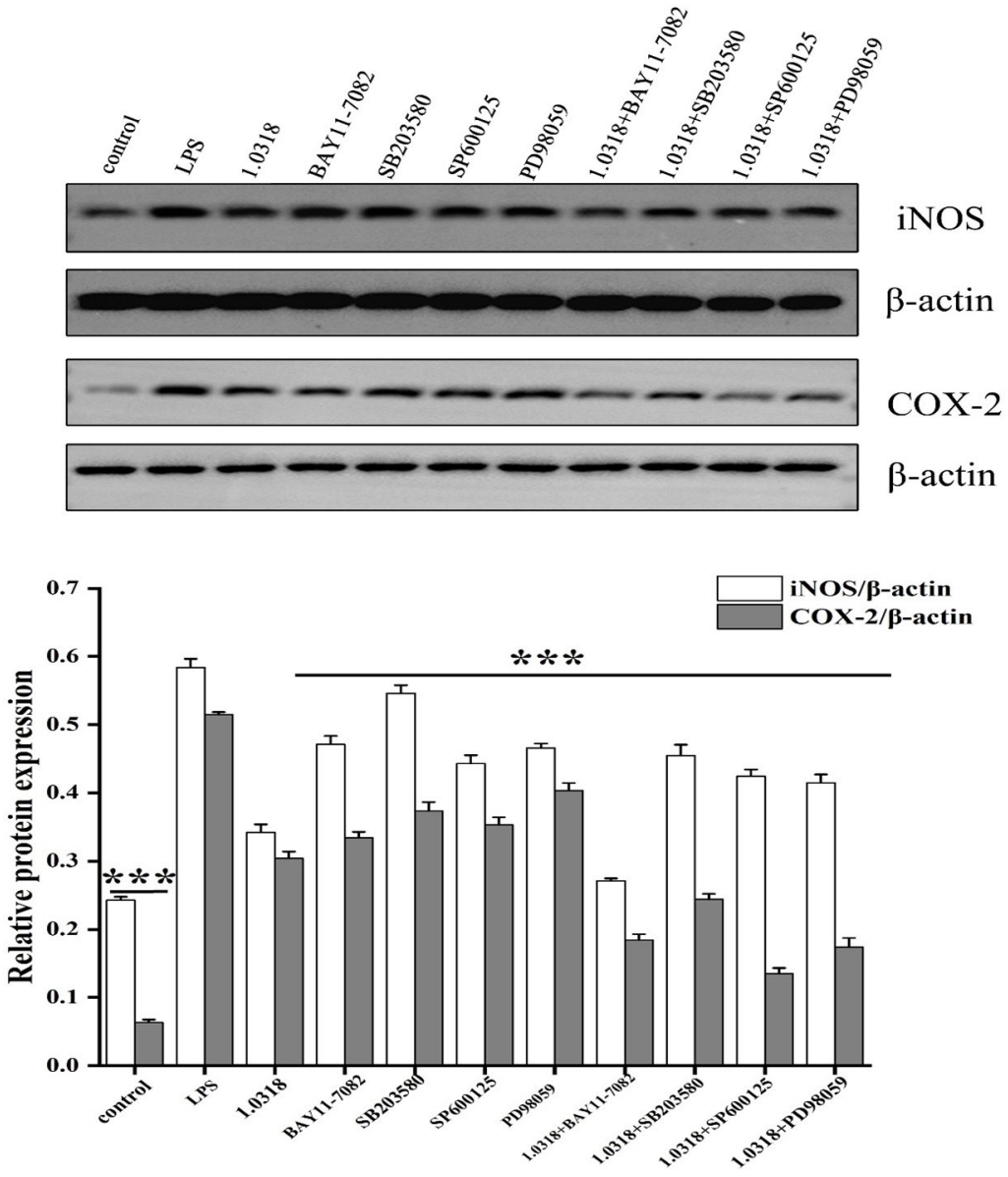

Figure 7. Effects of L. plantarum KLDS1.0318 and NF-κB and MAPKs inhibitors on LPS-induced iNOS and COX-2 protein relative expression in RAW 264.7 cells. Cells were pretreated with three strains for 1h, iNOS and COX-2 were determined by LPS stimulation for 24 h, respectively. Data are expressed as means ± SD (n = 3). ***p <0.001 vs. the LPS group.

