Brazilian Journal of Poultry Science
Revista Brasileira de Ciência Avícola

ISSN 1516-635X Oct - Dec 2019 / v.21 / n.4 / 001-010

http://dx.doi.org/10.1590/1806-9061-2018-0976

Original Article

Immunomodulation Properties of Solid-State Fermented Laetiporussulphureus Ethanol Extracts in Chicken Peripheral Blood Monocytes In Vitro

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■Keywords

Laetiporussulphureus, in vitro, immunomodulatory, solid-state fermentation.

Submitted: 21/December/2018
Approved: 27/June/2019

ABSTRACT

Laetiporus sp. is recognized as a fungal species traditionally used for medicinal purposes. This study investigated the *in-vitro* effects of solid-state fermented *Laetiporussulphureus* ethanol extracts (LSE) for their immunomodulatory potential. Bioactive levels detected in the LSE on different days throughout the fermentation period revealed that the 12th day was the most efficient, with 7.19 ± 0.66 GAE/g DM crude phenolic content, 2.71 ± 0.03 UAE/g DM crude triterpenoid content, 12.93 ± 0.88 GCE/g DM crude polysaccharides, and 96.44 ± 0.2 mg/g DM ergosterol content. *In-vitro*LSE tests on chPBMC showed no cytotoxicity within a range of 0.05-1 mg/mL, but LPS-inhibited cell viability was improved, as well as LPS-induced nitric oxide (NO) production and mRNA levels of nuclear factor kappa B (NFκB), Toll-like receptor 4 (TLR4), inducible nitric oxide synthase (iNOS), and interleukin (IL)-1βwere attenuated Furthermore, the direct application of LSE on chPBMC showed a small but not significant increase in NFκB, TLR4, and iNOS mRNA expression compared with the control group. These results indicate the potential of LSE to modulate LPS-triggered inflammation processes involving TLR4 and NFκB mediation. However, further experiments are required to determine the specific pathway.

INTRODUCTION

Laetiporus sp. is a medicinal fungus traditionally used by Europeans to treat pyretic diseases, coughs, gastric cancer, and rheumatism (Ríos *et al*., 2012). Recent studies show that the fruit bodies of *Laetiporus* sp. contain various pharmaceutical compounds, including polysaccharides (Alquini *et al*., 2004), triterpernoids (He *et al*., 2015) and euburicoic acids (Wang *et al*., 2017). Submerged mycelial cultured *Laetiporussulphureus* (LS) has been reported to produce functional polysaccharides (Jayasooriya *et al*., 2011; Lung *et al*., 2011) as well as mycophenolic acids (Fan *et al*., 2014). This research excludes the possible limitation of only the fruiting body form of LS being used. It also indicates the potential use of fast-producing mycelial culture in the health promotion field.

The rapid-growth of global population has increased the demand of grains, which considerably raised feedstuff costs, and, therefore, it is very important to develop alternative feeds. The processing of agricultural products yields many by-products, such as brans or grain husks. The low nutrition value of these by-products has always been an issue when it comes to feeding monogastric animals due to their high lignocellulosic content (Yu *et al*., 2008). Over 6.5 million tons of wheat are produced globally per year, resulting in a large amount of wheat bran as a by-product of flour production. However, it is very

challenging to include wheat bran in feed formulation due its low energy (approximately 1,300 kcal/kg of metabolizable energy) and high dietary fiber contents (44.0%) (Prückler *et al*., 2014).

Solid-state fermentation (SSF) technique has been proven to have higher yields and productivities than submerged fermentation (SmF). Furthermore, SSF process utilize low-cost agricultural and agro-industrial waste as substrates, which make it more efficient and cost-effective than SmF when producing bioactive compounds (Hölker *et al*., 2004). Filamentous fungi, which are capable of enduring low-moisture fermentation environments, are reported to be the most suitable for this purpose (Vattem *et al*., 2003; Hernández *et al*., 2008). Solid-state fermented wheat bran by *Pleurotuseryngii* had been shown to exhibit antioxidant properties and improved nutrition value, making it a potential candidate as a low-cost feedstuff (Wang *et al*., 2016). Wen *et al*. (2016) reported that polysaccharide production by *Ganoderma atrum* was enhanced using SSF versus SmF. Ren *et al*. (2014) also found that the optimal conditions for *Paecilomyces cicadae* to produce polysaccharides using SSF. Besides polysaccharides, triterpenoid production was also viable, according to Yang *et al*. (2012), who reported grapefruit peel to be the most suitable SSF substrate for *Antrodiacinnamomea.* These researches suggest the potential of SSF for the utilization of agro-industrial by-products to produce bioactive compounds and functional feedstuffs.

Toll-like receptors (TLRs) are capable of recognizing various kinds of pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharides (LPS) and lipoteichoic acid (LTA). TLR4 is capable of recognizing LPS and triggering the downstream activation of nuclear factor kappa B (NFκB). Under normal circumstances, inactive NFκB is sequestered in the cytoplasm by binding to the kappa B inhibitor (IκB), which is regulated by IκB kinase (IκK). Upon activation by pathogens, oxidative stress, inflammatory stimuli, cytokines or the presence of carcinogens, IκB is degraded, releasing NFκB which is translocated to the nucleus, causing the expression of various pro-inflammatory substances, such as interlukin-6 (IL-6), IL-1β, and inducible nitric oxide synthase (iNOS). Studies show that bioactive compounds produced by fungi can also be recognized by TLRs (Akira, 2003; Hsu *et al*., 2004; Medvedev *et al*., 2007), especially TLR4, which can be activated by fungal polysaccharides. NFκB is the key regulator of the inflammatory response, in addition to the innate and adaptive immune systems. As such, the regulation of TLR4 and NFκB is the key for the study of the immunomodulatory effects of medicinal fungi.

