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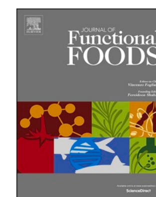

Fermentation of *Moringa oleifera* Lam. using *Bifidobacterium animalis* subsp. *lactis* enhances the anti-inflammatory effect in RAW 264.7 macrophages

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ABSTRACT

Moringa oleifera Lam. (MO) is traditionally used to treat various ailments, including swelling, hypertension, and diabetes. We investigated the anti-inflammatory effects of *Bifidobacterium animalis* subsp. *lactis* (*B. lactis*)-fermented MO (MO-B) on LPS-mediated RAW 264.7 cells. HPLC analysis showed that (+)-catechin, ellagic acid, and quercetin-3-glucuronide contents of MO-B were markedly higher than those of MO extract. MO-B contained kaempferol, which was not observed in MO. MO-B exhibited better inhibitory effects on the inflammatory factors NO and ROS in LPS-stimulated RAW 264.7 cells than MO and *B. lactis*, and the expression of pro-inflammation cytokine (*IL-6, IL-1β, TNFα*) was significantly downregulated in LPS-activated macrophages following MO-B-treatment. MO-B also suppressed the TLR4/NF-κB pathway cells and ameliorated the PI3K/AKT and MAPK pathways, involved in the regulatory mechanisms underlying NF-κB-mediated inflammation. Our results suggest that MO-B have the possibility to be a novel anti-inflammatory agent for use in therapeutics or as an ingredient in functional foods.

1. Introduction

Inflammation plays a key role in the response to infection and injury by signaling the immune system to heal and repair damaged tissues. However, excessive inflammatory responses could increase the risk of diseases such as diabetes, cardiovascular disease, arthritis, allergies, psoriasis, and rheumatoid arthritis. Macrophages are vital members of the innate immune system that can detect anti-inflammatory and immunoregulatory agents (Shou et al., 2019). LPS, an endotoxin found in cell walls of gram-negative bacteria, stimulates macrophages to induce pro-inflammatory factors such as nitric oxide (NO), *TNF-α, IL-1β,* and *IL-6*, through Toll-like receptor 4 (TLR4), expressed on the surface of the cell (Akira & Hemmi, 2003; Chae, 2018). TLR4 activates NF-κB to respond the redox-sensitive reactions and mediates the expression of pro-inflammatory genes, leading to the accumulation of pro-inflammatory cytokines and resulting in inflammation and injury (Fan et al., 2016). Preventing activation of the MAPK and PI3K/AKT signaling pathways is thus considered a potential strategy to block the NF-κB pathway and ameliorate inflammation-related diseases (Bist et al., 2017). In the present study, the anti-inflammatory potentials of MO, *B. lactis* and *B. lactis*-fermented MO were compared by evaluating the NF-κB, PI3K/AKT, and MAPK signaling pathways in LPS-stimulated murine macrophages.

MO belonging to the Moringaceae family is commonly known as the drumstick tree. It is a traditional herb that is widely cultivated in many tropical and subtropical countries worldwide, including India, Pakistan and Nepal (Razis, Ibrahim, & Kntayya, 2014). MO leaves have been traditionally used to treat various conditions such as asthma, bronchitis, blood impurities, blackheads, chest congestion, skin infections, cholera, diabetes and hypertension (Razis, Ibrahim, & Kntayya, 2014). MO is rich in bioactive compounds including polyphenols, flavonoids, phenolic acids, and isothiocyanates, which have anti-cancer, antibacterial, anti-inflammatory, antioxidant, hepatoprotective, and neuroprotective effects (Kooltheat et al., 2014; Kou et al., 2018), and has attracted the interest of researchers owing to its excellent anti-inflammatory properties that are the result of its of abundant bioactive compounds. Some studies have reported that MO can suppress lipopolysaccharide (LPS)-mediated RAW 264.7 cell activation, decrease the cytokines production,

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such *TNFα, IL-6*, and *IL-8* and inhibit the NF-κB signaling pathway involved in inflammation (Vergara-Jimenez et al., 2017). Various cytotoxic bioactive substances have been extracted using various of harmful solvents, including acetone, ethanol, ethyl acetate, and methanol (Cha et al., 2012).

Fermentation can enhance the biochemical and physiological activities of compounds by modifying the original molecule; it is, therefore, possible for the bacterial hydrolysis and structural degradation of plant cell walls involved in the fermentation process to increase the amount of polyphenol and other bioactive compounds present within fermented plant products, resulting in increased biological functions (Jeong et al., 2018; Wang et al., 2019). Previous studies have reported that fermented blueberry-blackberry has antioxidant, anti-inflammatory and antibacterial effects because the original phytochemical molecules are modified during the fermentation process (Johnson et al., 2013; Lavefve et al., 2020). The gastrointestinal microbiome plays a critical role in the human immune and metabolic systems (Luo et al., 2018; Strandwitz, 2018). Scientific studies have confirmed the health-promoting effects of fermented cabbage products *via* antioxidant, and anti-inflammatory activities (Peñas et al., 2012; Peñas, Martinez-Villaluenga, & Frias, 2017). *B. lactis* is a probiotic with several beneficial properties, including, immune response, anti-aging, anti-inflammation, anti-pathogenic and anti-tumor activities, all of which are of great benefit to human health (Delgado et al., 2020; Yu et al., 2019). Therefore in this study, we aimed to explore and confirm the therapeutic potential of *B. lactis*-fermented MO as an anti-inflammatory agent.

2. Materials and methods

2.1. Chemicals and reagents

GeneDEPOT company (San Antonio, TX, USA) provided DMEM medium, penicillin-streptomycin (PS; CA005-010), and fetal bovine serum (FBS; F0900-050) (made in USA). Dimethyl sulfoxide (DMSO; D2650-100 ML), and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; M6494), and lipopolysaccharide from *Escherichia* (LPS; L2880-10MG) reagents were obtained from Sigma-Aldrich (St Louis, MO, USA). DeMan, Rogosa, and Sharp (MRS; 9765) broth was procured from Company (BD, San Jose, CA USA). Macrogen company (Geumcheon-gu, Seoul) engineered primers for *IL-6, TNF-α, IL-1β, NF-κB*, and *IKKα*. Primary antibodies against: p38 (#8690), p-p38 (#4511), ERK (#9102), p-ERK (#9101), JNK (#9252), p-JNK (#9251), p65, IκBα (#9242), p-IκBα (#9246), p-p65 (#3033) and β-actin (#3700) antibodies were obtained by Cell Signaling Technology (CST, MA, USA). Standard references, such as (+)-catechin (CAS No. 154-23-4), quercetin-3-glucuronide (CAS No. 22688-79-5), ellagic acid (CAS No. 476-66-4) and kaemferol (CAS No. 520-18-3) were provided by Natural Product Institute of Science and Technology (Anseong, South Korea).