The previous study demonstrated that Hwangryunhaedoktang weakly inhibited various inflammatory mediators (NO, PGE2, iNOS, and COX-2) induced by LPS. However, fermentation with lactobacilli significantly increased the herbal compound's inhibitory effect, Hwangryunhaedoktang, on inflammatory mediator expression (Han et al., 2017). Especially among the tested strains, *L. plantarum* KLDS 1.0318 had the most potent inhibitory effect.

LPS-induced murine RAW264.7 cells produce several proinflammatory cytokines, including TNF-α, IL-1β, and IL-6. Excessive release of proinflammatory cytokines can cause acute or chronic inflammatory diseases. TNF-α is a key cytokine that stimulates other cytokines' production in inflammatory responses and promotes the immune response process (Shou et al., 2019). IL-1β involves in local and systemic immune responses and is a common inflammatory cytokine that can secrete other inflammatory cytokines, such as IL-6, IL-8, and TNF-α (Zhang et al., 2016). IL-6 perpetuates and amplifies the inflammatory response and plays an important role in innate and adaptive immunity (Singh et al., 2016). In the present study, the levels of pro-inflammatory cytokines levels (TNF-α, IL-1β, IL-6, and PGE2) were higher after LPS treatment than that in the control group. However, compared to the LPS group, we discovered a remarkable reduction in the production and mRNA expression of those cytokines in *L. acidophilus* KLDS 1.0901, *L. helveticus* KLDS 1.8701, and *L. plantarum* KLDS 1.0318-treated groups. In agreement with our observations, *L. plantarum* 10hk2 isolated from fermented vegetables reduced

IL-1β, IL-6, and TNF-α levels and increased the secretion of IL-10 in LPS-induced RAW 264.7 cells (Chon et al., 2010). The above results established the anti-inflammatory potential of *L. acidophilus* KLDS 1.0901, *L. helveticus* KLDS 1.8701, and *L. plantarum* KLDS 1.0318.

To further identify the possible mechanisms by which these strains confer the reported beneficial properties, we investigate whether TLR4-mediated NF-κB and MAPKs signaling pathways are associated with the anti-inflammatory ability *L. acidophilus* KLDS1.0901, *L. helveticus* KLDS1.8701, and *L. plantarum* KLDS1.0318. TLR4 is an integral receptor for LPS to activate NF-κB and MAPK pathways (Wang et al., 2021). NF-κB signal plays a crucial role in gene expression in macrophages. LPS induces degradation and ubiquitylation of IκB and NF-κB transfer into the nucleus; afterward, NF-κB regulates downstream gene transcription to promote the expression of proinflammatory cytokines (TNF-α, IL-6, and IL-1β) and inflammatory mediators (NO and PGE2) (Wang et al., 2018; Wu et al., 2017; Xu et al., 2014; Xue et al., 2008). In this study, the three strains studied showed significant inhibitory effects on the nuclear translocation of NF-κB. Similarly, *Lactobacillus*-fermented adlay-soymilk could suppress the production of PGE2 and NO and the synthesis of IL-1β, IL-6, and TNF-α by down-regulated translocation of NF-κB p65 in the LPS-induced RAW164.7 cells (Wu et al., 2013).

The MAPKs are intracellular signaling molecules that regulate inflammatory responses through NF-κB activation and transcription. Currently, the MAPKs family includes three primary members, notably p38, JNK, and ERK regulating gene expression (Shanura Fernando et al., 2018; Shin et al., 2021). For example, COX is the rate-limiting enzyme for prostaglandin (PG) synthesis, and p38, JNK, and ERK are involved in the expression of COX-2. Furthermore, the MAPKs signaling pathway could regulate cytokine secretion through p38, JNK, and ERK phosphorylation. The present study showed that treatment with all tested strains suppressed MAPKs signal pathway activation by reducing the phosphorylation of p38, JNK, and ERK factors. Previously, *Lactobacillus brevis* G-101 down-regulated TNBS-induced inflammatory signatures by suppressing the MAPKs phosphorylation in mice colitis (Jang et al., 2013).