In this study, the optimal length of fermentation of LS using wheat bran (WB) as substrate was first determined byexamining the functional compounds. Following the decision on the optimal number of days of fermentation, the ethanolic extracts of the desired product were tested both in blank chicken peripheral blood monocytes (chPBMC) and LPS-stimulated chPBMC. mRNA expression of TLR4 and NFκB was also investigated along with IL-1β and iNOS for better understanding of their immunomodulatory effects.

MATERIALS AND METHODS

Microorganisms

The *Laetiporussulphureus* BCRC 35305 used for this study was purchased from the Bioresource Collection and Research Center (BCRC), Food industry Research and Development Institute (Hsinchu, Taiwan). The microorganism was routinely maintained on a malt extract agar (MEA, glucose 2%, malt extract 2%, peptone 1% and agar 2%) plate at 25°C with regular sub-cultivation (not longer than 1 week).

Inoculum preparation

The inoculum for solid-state fermentation was prepared by shake flask culture with malt extract broth (MEB). Briefly, 250mL Erlenmeyer flasks filled with 100 mL of MEB were covered with tin foil and autoclaved at 121 ± 1°C for 30 min. LS was transferred to the medium by punching out round-shaped agar pieces (about 1cm in diameter) from MEA plates; 5 pieces of agar were used for the inoculation of 100 mL of liquid media. Flasks with agar pieces in MEB were incubated at 25°C in a rotary shaker incubator at 120 rpm for 5 d.

Solid-state fermentation (SSF)

Solid-state fermentation of LS was performed in a heat-resistant plastic bag containing50g of wheat bran; the moisture content was adjusted to 50% with distilled water. The contents of each bag were thoroughly mixed before autoclaving at 121 ± 1°C for 30 min. The autoclaved wheat bran was inoculated with 0.2 mL of homogenized LS inoculum per gram of wheat bran. The wheat bran was aerobically fermented under environmentally-controlled conditions and maintained at 25°C for 16 d. Samples were collected on days 4, 8, 12, and 16 and dried at 40°C for 2 d

before ground in a mill and stored at -20°C prior to extraction.

Preparation of fermented LS ethanolic extracts (LSE)

To perform the extraction of fermented LS, 5.0 g of samples were weighed into tubes and extracted with 70% ethanol by ultrasonication (DC 300, DELTA) at 40°C for 2 h. After centrifuging at 5,000 rpm for 5 min, the supernatant was collected and stored at -20°C for subsequent analysis. For the *in-vitro* chPBMC test, LSE was vacuum-dried and resuspended in 0.1% DMSO.

Crude phenolic acid content

The crude phenolic acid content was determined as per the methods described by Kujala et al. (Kujala et al., 2000), with minor modifications. Briefly, an aliquot of 50 µL LSE was mixed with 0.5 mL Folin-Ciocalteu phenol reagent (Sigma) and 1 mL of 7.5% sodium carbonate, and allowed to react for 30 min at room temperature (RT).Subsequently, an equation obtained from the standard gallic acid (GA) graph was used to determine LSE phenolic compounds (milligram of GA equivalent, mg GAE), via comparison with a GA standard.

Crude triterpenoid content

The crude triterpenoid content of LSE was determined according to the methods of Lu *et al*. (Lu *et al*., 2011), with minor modifications. Briefly, after heating a 200-µL sample solution in a test tube to evaporation in water bath, 1 mL of newly mixed 5% (W/V) vanillin-acetic solution and 1.8 mL sulfuric acid were added to the mix before incubation at 70°C for 30 min. The solution was then cooled and diluted to 10 mL with acetic acid. The absorbance was measured colorimetrically at 573 nm against a blank. The blank consisted of all reagents and solvents without a sample solution. The content was determined using the standard ursolic acid (Sigma) calibration curve.

Crude polysaccharides

The total amount of polysaccharides was determined by phenol-sulfuric acid assay, as per Dubois *et al*. (Dubois *et al*., 1956). Briefly, 1 mL samples were pipetted into dilution tubes, and 1 mL 5% (v/v) phenol and 5 mL of 18M sulfuric acid were added, and the mixture was left to stand for 10 min at RT. The tubes were then thoroughly vortexed and left for 30 min at RT, before being immersed in ice-cold water to stop the reaction. The absorbance was measured at 490 nm using a spectrophotometer (Ultrospec 2100 Pro, Mersham, Hong Kong). The content was determined using the standard glucose calibration curve and expressed as mg GCE (milligram of the glucose equivalent)/g DM.

Ergosterol content

Ergosterol content was determined using high-performance liquid chromatography (HPLC). LSE was filtered through a 0.22-µm membrane filter and subsequently analyzed using an HPLC instrument (HITACHI, Kyoto, Japan) equipped with a pump (L-2130), UV detector (L-2490), column (Transgenomic CARBOS ep CH0682 Pb, 300 mm × 7.8 mm), and computer system with HPLC D-2000 Elite. The sample injection volume was 20 µL. Chromatographic peaks in the samples were identified by comparing their retention times and UV spectra with the reference standard (Ergosterol, Sigma). Working standard solutions (20 µL) were injected into the HPLC instrument to obtain peak-area responses. A standard curve and calibration formula for ergosterol was prepared by plotting concentration versus area. Quantification was conducted according to the integrated peak areas of the sample and corresponding standard curves.