2.2. MO extraction

MO leaves were purchased from a local market in Coimbatore, Tamil Nadu, India, and their identity verified by Professor P. Jayaraman (Plant Anatomy Research Center). The specimen voucher number is PARC/2017/3906. MO leaves were completely washed, dried and then ground. 10 g of the resulting MO powder was soaked in 50 mL of ethanol (70%) at 37 °C for 24 h under dark conditions, and repeated three times. The extracted solution was then filtered through a Whatman No. 1 filter paper, and a rotary vacuum evaporator was used to evaporate the solvent at 50 °C. The resulting sample was kept at 4 °C. The extract was first dissolved in distilled water before use. A total yield of approximately 5–6% was obtained.

2.3. MO and B. lactis fermentation (MO-B)

The 16S rRNA sequence of *B. lactis* was obtained from a Korean company and deposited in NCBI GenBank under the accession number CP001606. *B. lactis* was anaerobically cultured in sterile MRS broth containing 0.05% L-cysteine-HCl at 37 °C in the non-shaking incubator. The culture reached an optical density of 1.2 at 600 nm, or reached stationary phase, the cells 10^7 CFU/mL after 48 h. MO (2 mg/mL) was fermented with *B. lactis* at 37 °C in incubator. Samples were sonicated for 40 min, then centrifuged at 4,000 rpm, 4 °C for 15 min after 24 h. The supernatant was collected and freeze dried for further experiments.

2.4. Determination of total polyphenol and total flavonoid content

The total phenol contents of MO-B and MO were determined using the Folin-Ciocalteau method (Baba & Malik, 2015) with some modifications. Briefly, 10 μL (1 μg/mL) samples solution was mixed with 10 μL Na_2CO_3 2% with 200 μL FolinCiocalteu reagent 50% in every well of a 96-well plate, and kept in the dark at room temperature. After 30 min the microplate was measured by SpectraMax® ABS plus machine at 750 nm. Gallic acid equivalents (0–500 μg/mL) were used to calculate total phenols, with the mean represented in mg of gallic acid equivalents per gram (mg GAE/g).

The aluminum chloride technique was used to determine total flavonoid content (Zhishen, Mengcheng, & Jianming, 1999) with slight modifications. Briefly, 10 μL (1 mg/mL) samples and 80 μL EtOH 80% were mixed into microplate wells and 20 μL of $AlCl_3$ 10%, 20 μL of $NaNO_2$ (1 M), and 80 μL EtOH 80% were added to the reaction mixture, which was then shaken, and the absorbance was determined at 415 nm. Various quercetin (0–200 μg/ mL) concentrations were prepared as a standard. The mean was presented as mg of quercetin equivalent per gram (mg QE/g).

2.5. HPLC analysis

(+)-Catechin, ellagic acid, quercetin-3-glucuronide, and kaempferol were considered as major compounds in MO, so their contents in MO and MO-B were quantitatively analyzed. The extract of MO (20 mg) and MO-B (20 mg) were dissolved in 1 mL of MeOH, respectively. MO and MO-B (20 mg/mL) were dissolved using an ultrasonic bath for 20 min. The suspension was filtered with a 0.45 μm PVDF membrane and the supernatant was used for HPLC analysis. The standard compounds including (+)-catechin, ellagic acid, quercetin-3-glucuronide, and kaempferol were dissolved in MeOH with concentration at 1 mg/mL and processed under the same conditions.

The contents of (+)-catechin, ellagic acid, quercetin-3-glucuronide, and kaempferol in MO and MO-B were analyzed using the Waters Alliance HPLC System (USA). A (0.1% trifluoroacetic acid in H_2O) and B (MeCN) were used to prepare the mobile phases. The gradient program is presented in Table S1. The sample volume was 10 μL, and the flow rate was set to 1.0 mL/min. The standard curves of (+)-catechin, ellagic acid, quercetin-3-glucuronide, and kaempferol were showed in Fig. S1.

2.6. Cell culture and viability examination

RAW 264.7 cells were cultured in full DMEM medium (10% FBS and1% PS) in a 5% CO_2 humidified incubator at 37 °C. The cytotoxicity of MO-B, MO, and *B. lactis* was assessed using an MTT assay. Cells were separated and cultured overnight in 96-well plates at a density of $1x10^5$ cells/mL. Cells were activated with LPS (1 μg/mL) for 1 h, and treated with different concentrations of MO-B, MO, and *B. lactis* (50, 75, 100, 150, and 200 μg/mL). After 24 h, MTT (0.5 mg/mL) solution was added, and the cells were kept in an incubator at 37 °C for 2 h. Formazan crystals were dissolved in DMSO. The absorbance was analyzed at 560 nm (SpectraMax® ABS plus) for each well.

2.7. NO determination

RAW 264.7 cells were treated with several concentrations of MO-B,

MO, and *B. lactis* (50, 75, 100, 150 and 200 μg/mL) and LPS (1 μg/mL) for 1 h. After 24 h, 100 μL of culture medium supernatant was transferred to another 96 well-plate and mixed with 100 μL Griess reagent (Thermo Fisher Scientific). The plate was then incubated at room temperature for 10 min, and the absorbance was measured at 570 nm by spectrophotometric microplate. Nitric production ($NaNO_2$) was used as standard to determine NO production.

2.8. ROS production

RAW 264.7 cells were cultured on glass slides in a small plate and treated with MO-B (100, 150, and 200 μg/mL), MO, and *B. lactis* (200 μg/mL) with LPS (1 μg/mL) over 24 h. Cells were then washed and treated using a cellular ROS/Superoxide Detection Assay Kit (Abcam, Cambridge, UK). A Leica DMLB fluorescence microscope was used to measure the fluorescence using rhodamine (Ex/Em = 550/620 nm) and fluorescein (Ex/Em = 490/525 nm) filter sets.

2.9. qRT-PCR analysis

RAW 264.7 cells were treated with MO, MO-B, *B. lactis*, or LPS (1 μg/mL) for 24 h. TRIsure^{TM} (Bioline, Luckennwalde, Germany) reagent was used to extract total RNA, which was diluted to 500 ng and mixed with amfiRivert cDNA Synthesis Platinum Master Mix (GenDEPOT, TX, USA) for transcription into cDNA. Each qRT-PCR reaction was performed in a 20 μL volume mixture containing 500 ng cDNA, primer and amfiSure qGreen Q-PCR Master Mix (GenDEPOT, TX, USA). Table S2 showed the list of inflammation-related genes. Relative gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method. The housekeeping gene, GAPDH, was used as a standard to analyze the expression levels of related genes.