Generally, the maximum phosphorylation of LPS-induced MAP kinase is achieved within 30 min after LPS treatment and then rapidly decreased. Therefore, we measured the phosphorylation changes of MAP kinase after LPS stimulation for 20 min. The production of inflammatory mediators (NO and PGE2) and proinflammatory cytokines (TNF-α, IL-1β, and IL-6) was measured for 24 h. However, the data did not specifically show NF-κB and MAP kinase activity changes in the late status of LPS-stimulated RAW 264.7 cells. Therefore, we aimed to determine LPS treatment's effect on NF-κB and MAPKs signaling pathways in the late stage, using strategic co-administration of *L. plantarum* KLDS 1.0318 with specific inhibitors, thereby decreasing the protein level of the downstream target genes (iNOS and COX-2) (Wang et al., 2018). The results showed that each combined treatment (*L. plantarum* KLDS 1.0318 + a specific inhibitor) exhibited better inhibition on LPS- stimulated iNOS and COX-2 expressions. Specifically, LPS-induced iNOS expression was markedly suppressed by the combinations of *L. plantarum* KLDS 1.0318 with BAY 11-708 and SB203580. Moreover, LPS-stimulated COX-2 expression was significantly inhibited by *L. plantarum* KLDS 1.0318 with PD98059 and BAY 11-708.

We investigated the anti-inflammatory potential of *L. acidophilus* KLDS 1.0901, *L. helveticus* KLDS 1.8701, and *L. plantarum* KLDS 1.0318 isolated from traditional dairy products in LPS-stimulated murine RAW264.7 cells. All tested strains could inhibit inflammatory mediators (NO and PGE2) and proinflammatory cytokines (TNF-α, IL-1β, and IL-6) though down-regulating their relative gene expressions. This inhibitory function was related to reduced translocation of NF-κB into the nucleus and the phosphorylation of MAP kinase. This study provides that *L. acidophilus* KLDS 1.0901, *L. helveticus* KLDS 1.8701, and *L. plantarum* KLDS 1.0318 could be functional probiotics to prevent inflammation-associated disorders.

Conflict of interest

The authors declare that they have no conflicts of interest.

Author contributions

Bailiang Li and Guicheng Huo planned and supervised the experiments; Jialu Shi Huizhen Li and Shengnan Liang carried out the experiments; Jialu Shi, Shengnan Liang, Huizhen Li analyzed the data; Jialu Shi and Smith Etareri Evivie prepared the figures; Jialu Shi wrote the manuscript; Bailiang Li and Guicheng Huo revised the manuscript; all authors have read and agreed to the final manuscript draft.

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References

Bock, P. M., Telo, G. H., Ramalho, R., Sbaraini, M., Leivas, G., Martins, A. F., & Schaan, B. D. (2021). The effect of probiotics, prebiotics or synbiotics on metabolic outcomes in individuals with diabetes: a systematic review and meta-analysis. *Diabetologia*, 64(1), 26-41. http://dx.doi.org/10.1007/s00125-020-05295-1. PMid:33047170.

Castagliuolo, I., Galeazzi, F., Ferrari, S., Elli, M., Brun, P., Cavaggioni, A., Tormen, D., Sturniolo, G. C., Morelli, L., & Palù, G. (2010). Beneficial effect of auto-aggregating Lactobacillus crispatus on experimentally induced colitis in mice. *FEMS Immunology and Medical Microbiology*, 43(2), 197-204. http://dx.doi.org/10.1016/j.femsim.2004.08.011.

Chen, L., Zou, Y., Peng, J., Lu, F., Yin, Y., Li, F., & Yang, J. (2015). *Lactobacillus acidophilus* suppresses colitis-associated activation of the IL-23/Th17 axis. *Journal of Immunology Research*, 2015, 909514. http://dx.doi.org/10.1155/2015/909514. PMid:25973440.

Chon, H., Choi, B., Jeong, G., Lee, E., & Lee, S. (2010). Suppression of proinflammatory cytokine production by specific metabolites of Lactobacillus plantarum 10hk2 via inhibiting NF-κB and p38 MAPK expressions. *Comparative Immunology, Microbiology and*

Infectious Diseases, 33(6), e41-e49. http://dx.doi.org/10.1016/j.cimid.2009.11.002. PMid:19954847.