Chicken peripheral blood mononuclear cell isolation

Whole blood (5 mL) was collected from 35-d-old broiler chickensby wing vein puncture into a tube containing 1% EDTA. The blood was layered on 1077 Histopaque (Sigma, 10771) and centrifuged at 200 × g for 10 min. Peripheral blood mononuclear cells (PBMCs) were collected from the gradient interface; the plasma suspension was combined and washed three times with phosphate buffered saline and then centrifuged at 200 × g for 10 min. After the suspension was removed, chPBMCs were resuspended in RPMI-1640 with 10% FBS and adjusted to 10^8 cells/mL. Two mL were then pipetted into 6 well plates and cultured in an incubator for 2h at 37°C in 5% CO_2 mixed with 95% air. After incubation, the cells were treated with LSE (0.05, 0.1, 0.5, 1 mg/mL) in the presence and absence of LPS (100 ng/mL) for 24 h; PBS was used as a control.

chPBMCs viability assay and nitric oxide (NO) assay

The chPBMCs harvested from whole blood density gradient centrifugation (Ficoll-Hypaque) were seeded in 96-well plates, which were incubated with PBS or

LPS in the absence or presence of LSE at the indicated concentration in an air incubator at 37°C. For the cell viability assay, cells were incubated for 48h before adding 20µL of MTT (3- (4, 5-dimethylthiazol-2-yl) -2, 5-diphenyl tetrazolium bromide) solution and incubated at 37°C for 4 h. The medium was then removed and DMSO added to dissolve the formazan crystals. The absorbance of the solution was measured by a microplate reader at 517 nm. NO production by chPBMC was measured according to the Griess reaction kit (Molecular Probes, Inc., USA), as per the manufacturer's protocol.

RNA Isolation and Quantitative Reverse Transcription–Polymerase Chain Reaction

Total RNA was isolated from cultured chPBMCs using the Trizol reagent (Invitrogen, USA) according to the manufacturer's protocol for the determination of mRNA expression. For determining total RNA concentration and purity, cDNA synthesis and qPCR analysis were performed and modified as per the methods of Lin *et al*. (2014). The designs of gene-specific primers were implemented according to *Gallus gallus* (chicken) genes; Table 1 lists the features of the primer pairs. After the normalization of the gene-expression data using the calculated GeNorm normalization factor, the means and standard deviations (SDs) were calculated for the samples of the same treatment groups.

Table 1 – Characteristics and performance data of the primers used for q-PCR analysis.

Gene	Forward primer (from 5′ to 3′) Reverse primer (from 5′ to 3′)	PCR product size (bp)	NCBI GenBank
β-actin	CTGGCACCTAGCACAATGAA ACATCTGCTGGAAGGTGGAC	109	X00182.1
NFκB[1]	GAAGGAATCGTACCGGGAACA CTCAGAGGGCCTTGTGACAGTAA	131	NM_205134
IL-1β[2]	GCTCTACATGTCGTGTGTGATGAG TGTCGATGTCCCGCATGA	80	NM_204524
iNOS[3]	TACTGCGTGTCCTTTCAACG CCCATTCTTCTTCCAACCTC	108	U46504
TLR4[4]	TGCACAGGACAGAACATCTCTGGA AGCTCCTGCAGGGTATTCAAGTGT	347	NM_001030693

[1]NFκB: Nuclear factor of kappa light polypeptide gene enhancer in B-cells p50.

[2] IL-1β: Interleukin 1, beta.

[3]iNOS: Inducible nitric oxide synthase

[4]TLR4: Toll like receptor 4

Statistical analysis

Data were subjected to analysis of variance (ANOVA) as a completely randomized design using the GLM function of the SAS software (SAS, 2004). Significant statistical differences among the various treatment group means were determined using Tukey's honestly significant difference test. The effects of the experimental diet on response variables were considered to be significant at $p<0.05$.

RESULTS

Changes of functional metabolites of LS during SSF

Fig. 1 demonstrates the increase in selected bioactive compounds in LS during SSF. The greatest increment was consistently seen between d8-d12 compared with d12-d16, where the level seemed to reach a plateau. Therefore, d12 was chosen as the optimal time for LS fermentation. Crude phenolic content (1A), crude triterpenoid (1B), crude polysaccharide (1C) and ergosterol (1D) contents of LS on d 12 of fermentation were 7.19 ± 0.66 GAE/g DM, 2.71 ± 0.03 UAE/g DM, 12.93 ± 0.88 GCE g DM, and 96.44 ± 0.2 mg/g DM, respectively.

Effects of LSE on release of NO by chPBMC

Fig. 2A shows that LPS treatment significantly increased NO concentration in the chPBMC culture medium compared with the control group, while LSE had no significant effect. Further investigation of LSE on NO release by LPS-induced chPBMC is summarized in Fig. 2B. The results indicate that LSE at all concentrations (0.05–1 mg/mL) significantly inhibited the release of NO due to LPS stimulation.

Effects of LSE on chPBMC cell viability

Fig. 3 illustrates the effects of LSE on chPBMC viability. LSE stimulation at all concentrations (0.05-1 mg/mL) had no effect on the cell viability of chPBMC; these findings are similar to the control group (Fig. 2A). However, the depressed cell viability of LPS-induced chPBMC was significantly improved by LSE stimulation (Fig. 2B).