2.10. ELISA and western blot analysis

RAW 264.7 cells were cultured in 60 mm dishes for 24 h and then treated with various concentrations of MO, MO-B, *B. lactis* and LPS (1 μg/mL) for 24 h. The culture supernatant was collected, and ELISA was used to quantify the *IL-6* (BD Biosciences, San Diego, CA, USA) and *TNF-α* (Thermo Fisher Scientific) levels in the supernatant. Cells were then washed in PBS, collected using a scraper and lysed using Pierce™ RIPA Buffer. The lysate as centrifuged at 12,000 rpm for 20 min at 4 °C. Bradford reagent was used to determine protein concentration. Total protein (50 ng) was run on a 10 % polyacrylamide gel electrophoresis, and the separated protein bands were transferred onto a PVDF membrane. After 1 h, the membrane was blocked with 5 % skim milk and probed with appropriate primary antibodies overnight at 4 °C. The membrane was then incubated with a horseradish peroxidase-linked secondary antibody (goat anti-mouse/rabbit IgG 1:2000) at room temperature for 2 h. Finally, an improved chemiluminescence (ECL) detection system (GenDEPOT, USA) was used with the Alliance Mini HD9 (Uvitec, Cambridge, UK) to detect the protein expression levels. ImageJ software was used to measure the intensity of the bands.

2.11. Statistical analysis

The experiment was repeated three times independently, and the results are presented as mean ± standard deviation or standard error for each experiment. The *p*-value of $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ was considered statistically significant.

3. Results

3.1. Quantification of phenolic content in MO-B and MO

The characteristics of MO-B and MO were identified to determine the biomedical properties of our sample (Table 1). The total polyphenol and flavonoid contents of MO were 11.75 ± 0.73 mg GAE/g and 4.72 ± 0.22 mg QE/g, respectively, whereas those of MO-B were 39.93 ± 3.09 mg GAE/g and 9.05 ± 0.1 mg QE/g, respectively, for MO-B, which is a significant increased.

Table 1
Total polyphenol, total flavonoid of MO-B and MO.

Samples	Total Polyphenol (mg GAE/g)	Total Flavonoid (mg QE/g)
MO-B	39.9 ± 3.09***	9.1 ± 0.1***
MO	11.8 ± 0.73	4.7 ± 0.22

Values are presented as mean ± standard deviation.
**P < 0.001 (*vs* MO).

Several phenolic compounds identified *via* HPLC analysis included (+)-catechin, ellagic acid, quercetin-3-glucuronide, and kaempferol as the main active compounds in MO and MO-B. The amount of (+)-catechin, ellagic acid, and quercetin-3-glucuronide (13.51 ± 0.16 mg/g, 0.59 ± 0.00 mg/g, and 9.88 ± 0.08 mg/g, respectively) was higher in MO-B than those in MO (Fig. 1). Kaempferol, not detected in the unfermented MO extract, was observed only after fermentation with *B. lactis.* These phenolic compounds have potent anti-inflammatory effects, indicating that fermentation may be the source of the enhanced anti-inflammatory properties observed.

3.2. Cytotoxicity of MO-B in RAW 264.7 cells

MTT assay was used to ascertain cytotoxicity in RAW 264.7 cells treated with MO-B. Fig. 2A indicates that no toxicity was associated with MO-B, MO, or *B. lactis* at the studied concentrations (from 50 to 200 μg/mL) in RAW 264.7 cells pretreated with LPS (1 μg/mL).

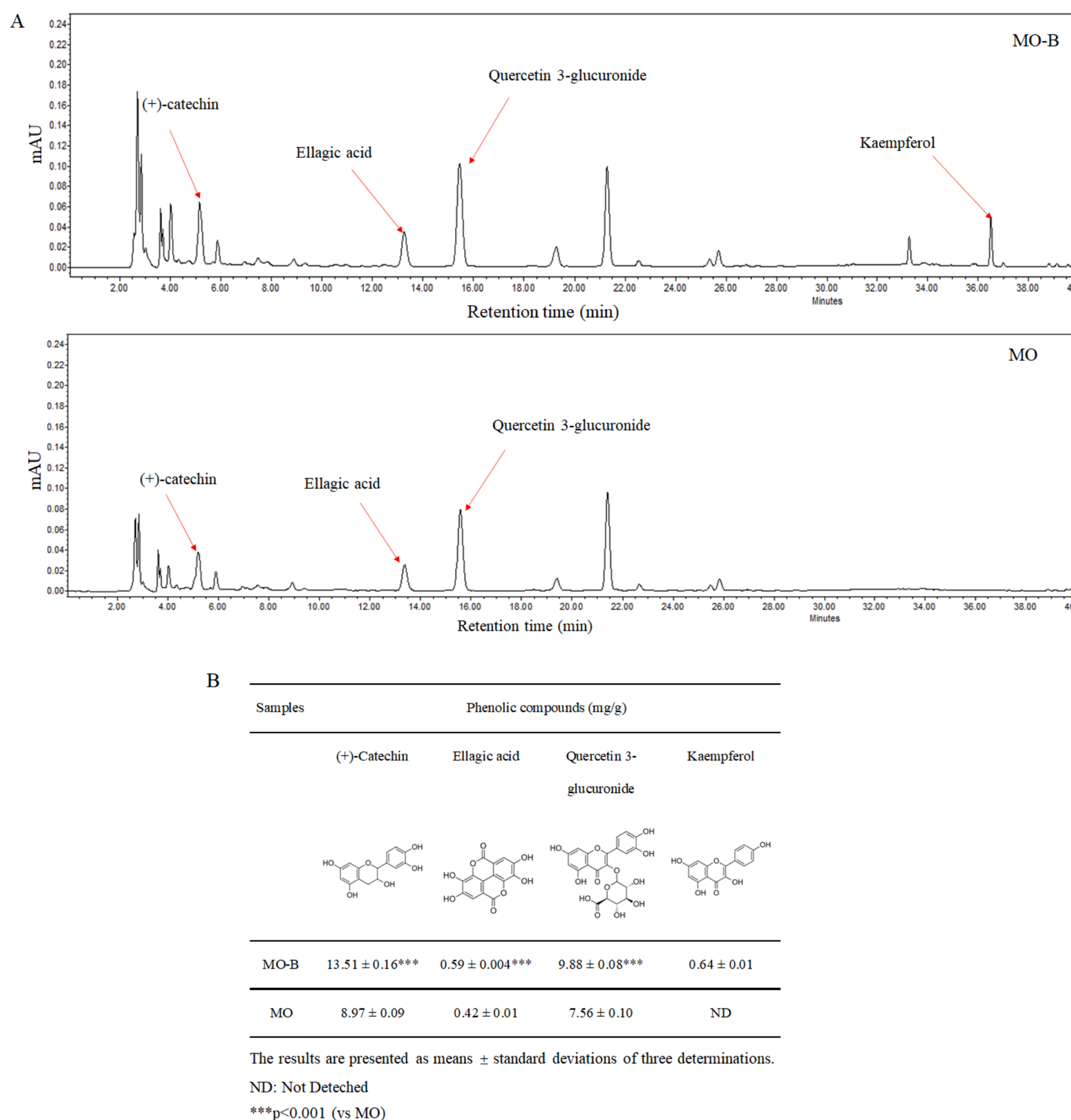
3.3. Effects of MO-B on NO production and iNOS expression in LPS-induced RAW 264.7 cells