Degagné, E., Grbic, D. M., Dupuis, A. A., Lavoie, E. G., Langlois, C., Jain, N., Weisman, G. A., Sévigny, J., & Gendron, F. P. (2009). P2Y2 receptor transcription is increased by NF-κB and stimulates cyclooxygenase-2 expression and PGE2 released by intestinal epithelial cells. Journal of immunology, 183(7), 4521-4529. http://dx.doi.org/10.4049/jimmunol.0803977. PMid:19734210.

Du, J. C., Fei, L., Li, B. L., Xin, B., Evivie, S. E., Min, X., & Huo, G. C. (2017). In Vitro Assessment of Probiotic Potential of Lactobacillus acidophilus and Antagonistic Activity Against Escherichia coil O157:H7. *The Journal of Northeast Agricultural University*, 024(001), 59-69.

Fedorak, R. N., Gionchetti, P., Campieri, M., Madsen, K., Isaacs, K., Desimone, C., & Sartor, B. (2003). VSL3 Probiotic mixture induces remission in patients with active ulcerative colitis. *Gastroenterology*, 124(4), A377-A377. http://dx.doi.org/10.1016/S0016-5085(03)81909-1.

Gorbach, S. L. (2000). Probiotics and gastrointestinal health. *The American Journal of Gastroenterology*, 95(1 Suppl. 1), S2-S4. http://dx.doi.org/10.1016/S0002-9270(99)00806-0.

Guo, X., & Chen, J. (2019). The protective effects of saxagliptin against lipopolysaccharide (LPS)-induced inflammation and damage in human dental pulp cells. *Artificial Cells, Nanomedicine, and Biotechnology*, 47(1), 1288-1294. http://dx.doi.org/10.1080/21691401.2019.1596925. PMid:30942641.

Han, B. H., Lee, Y. J., Yoon, J. J., Choi, E. S., Namgung, S., Jin, X. J., Jeong, D. H., Kang, D. G., & Lee, H. S. (2017). Hwangryunhaedoktang exerts anti-inflammation on LPS-induced NO production by suppressing MAPK and NF-κB activation in RAW264.7 rmacrophages. *Journal of Integrative Medicine*, 15(4), 326-336. http://dx.doi.org/10.1016/S2095-4964(17)60350-9. PMid:28659238.

Jang, S. E., Hyam, S. R., Han, M. J., Kim, S. Y., Lee, B. G., & Kim, D. H. (2013). *Lactobacillus brevis* G-101 ameliorates colitis in mice by inhibiting NF-κB, MAPK and AKT pathways and by polarizing M1 macrophages to M2-like macrophages. *Journal of Applied Microbiology*, 115(3), 888-896. http://dx.doi.org/10.1111/jam.12273. PMid:23742179.

Lawrence, T., Willoughby, D. A., & Gilroy, D. W. (2002). Anti-inflammatory lipid mediators and insights into the resolution of inflammation. *Nature Reviews. Immunology*, 2(10), 787-795. http://dx.doi.org/10.1038/nri915. PMid:12360216.

Lee, H. N., Na, H. K., & Surh, Y. J. (2013). Resolution of inflammation as a novel chemopreventive strategy. *Seminars in Immunopathology*, 35(2), 151-161. http://dx.doi.org/10.1007/s00281-013-0363-y. PMid:23370700.

Li, B., Du, P., Smith, E. E., Wang, S., Jiao, Y., Guo, L., Huo, G., & Liu, F. (2019). In vitro and in vivo evaluation of an exopolysaccharide produced by Lactobacillus helveticus KLDS1.8701 for the alleviative effect on oxidative stress. *Food & Function*, 10(3), 1707-1717. http://dx.doi.org/10.1039/C8FO01920G. PMid:30839970.

Lu, Y. C., Yeh, W. C., & Ohashi, P. S. (2008). LPS/TLR4 signal transduction pathway. *Cytokine*, 42(2), 145-151. http://dx.doi.org/10.1016/j.cyto.2008.01.006. PMid:18304834.

Lu, Y., Suh, S. J., Kwak, C. H., Kwon, K. M., Seo, C. S., & Ying, L. (2012). Saucerneol F, a new lignan, inhibits iNOS expression via MAPKs, NF-κB and AP-1 inactivation in LPS-induced RAW264.7 cells. *International Immunopharmacology*, 12(1), 175-181. http://dx.doi.org/10.1016/j.intimp.2011.11.008.