Effect of LSE on mRNA levels of NFκB, TLR4, iNOS and IL-1β on chPBMC

Fig. 4 presents the effects of LSE on the mRNA levels of selected immune-related genes of chPBMC. The graph shows that LPS stimulation significantly induced the mRNA expression levels of NFκB (4A), TLR4 (4B), iNOS (4C) and IL-1β (4D) comparedwiththe control and other treatment groups. Despite the lack of statistical significance, LSE stimulation slightlyincreased mRNA levels of NFκB, TLR4 and iNOS. The only significanteffect of LSE was on the mRNA level of IL-1β, in that 1 mg/mL

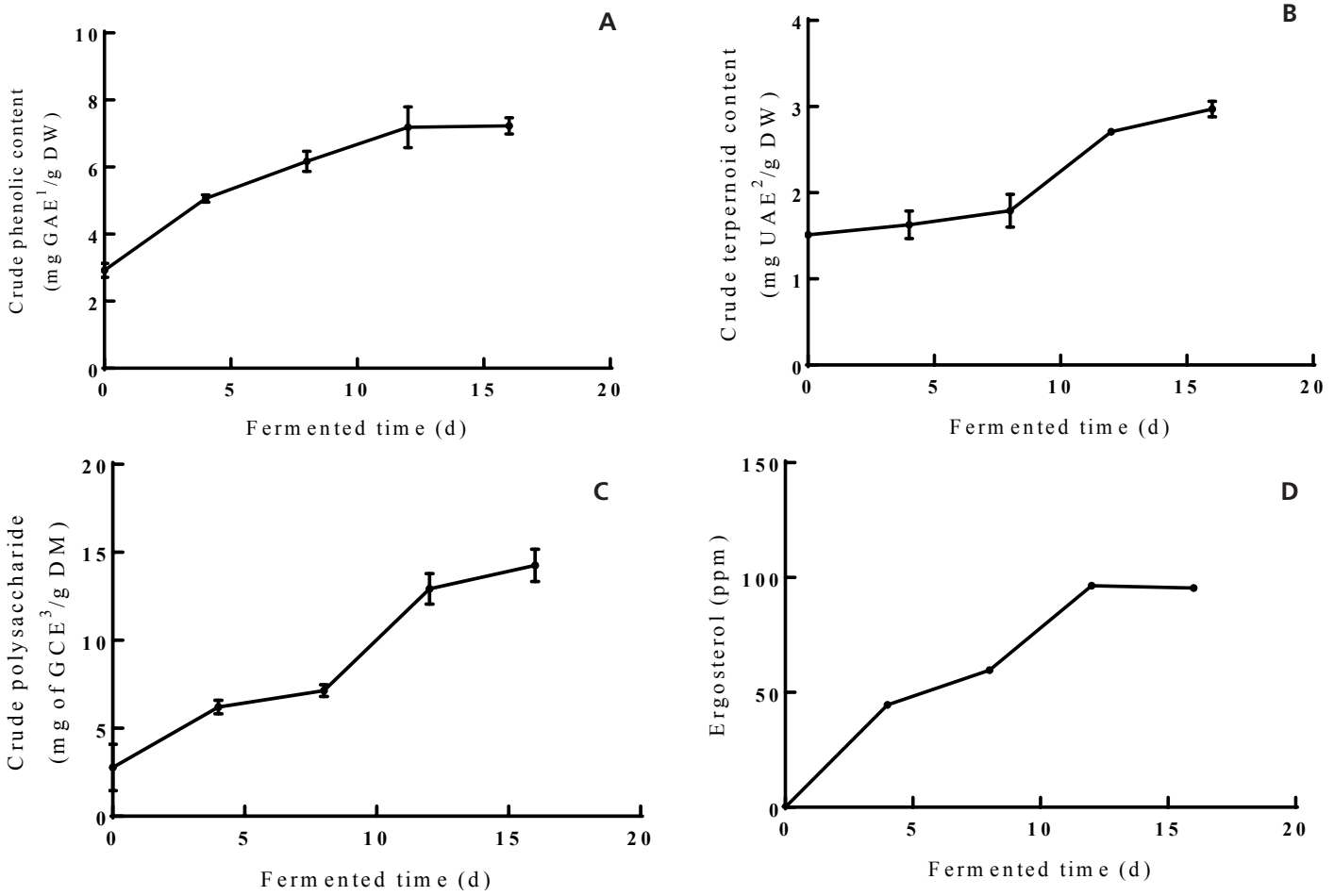

Figure 1 – Changes of crude phenolic (A), crude terpenoid (B), crude polysaccharide (C), and ergosterol (D) content of WB ethanolic extracts during the 16-day fermentation by *L. Sulphureus*. [1]GAE: Gallic acid equivalent, [2]UAE: Ursolic acid equivalent, [3]DW: Dry weight, [4]GCE: Glucose equivalent. Values are expressed as mean ± standard deviation (n=4).