Nitrite levels were evaluated using Griess to determine the extent to which NO production was inhibited by MO-B in LPS-activated RAW 264.7 cells. LPS (1 μg/mL) led to a marked increase in excess NO; however, NO decreased significantly (from 34.34 μM to 9.18 μM) in macrophages following treatment with MO-B (50–200 μg/mL) as compared to that in macrophages treated with same concentrations of MO and *B. lactis* (Fig. 2B).

iNOS has previously been found to regulate NO secretion in RAW 264.7 cells after LPS treatment (Xie et al., 2019). We, therefore, assessed the iNOS mRNA expression following treatment with MO-B, MO, and *B. lactis*, with results suggesting that MO-B significantly suppressed iNOS mRNA expression (1.3-, 2.3-, and 2.5-fold) in LPS-stimulated RAW 264.7 cells (Fig. 2C), while MO and *B. lactis* reduced iNOS mRNA expression only slightly (1.0- and 1.1-fold, respectively) in LPS-activated macrophages. These results indicate that MO-B suppresses NO production by inhibiting the production of iNOS mRNA in LPS-activated RAW 264.7 cells.

3.4. Effect of MO-B on ROS production

ROS is considered necessary for mediating LPS-induced inflammation (J. Park et al., 2015). The ROS levels in RAW 264.7 cells pretreated with LPS showed a significant increase (10.5-fold) compared to those in control cells (Fig. 2D). However, the fluorescence intensity of LPS-stimulated RAW 264.7 cells decreased 4.2-, 4.3-, and 1.8-fold following MO-B treatment at the three concentrations 50, 100, and 200 μg/mL, respectively, and the effects obtained using MO-B were more significant than those for MO (9.3-, 7.2-, and 6.3-fold) and *B. lactis* (10.1-fold) at 200 μg/mL under the same conditions.



Samples	Phenolic compounds (mg/g)			
	(+)-Catechin	Ellagic acid	Quercetin 3-glucuronide	Kaempferol
MO-B	13.51 ± 0.16***	0.59 ± 0.004***	9.88 ± 0.08***	0.64 ± 0.01
MO	8.97 ± 0.09	0.42 ± 0.01	7.56 ± 0.10	ND

The results are presented as means ± standard deviations of three determinations.
ND: Not Detected
***p<0.001 (vs MO)

Fig. 1. The HPLC analysis of (A) MO-B and MO, (B) The content of (+)-Catechin, Ellagic, Quercetin 3-glucuronide, and Kaempferol in MO-B and MO.

3.5. Effects of MO-B on pro-inflammatory cytokines

Pro-inflammatory cytokines, such as *IL-6, TNF-α*, and *IL-1β*, play a vital role in the inflammatory process. To investigate the effects of the fermented plant on these cytokines, RNA was isolated from LPS-stimulated RAW 264.7 cells treated with MO-B, MO, and *B. lactis*. As shown in Fig. 3A–C, the mRNA expression of *IL-1β, TNF-α*, and *IL-6* significantly increased after LPS stimulation. However, the mRNA expression of these cytokines in the MO-B group decreased in a dose-dependent manner compared to that observed in the MO and *B. lactis* treatment groups. *IL-1β, TNF-α*, and *IL-6* mRNA levels were dramatically reduced in the MO-B treatment group at a concentration of 200 μg/mL (3-, 5-, and 23-fold, respectively). Furthermore, MO-B strongly suppressed the secretion of *TNFα* and *IL-6* in a dose-dependent manner compared to MO or *B. lactis* (Fig. 3D-E). Interestingly, *IL-6* expression in the MO-B group was highly suppressed according to the qRT-PCR and ELISA results, suggesting that *IL-6* may be the most significant target of MO-B. These results indicate that MO-B may mediate anti-inflammatory effects by inhibiting pro-inflammation cytokines.

3.6. Effect of MO-B on the TLR4/NF-κB signaling pathway

TLR4, a member of the TLR family, is considered an indispensable component of the immune system (Tao et al., 2020). Previous studies have indicated that inflammatory responses occur when antigens activate the TLR4/NF-κB signaling pathway by binding to TLRs (Tao et al., 2020). The mRNA expression of *TLR4* in RAW264.7 cells activated with LPS was markedly increased; however, treatment with MO-B attenuated the *TLR4* mRNA expression level as the concentration was increased from 100 μg/mL to 200 μg/mL (Fig. 4A). To further investigate the anti-inflammatory mechanisms of MO-B in terms of the NF-κB pathway, IKKα was used to regulate IκBα, which was downregulated by MO-B as the dose increased in LPS-stimulated RAW 264.7 cells, leading to active phosphorylation via IκBα and NF-κB. Fig. 4B-C shows the dose-dependent attenuation in the mRNA expression of *IKKα* and *p65* in LPS-activated RAW macrophages compared to the MO or *B. lactis*