Meng, Y., Wang, J., Wang, Z., Zhang, G., Liu, L., Huo, G., & Li, C. (2019). *Lactobacillus plantarum* KLDS1.0318 ameliorates impaired intestinal immunity and metabolic disorders in cyclophosphamide-treated mice. *Frontiers in Microbiology*, 10, 731. http://dx.doi.org/10.3389/fmicb.2019.00731. PMid:31031723.

Park, J. W., Kwon, O. K., Kim, J. H., Oh, S. R., Kim, J. H., Paik, J. H., Marwoto, B., Widjhati, R., Juniarti, F., Irawan, D., & Ahn, K. S. (2015). Rhododendron album Blume inhibits iNOS and COX-2 expression in LPS-stimulated RAW264.7 cells through the downregulation of NF-κB signaling. *International Journal of Molecular Medicine*, 35(4), 987-994. http://dx.doi.org/10.3892/ijmm.2015.2107. PMid:25784296.

Rama, G. R., Dullius, D., Agnol, W. D., Esquerdo, V. M., Lehn, D. N., & Souza, C. F. V. (2020). Ricotta whey supplemented with gelatin and collagen for the encapsulation of probiotic lactic acid bacteria. *Food Science and Technology (Campinas)*, 41(3), 576-586. http://dx.doi.org/10.1590/fst.19720.

Rauta, P. R., Samanta, M., Dash, H. R., Nayak, B., & Das, S. (2014). Toll-like receptors (TLRs) in aquatic animals: signaling pathways, expressions and immune responses. *Immunology Letters*, 158(1–2), 14-24. http://dx.doi.org/10.1016/j.imlet.2013.11.013. PMid:24291116.

Rong, J., Zheng, H., Liu, M., Hu, X., Wang, T., Zhang, X., Jin, F., & Wang, L. (2015). Probiotic and anti-inflammatory attributes of an isolate Lactobacillus helveticus NS8 from Mongolian fermented koumiss. *BMC Microbiology*, 15(1), 196. http://dx.doi.org/10.1186/s12866-015-0525-2. PMid:26428623.

Ryu, H. W., Lee, S. U., Lee, S., Song, H. H., Son, T. H., Kim, Y. U., Yuk, H. J., Ro, H., Lee, C. K., Hong, S. T., & Oh, S. R. (2017). 3-Methoxy-catalposide inhibits inflammatory effects in lipopolysaccharide-stimulated RAW264.7 macrophages. *Cytokine*, 91, 57-64. http://dx.doi.org/10.1016/j.cyto.2016.12.006. PMid:28011397.

Satish Kumar, C. S., Kondal Reddy, K., Reddy, A. G., Vinoth, A., Ch, S. R., Boobalan, G., & Rao, G. S.. (2015). Protective effect of *Lactobacillus plantarum* 21, a probiotic on trinitrobenzenesulfonic acid-induced ulcerative colitis in rats. *International Immunopharmacology*, 25(2), 504-510. http://dx.doi.org/10.1016/j.intimp.2015.02.026. PMid:25727887.

Sautebin, L. J. F. (2000). Prostaglandins and nitric oxide as molecular targets for anti-inflammatory therapy. *Fitoterapia*, 71(Suppl. 1), S48-S57. http://dx.doi.org/10.1016/s0367-326x(00)00181-7.

Shanura Fernando, I. P., Asanka Sanjeewa, K. K., Samarakoon, K. W., Lee, W. W., Kim, H. S., Ranasinghe, P., Gunasekara, U. K. D. S. S., & Jeon, Y. J. (2018). Antioxidant and anti-inflammatory functionality of ten Sri Lankan seaweed extracts obtained by carbohydrase assisted extraction. *Food Science and Biotechnology*, 27(6), 1761-1769. http://dx.doi.org/10.1007/s10068-018-0406-1. PMid:30483441.

Shin, K. S., Seo, H., Lee, S. J., Sung, S., & Hwang, D. (2021). Polysaccharide isolated from Korean-style soy sauce activates macrophages via the MAPK and NK-κB pathways. *Food Science and Technology*, 41(4), 817-824. http://dx.doi.org/10.1590/fst.06121.