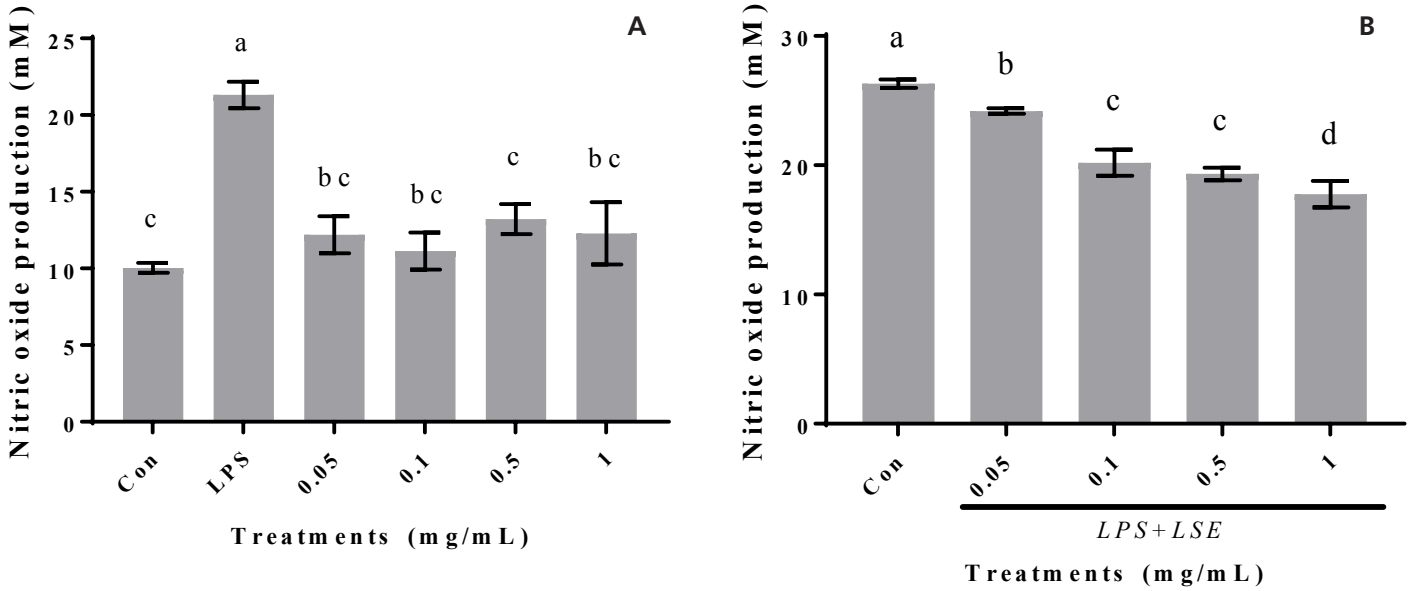

Figure 2 – Effects of*Laetiporussulphureus*fermented WB ethanolic extracts on NO production bychPBMC. (A) NO production bychPBMC was tested after incubation with PBS (Con)/LPS/ or LSE (0.05-1 mg/mL) for 24 h. (B). NO production bychPBMC was tested after pre-stimulation with LSE (0.05-1 mg/mL) following co-incubation with LPS for 24h. Values are expressed as mean ± standard deviation (n=4). a-d Means within the same rows without the same superscript letter are significantly different ($p<0.05$).

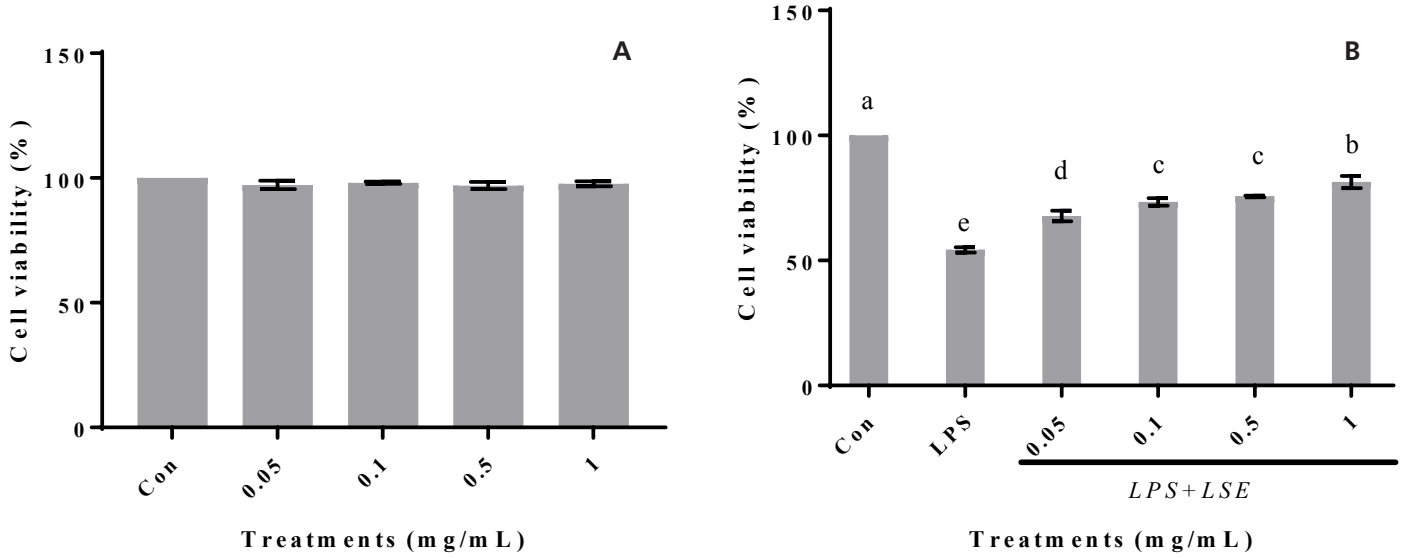

Figure 3 – Effects of LSE on the cell viability of chPBMC. (A) Cytotoxicity of FWBE was tested after incubation with PBS (Con)/ LPS/ or LSE (0.05-1 mg/mL) for 24 h. (B). Cytotoxicity of LSE was tested after pre-stimulation with LSE (0.05-1.0 mg/mL) following co-incubation with LPS for 24h. Values are expressed as mean ± standard deviation (n=4). a-e Means within the same rows without the same superscript letter are significantly different (p<0.05).