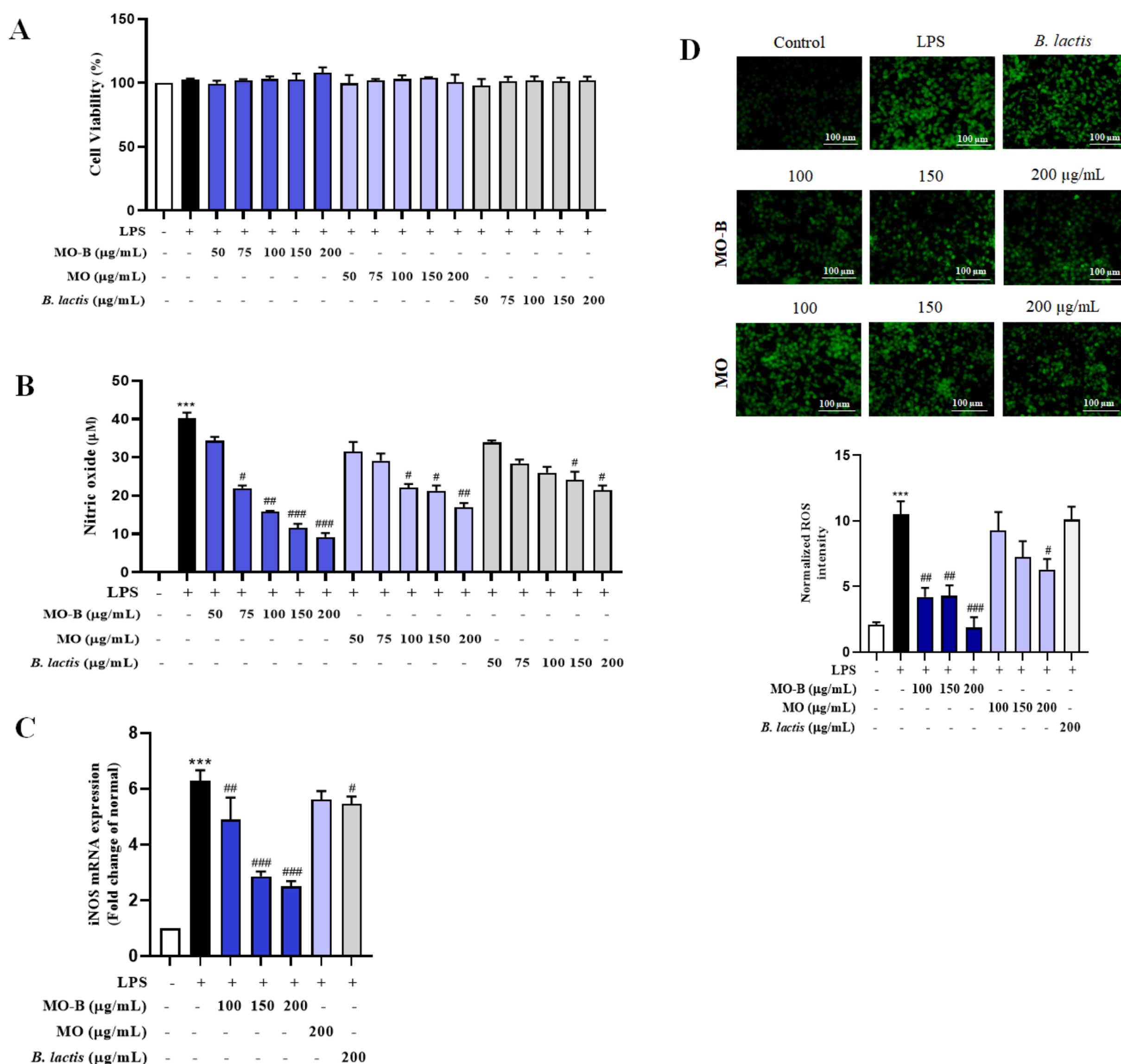


Fig. 2. The inhibition in inflammation of MO-B, MO, *B. lactis* in LPS-activated macrophages. (A) MTT assay, (B) NO production, (C) iNOS mRNA expression, and (D) ROS staining. The data was presented as a mean S.D. $^{***}p < 0.001$ compared *vs.* control. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared *vs.* LPS group.

groups. LPS activated IκBα and p65 phosphorylation (Fig. 4D–E); however, the activation of these pathways was decreased by MO-B in a dose-dependent manner. Specifically, the protein expression of phosphorylated IκBα and p65 in the MO-B (200 μg/mL) group was dramatically reduced (5- and 2-fold, respectively) in the LPS-activated RAW macrophages, suggesting that IκBα inhibition prevented p65 activation. These results indicate that MO-B may mediate anti-inflammatory effects by blocking the NF-κB signaling pathway.

3.7. Effect of MO-B on the MAPK signaling pathway

To investigate whether MO-B could inhibit the MAPK pathway, the expression of several proteins related to the MAPK family, such as ERK, JNK, and p38, was evaluated in LPS-stimulated RAW 264.7 cells treated with MO-B. As shown in Fig. 5, western blotting results confirmed a significant decrease in the levels of p-JNK, p-p38, and p-ERK in the LPS-stimulated RAW 264.7 cells following treatment with MO-B (100, 150, and 200 μg/mL), whereas negligible inhibition of the MAPK signaling pathway was observed in the MO and *B. lactis* treatment groups. Thus, MO-B significantly suppressed JNK, p38, and ERK phosphorylation in LPS-activated RAW 264.7 cells, and this phenomenon may subsequently suppress NF-κB activation.

3.8. Effect of MO-B on the PI3K/AKT signaling pathway

PI3K/AKT plays a critical role in inflammation by activatingv the NF-κB pathway. As shown in Fig. 6 A–D, western blotting and qRT-PCR analyses showed that treatment with MO-B at different concentrations suppressed the expression of AKT and PI3K in LPS-stimulated RAW 264.7 cells compared to those under MO or *B. lactis* treatment. In particular, p-PI3K protein expression was significantly decreased in the MO-B treatment group but not in the MO or *B. lactis* treatment groups. These results indicate that MO-B successfully inhibited the PI3K/AKT signaling pathway. We, therefore, assume that MO-B significantly suppresses the phosphorylation of PI3K and AKT in LPS-stimulated RAW 264.7 cells.

4. Discussion

Fermentation has been used as a method of preserving food for millennia. The microorganisms involved in fermentation produce substances that are useful to the body (Lee et al., 2020), and several studies