Shou, J., Kong, X., Wang, X., Tang, Y., Wang, C., Wang, M., Zhang, L., Liu, Y., Fei, C., Xue, F., Li, J., & Zhang, K. (2019). Tizoxanide Inhibits Inflammation in LPS-Activated RAW264.7 Macrophages via the Suppression of NF-κB and MAPK Activation. *Inflammation*, 42(4), 1336-1349. http://dx.doi.org/10.1007/s10753-019-00994-3. PMid:30937840.

Singh, U. P., Singh, N. P., Murphy, E. A., Price, R. L., Fayad, R., Nagarkatti, M., & Nagarkatti, P. S. (2016). Chemokine and cytokine levels in inflammatory bowel disease patients. *Cytokine*, 77, 44-49. http://dx.doi.org/10.1016/j.cyto.2015.10.008. PMid:26520877.

Vanderhoof, J. A., & Mitmesser, S. H. (2010). Probiotics in the management of children with allergy and other disorders of intestinal inflammation. *Beneficial Microbes*, 1(4), 351-356. http://dx.doi.org/10.3920/BM2010.0034. PMid:21831774.

Wang, D. D., Pan, W J., Shomaila, M., Chen, X. D., & Yan, C. (2018). Polysaccharide isolated from Sarcodon aspratus induces RAW264.7 activity via TLR4-mediated NF-κB and MAPK signaling pathways. *International Journal of Biological Macromolecules*, 120(Pt A), 1039-1047. http://dx.doi.org/10.1016/j.ijbiomac.2018.08.147.

Wang, M. L., Wang, W. D., Wang, J. S., & Zhang, J. (2021). MiR-155 contribute to airway inflammation in COPD by regulating autophagy via targeting TLR4/NF-KB. *Food Science and Technology*, In press. http://dx.doi.org/10.1590/fst.44321.

Wu, S. J., Chen, Y. W., Wang, C. Y., & Shyu, Y. T. (2017). Anti-inflammatory properties of high pressure-assisted extracts of Grifola frondosa in lipopolysaccharide-activated RAW 264.7 macrophages. *International Journal of Food Science & Technology*, 52(3), 671-678. http://dx.doi.org/10.1111/ijfs.13320.

Wu, S. J., Fang, J. Y., Ng, C. C., Wang, C. Y., & Shyu, Y. T. (2013). Anti-inflammatory activity of Lactobacillus-fermented adlay-soymilk in LPS-induced macrophages through suppression of NF-κB pathways. *Food Research International*, 52(1), 262-268. http://dx.doi.org/10.1016/j.foodres.2013.02.053.

Xin, L. I., Chen, M. K., Jin, J. L., Zhou, H. Y., & Tian, Z. (2016). Clinical effect of probiotics on the ulcerative colitis and its mechanisms. *Xiandai Shengwu Yixue Jinzhan*, 16(27), 5361-5363. http://dx.doi.org/10.13241/j.cnki.pmb.2016.27.044.

Xu, X., Yin, P., Wan, C., Chong, X., Liu, M., Cheng, P., Chen, J., Liu, F., & Xu, J. (2014). Punicalagin inhibits inflammation in LPS-induced RAW264.7 macrophages via the suppression of TLR4-mediated MAPKs and NF-κB activation. *Inflammation*, 37(3), 956-965. http://dx.doi.org/10.1007/s10753-014-9816-2. PMid:24473904.

Xue, Y., Wang, Y., Feng, D. C., Xiao, B. G., & Xu, L Y. (2008). Tetrandrine suppresses lipopolysaccharide-induced microglial activation by inhibiting NF-κB pathway. *Acta Pharmacologica Sinica*, 29, 245-251. http://dx.doi.org/10.1111/j.1745-7254.2008.00734.x.

Yang, S. F., Zhuang, T. F., Si, Y. M., Qi, K. Y., & Zhao, J. (2015). Coriolus versicolor mushroom polysaccharides exert immunoregulatory effects on mouse B cells via membrane Ig and TLR-4 to activate the MAPK and NF-κB signaling pathways. *Molecular Immunology*, 64(1), 144-151. http://dx.doi.org/10.1016/j.molimm.2014.11.007.

Zhang, H. F., Zhou, G. X., & Zhang, B. (2016). Expression and significance of IL-1β in ulcerative colitis. *Journal of Nantong University*, 36(5), 417-420. http://dx.doi.org/10.16424/j.cnki.cn32-1807/r.2016.05.014.