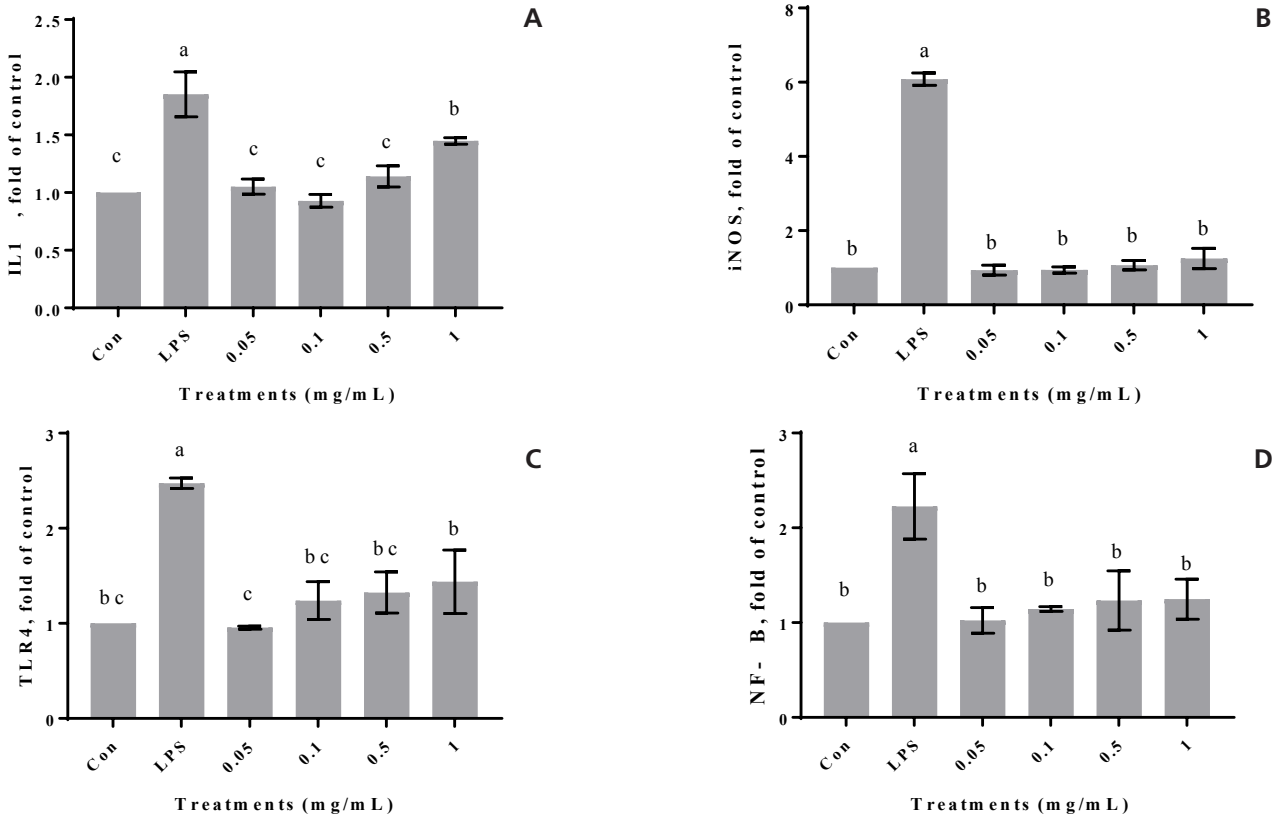

Figure 4 – Relative mRNA expression of NFkB (A),TLR4 (B), iNOS (C), and IL-1b (D) of chPBMC stimulated with LSE. Values are expressed as mean ± standard deviation (n=4). a-c Means within the same rows without the same superscript letter are significantly different (p<0.05).