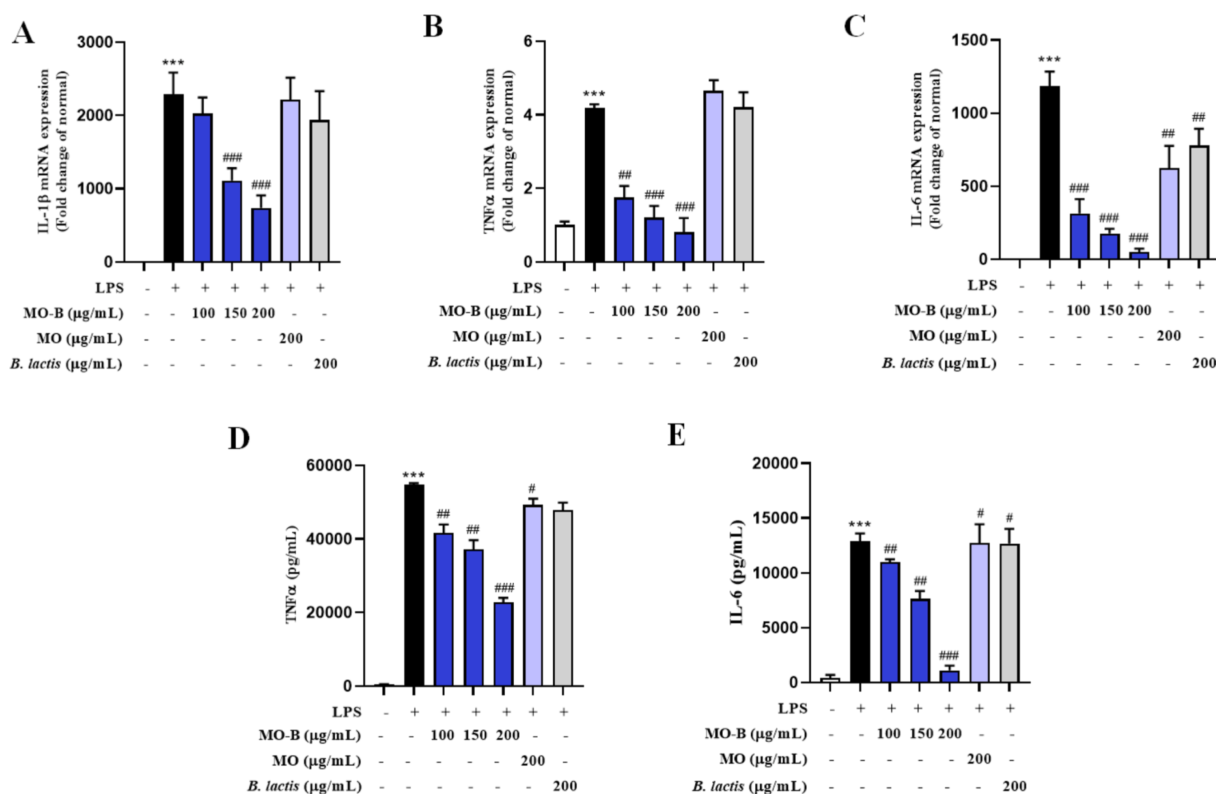


Fig. 3. The inhibition of MO-B, MO, *B. lactis* on pro-inflammation in macrophage treated with LPS. (A) *IL-1β*, (B) *TNFα*, (C) *IL-6* mRNA expression by qRT-PCR, (D) TNFα and (E) IL-6 secretion by ELIZA kit. The data was presented as a mean S.D. $^{***}p < 0.001$ compared *vs.* control. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared *vs.* LPS group.

have suggested that fermented foods may improve the absorption rate and enrich phytochemicals and bioactive compounds in health-promoting foods (Lee et al., 2020; Shahbazi et al., 2021). Lee et al. indicated that fermented *Zanthoxylum schinifolium* has enriched polyphenol content, resulting in improved antioxidant and anti-inflammatory effects (Lee et al., 2020). Hur et al. reported that fermented MO utilizes *Rhizopus oligosporus* to alleviate atopic dermatitis, an inflammation-associated skin disease (Hur et al., 2018); however, the mechanism by which this occurs was not studied in depth. In this study, we fermented MO with *B. lactis* to investigate its anti-inflammatory activity and the underlying mechanisms of LPS-stimulated RAW 264.7 macrophages.

Fermentation can convert phenolic substances into plant components, enhancing their bioactivity (Le, Thi, Anh, & Yang, 2020). During fermentation process, probiotics produce a large number of enzymes that catalyze the hydrolysis of glycosidic bonds in some phenolic compounds. It is believed that enzymatic hydrolysis could enhance the amount of free polyphenols present (Lee et al., 2015). Haile et al. reported that the levels of polyphenols and flavonoids in green coffee beans significantly increased after fermentation with yeast (Haile & Kang, 2019). Our results showed a notable increase in the total polyphenol and total flavonoid contents of MO-B as compared to MO. These results clearly suggested that fermentation (with *B. lactis*) made the MO rich in polyphenols and flavonoids contents *via* different enzymic reaction that could stimulate the synthesis of polyphenols or flavonoids (Chiu et al., 2019). The detailed compositions in MO and MO-B were further analyze using HPLC. The HPLC analysis results of moringa extract and *Moringa* extract fermented with *B. lactis* were also compared, with results indicating that similar to the changes in total polyphenol and flavonoid content, the content of several phenolic compounds, such as (+)-catechin, ellagic acid, and quercetin-3-glucuronide were significantly increased after fermentation. Moreover, we observed that the fermented MO contains kaempferol, which could not be detected in the MO extract. These results imply that *B. lactis* enhances the biological properties of MO extract by producing phytochemicals. Several studies suggest that (+)-catechin, ellagic acid, kaempferol, and quercetin-3-glucuronide can suppress inflammation (Boesch-Saadatmandi et al., 2011; Cheng et al., 2019; Devi et al., 2015; Kassim et al., 2010). In particular, catechin exhibited significant anti-inflammation effect in LPS-activated RAW 264.7 by regulating ferroptosis pathway (Kuang et al., 2022). Furthermore ellagic acid, kaempferol, and quercetin-3-glucuronide remarkably reduced inflammation in LPS-activated RAW 264.7 cells through inhibiting NO production and pro-inflammation cytokines expression (BenSaad et al., 2017; Lin et al., 2003; Nishikawa et al., 2022). Accordingly, the increase of these phenolic compounds may improve the anti-inflammatory properties of fermented *Moringa*. Therefore, MO fermented with *B. lactis* might have anti-inflammatory properties and could be used as a food additive. We speculate that MO-B exerts stronger activity than MO or *B. lactis* alone.

Macrophages, the main modulators of inflammation, are associated with the autoimmune and autoinflammatory processes that lead to chronic inflammation-related diseases (Saqib et al., 2018). Macrophages release pro-inflammatory cytokines and inflammatory mediators, such as *IL-1β, TNF-α, IL-6*, NO, *iNOS*, and ROS, inducing cell damage (H. H. Lee et al., 2020). In our study, MO-B remarkably inhibited the production of *IL-1β, TNF-α, IL-6*, NO, and *iNOS* in LPS-stimulated RAW 264.7 cells compared to MO or *B. lactis*. One previous study suggested that fermentation can elevate anti-inflammatory activity by converting phenolic compounds (Le, Thi, Anh, & Yang, 2020). Following the fermentation of MO with *B. lactis,* the contents of (+)-catechin, ellagic acid, and quercetin-3-glucuronide were markedly higher than those obtained from MO alone. These phenolic compounds show anti-inflammatory ability by modulating the secretion of pro-inflammatory cytokines (*IL-1β, TNF-α*, and *IL-6*) and inflammatory mediators (NO and *iNOS*) (Boesch-Saadatmandi et al., 2011; Du et al., 2018; Yamaguchi & Levy, 2019). Thus, the elevation of these chemical components may