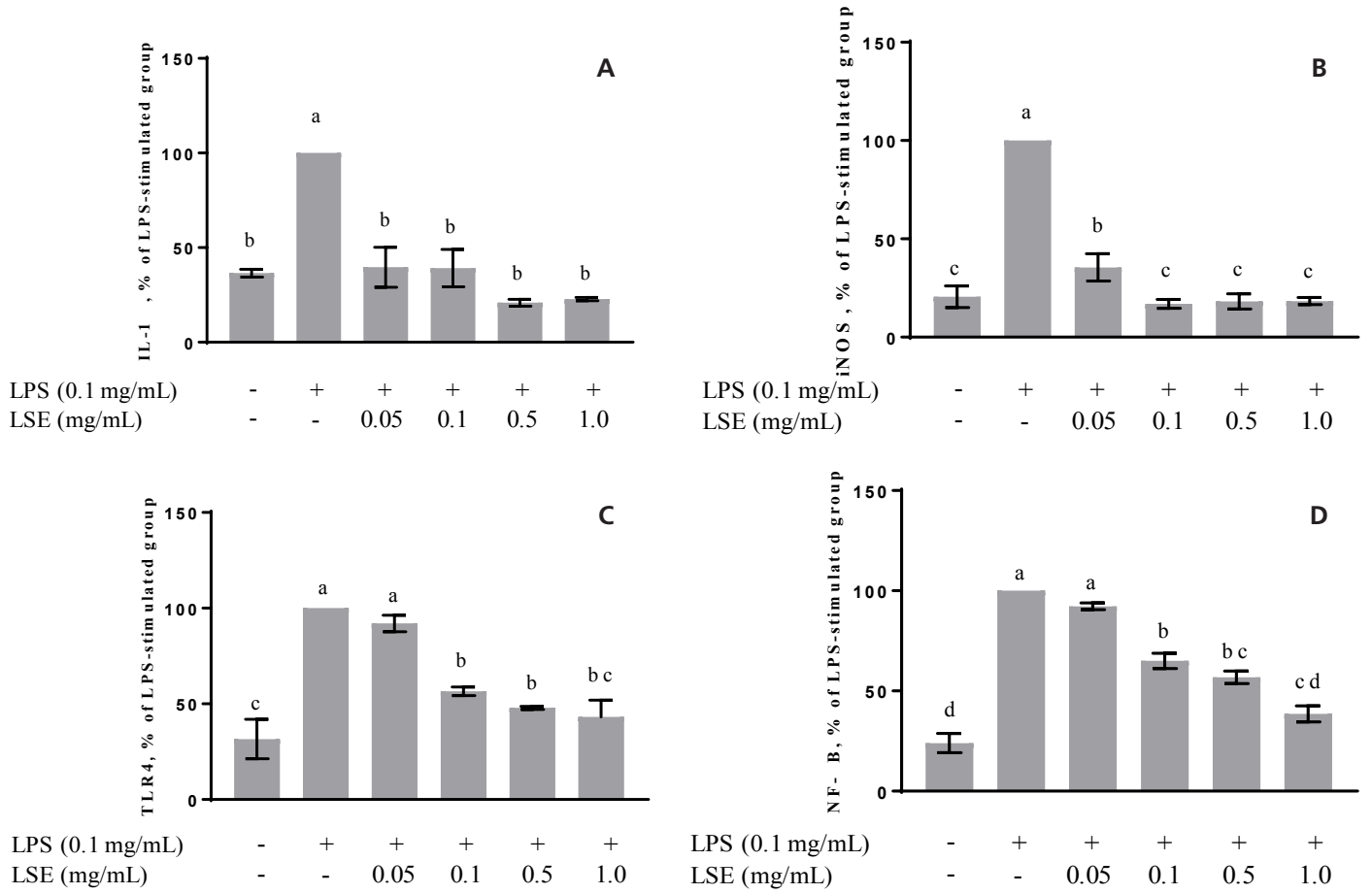

Figure 5 – NFkB (A),TLR4 (B), iNOS (C), and IL-1b (D) mRNA expression by chPBMC treated with LSE (0.05, 0.1, 0.5, 1 mg/mL) in the presence or absence of LPS (100 ng/mL) for 24 h. Values are expressed as mean ± standard deviation (n=4). a-d Means within the same rows without the same superscript letter are significantly different (p<0.05).

of LSE significantly elevated its expression compared with the control group (Fig. 4D).

Effects of LSE on mRNA levels of NFκB, TLR4, iNOS and IL1β on LPS-induced chPBMC

In order to further explore the immunomodulatory effects of LSE on chPBMC, its impact on the mRNA levels of NFκB, TLR4, iNOS and IL-1β on LPS-induced chPBMC was measured; the effects are summarized in Fig 5. LSE significantly reduced the expression of NFκB (5A) and TLR4 (5B) in a dose-dependent manner, although 0.05 mg/mL LSE had no effect on TLR4 levels (5B). iNOS level was significantly suppressed; 0.1-1 mg/mL LSE consistently had the best overall effect (5C). All LSE groups inhibited IL-1β mRNA expression (5D); however, significant differences were not found among treatment groups.

DISCUSSION

Several studies have previously investigated SmF conditions of *L. sulphureus* by using the exopolysaccharide as an index to determine optimal growth conditions (Lung *et al*., 2011; Hwang *et al*., 2008; Seo *et al*., 2010; Luangharn *et al*., 2014). However, no report to date has supplied any data regarding SSF conditions for this fungus species. This paper has provided a glimpse into the SSF of *L. sulphureus*. Since determining the numerous factors that contribute to the optimal cultivation of a specific fungus is very time-consuming, and the interaction between several of these factors would not be discernible(Yang *et al*., 2003), this study used several selected bioactive compounds (i.e., crude phenolic, crude triterpenoid, crude polysaccharide, and ergosterol levels) as approximate indicators of optimal SSF conditions. This was also out of consideration for the versatile medicinal functions and components of *L. sulphureus* mentioned in past reports (He *et al*., 2015; Wang *et al*., 2017a; Green *et al*., 1994). These chosen indicators showed a decrease in growth from d12-d16; therefore, the optimal harvesting time was established as d12.

LPS-induced activation of macrophages results in NO production (Lee *et al*., 1992), which is important in immune functions such as the killing of bacterial parasites (Hibbs *et al*., 1988). However, excessive immune reactions cause an overproduction of NO that further generates oxidants and nitrating agents, which interact with biological molecules, damaging cell membranes and causing cell death (Choi *et al*., 2006). When observing the reaction of LSE to LPS stimulation, we found that a specific dosage (0.01-1mg/mL) improved chPBMC viability and NO production. Jayasooriya *et al*. (2011) reported that exopolysaccharides purified from the LS culture medium significantly decreased NO production of LPS-induced BV2 microglia cells at a dosage of 2.0 mg/mL; no cytotoxicity was observed. Saba *et al*. (2015) and Wang *et al*. (2017a) both applied triterpenes purified from LS to RAW 264.7 cells and reported an improvement in the viability and NO production at dosages of 25-100 μg/mL and 0.02-0.08 μM, respectively. These studies confirmed that they were able to attenuate the excessive immune activation of the selected cells without causing cytotoxicity within the chosen dosage range. However, it should be noted that the cytotoxicity of LSE exceeded the reported dosage; the exposure time was unknown.

TLR4 is capable of recognizing PAMPs, including LPS. This triggers the downstream activation of transcription factor NFκB p65, which is translocated into the nucleus, and the expression of various pro-inflammatory substances, including IL-1β and iNOS. Fungal-derived polysaccharides have been shown to potentially activate TLR4 (Li and Xu, 2011), stimulating cell immune function to aid in pathogen defense. Therefore, LSE was first tested for its ability to trigger the mRNA expression of TLR4 and NFκB. After testing on mice peritoneal macrophages, Li *et al*. (2011) reported that polysaccharides from *Polyporusumbellatus*are able to exert immunostimulatory effects via TLR-4 activation of the signaling pathway. Lung *et al*. (2011) investigated the polysaccharides of LS and found that RAW 264.7 caused the release of tumor necrosis factor-α (TNF-α), a pro-inflammatory cytokine released by the activation of TLR4 that can also cause the activation of NFκB. In this study, LSE showed no significant effect on the TLR4 and NFκB activation or downstream iNOS levels. However, the mRNA levels of the pro-inflammatory cytokine IL-1βwere significantly elevated when the cells were stimulated with 1 mg/mL LSE; TLR4, NFκB and iNOS also showed a slight increase in mRNA levels after receiving LSE. These results indicate that LSE has the potential to trigger the pro-inflammatory status of chPBMC by activating TLR4 and NFκB. Higher dosage stimulation, time-course mRNA expression and the clarification of possible interactions in the LSE will be required in future studies to validate this statement.

The second part of the *in-vitro* cell experiment was to examine the anti-inflammatory properties of LSE by using LPS-induced chPBMC as a model. LPS is a virulence factor of gram-negative bacteria that acts as a PAMP to be recognized by TLR4 and trigger the downstream NFκB pathway, causing the release of IL-1β and iNOS. LPS causes the excessive release of pro-inflammatory mediators as well as lethal systemic disorders such as septic shock. IL-1βis highly inflammatory; its main function is to activate the immune system as part of the acute phase response. iNOS is the major enzyme to catalyze NO synthesis (Zamora *et al*., 2000); excessive production of iNOS leads to reactive nitrogen species (RNS) accumulation, causing oxidative damage to cells. All of the bioactive components that were tested during the LS SSF trial exerted significant effects on the anti-inflammatory properties. Phenolics are a heterogenic group of compounds derived from the secondary metabolism of plants and fungi. The structure of hydroxyl groups bonded to aromatic rings gives these compounds strong antioxidant activities (Ambriz-Pérez *et al*., 2016; Decker, 1997), which can neutralize the inflammatory damage by scavenging RNS. Turkoglu *et al*. (Turkoglu *et al*., 2007) reported on the antioxidant properties of LS fruiting body ethanol extracts, and credited this effect to the existence of phenolic compounds in LS. Ma *et al*. (2013) mentioned that phenolic antioxidants are able to attenuate LPS-induced inflammation by suppressing transcription factor NFκB. Triterpenoids have been recognized as anti-inflammatory and anti-oncolytic agents which can be retrieved from plants and fungi. LS-derived triterpene eburicoic acid has been described by Wang *et al*. (2017a) as able to inhibit LPS-induced activation of NFκB pathways and downregulate the inflammatory response in RAW 264.7 cells. Saba *et al*. (2015) also reported that acetyl eburicoic acid from LS can reduce the pro-inflammatory cytokines secreted by LPS-induced RAW 264.7 cells. Ergosterol is a fungal sterol, known as pro-vitamin D2, which inhibits NFκB expression in RAW 264.7 cells and exerts an anti-inflammatory effect (Ma *et al*., 2013). In this study, the significant inhibition of LSE on LPS-induced pro-inflammatory cytokine secretion was due to the contribution of various biofunctional compounds, in addition to the inhibition ofTLR4 and NFκB mRNA expression.

In the current study, we focused on using ethanolic extracts of the solid-state fermented LS, or LSE, to directly stimulate the immune function and inhibit LPS-induced inflammation in chPBMCs. According to our results, LSE could potentially stimulate the TLR4 and NFκB related immune response in normal cells. Moreover, LSE is able to attenuate LPS-induced IL-1β and iNOS secretion by suppressing the TLR4 and NFκB expression. This is accompanied by an improvement in cell viability and reduced NO production, without any evidence of cytotoxicity within the tested dosage and exposure time. However, further investigations are needed regarding the hidden interactions between various biofunctional compounds present in LSE and the influence of SSF substrates and conditions on the properties of the final fermenting products.

CONCLUSION

The results in this study indicate that LSE has potential immunomodulatory applications by modulating TLR4- and NFκB-related pathways of chicken peripheral mononuclear cells.

ACKNOWLEDGEMENT

The authors thank the Ministry of Science and Technology (MOST107-2313-B-005 -037-MY2) and the iEGG and Animal Biotechnology Center from The Feature Areas Research Center Program within the framework of the Higher Education Sprout Project by the Ministry of Education (MOE) in Taiwan to Prof. Tzu-Tai Lee.

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