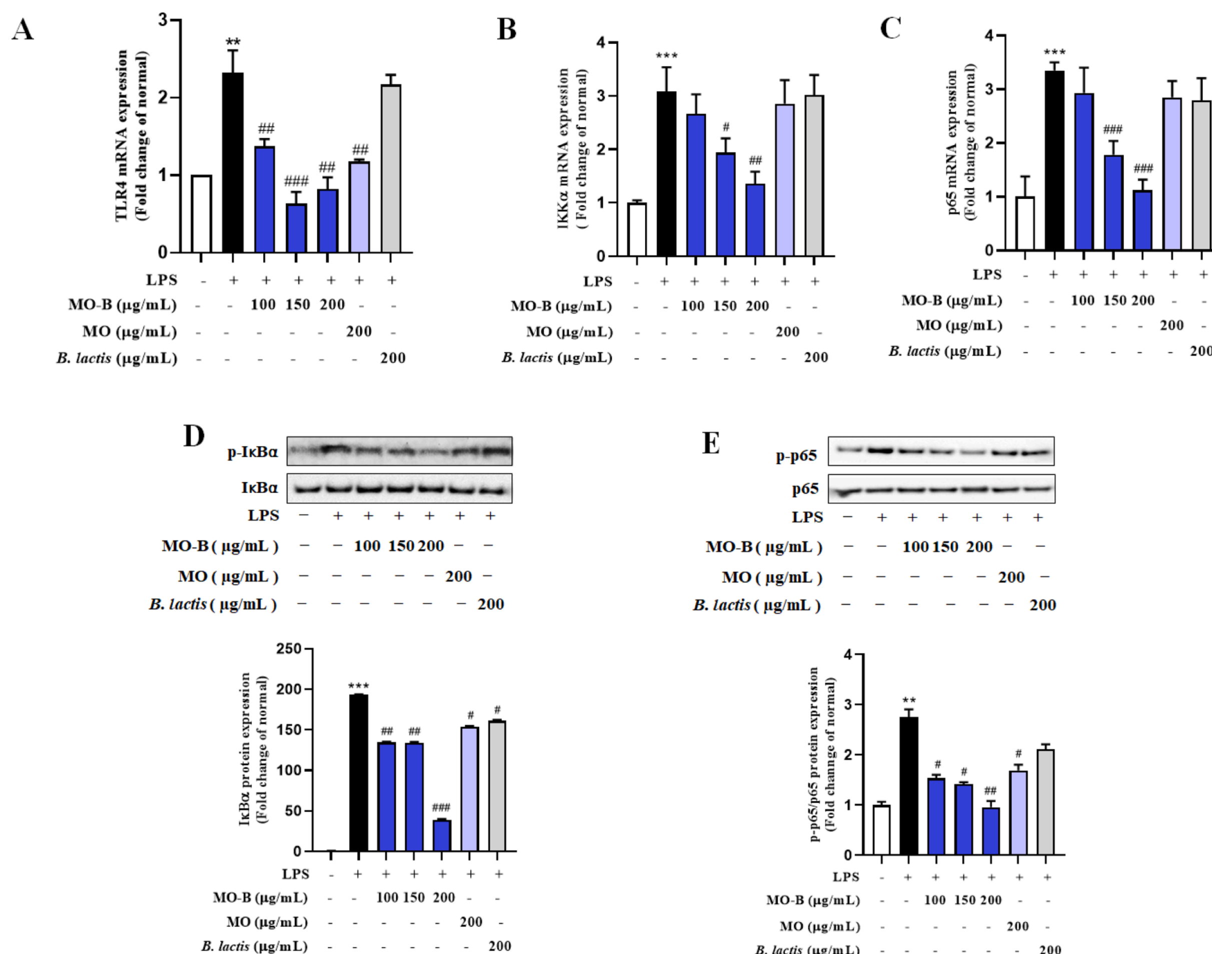


Fig. 4. Effect of MO-B, MO, *B. lactis* on TLR4/NF-κB signaling pathway, including IKKα, IκBα, NF-κB. (A) *TLR4*, (B) *IKKα*, and (C) *p65* mRNA expression, (D) IκBα and (E) p65 protein expression. The data was presented as a mean S.D. $^{***}p < 0.001$ compared *vs.* control. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared *vs.* LPS group.

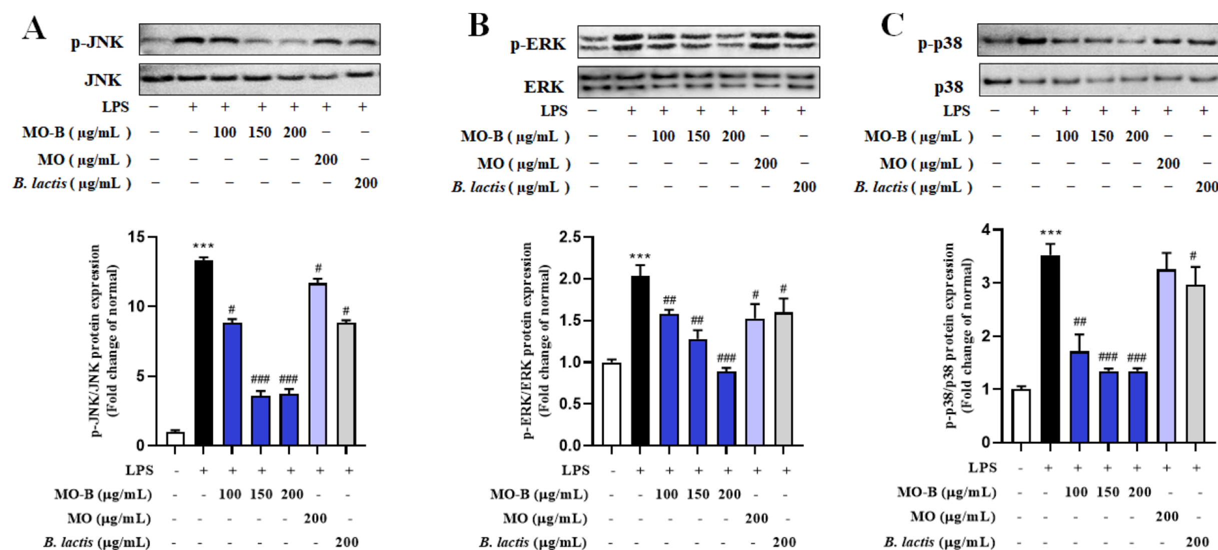


Fig. 5. Effect of MO-B, MO, *B. lactis* on *MAPK* signaling pathway. A) JNK, (B) ERK, (C) p38 protein expression. The data was presented as a mean S.D. $^{***}p < 0.001$ compared *vs.* control. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared *vs.* LPS group.

contribute to the improved anti-inflammatory activity of MO-B.

Inflammation is related to excess ROS in cells, which alter the pro-inflammatory cytokines by activating the transcription of inflammation-related genes (J. Park et al., 2015). Specifically, ROS are secreted mainly by the mitochondria, peroxisomes, and endogenously activated inflammatory cells (Mohsenzadegan & Mirshafiey, 2012). Our results indicate that LPS induces intracellular ROS accumulation in RAW264.7 cells and that elevated ROS production can be significantly

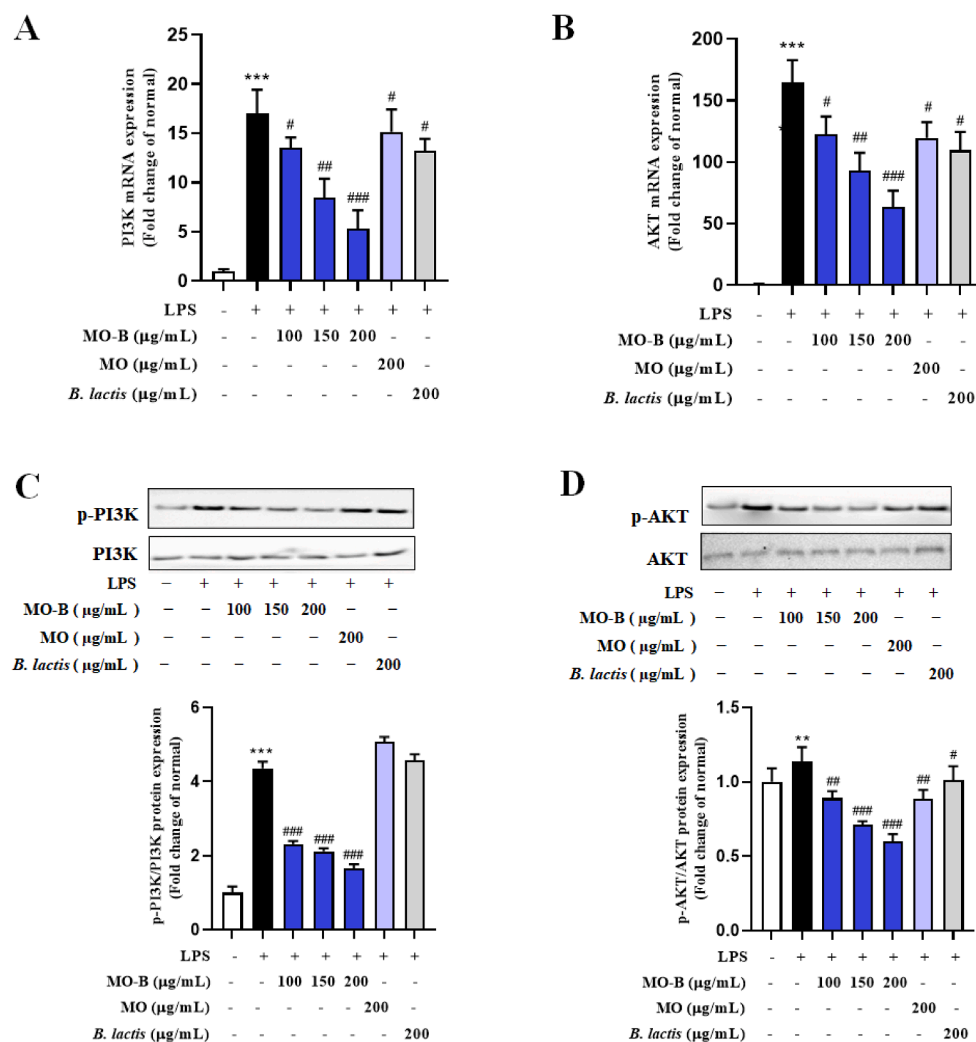


Fig. 6. Effect of MO-B, MO, *B. lactis* on PI3K/AKT in LPS-activated RAW 264.7 cells. (A) *PI3K*, (B) *AKT* mRNA expression, (C) PI3K and (D) AKT protein expression. The data was presented as a mean S.D. $^{***}p < 0.001$ *vs.* control, $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$, $^{\#\#\#}p < 0.001$ compared *vs.* LPS group.

reduced by treatment with *B. lactis*-fermented MO. Huang et al. confirmed that kaempferol is a potent flavonoid that reduces LPS-induced ROS in RAW264.7 macrophages (Huang et al., 2014). In this study, kaempferol appeared in MO fermented with *B. lactis*, whereas it was not observed in the MO extract. The inhibitory effect of MO-B on ROS production may thus be partially attributed to the presence of kaempferol.

NF-κB is the most important transcription factor that regulates the expression of inflammatory factors (C. Park et al., 2021). The binding of antigens onto TLRs activates the TLR4/NF-κB signaling pathway, leading to inflammation (Zusso et al., 2019). Park *et al.* determined that morroniside inhibited LPS-induced inflammation in LPS-activated RAW 264.7 cells by blocking the TLR4/NF-κB signaling pathway (C. Park et al., 2021). Therefore, the expression levels of TLR4, NF-κB, and IκBα were investigated to evaluate the mechanism by which the LPS-induced inflammatory response is prevented by MO-B. MO-B reduced the mRNA expression of *NF-κB* and *IKKα* dose-dependently. The protein activation of NF-κB and IκBα was also prevented by MO-B treatment in LPS-stimulated RAW 264.7 cells. These results suggest that MO-B can reduce LPS-mediated macrophages by inhibiting TLR4-induced NF-κB signaling via the binding of TLR4 and LPS.

Furthermore, the MAPK signaling pathway, activated in the LPS-activated macrophages, modulates the expression of inflammation mediators via NF-κB activity (Kaminska, 2005). A previous study reported that fermented *Asparagus cochinchinensis* could reduce activation of the MAPK signaling pathway in LPS-mediated RAW 264.7 cells (H. A. Lee et al., 2017). Our investigation revealed that MO-B significantly suppressed JNK, p38, and ERK phosphorylation in LPS-activated RAW 264.7 cells in a dose-dependent manner. Previous report has demonstrated that the PI3K/AKT signaling pathway is closely related to *NF-κB* activation and contributes to the production of inflammatory mediators (Nguyen et al., 2020). The activity of NF-κB is regulated *via* AKT phosphorylation, inducing the production of pro-inflammatory mediators. In this study, MO-B significantly suppressed the phosphorylation of PI3K and AKT in LPS-stimulated RAW 264.7 cells. Our results indicate that MO-B may inhibit LPS-induced NF-κB activation by reducing MAPKs and PI3K/AKT phosphorylation.

5. Conclusion

In summary, *B. lactis*-fermented MO-B inhibits the production of NO and pro-inflammatory cytokines, resulting in anti-inflammatory effects in the LPS-activated macrophages. MO-B showed greater anti-inflammatory activity than MO and *B. lactis*, which was likely attributed to high levels of ellagic acid, (+)-catechins, and quercetin-3-glucuronide. Kaempferol, a potent flavonoid, was found only in MO-B. Moreover, MO-B strongly inhibited the inflammatory response by blocking the TLR4/NF-κB signaling pathway and preventing activation of the PI3K/AKT and MAPK signaling pathways in LPS-stimulated RAW 264.7 cells, thus inhibiting inflammation. Overall, our results

demonstrate that *B. lactis*-fermented MO-B possesses anti-inflammatory properties and that it should be further investigated.

6. Ethics statement

There were no human subjects or animal experiments involved in this research.

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CRediT authorship contribution statement

Thi Hoa My Tran: . **Sanghyun Lee:** Investigation. **Jeong-Eun Huh:** Writing – review & editing. **Haribalan Perumalsamy:** Resources. **Sri Renukadevi Balusamy:** . **Yeon-Ju Kim:** Conceptualization, Methodology.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2023.105752.

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