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Research Article

Anti-Inflammatory Effects and Immunomodulatory Efficacy of Unitein (Fermented *Glycine max*, *Panax ginseng*, and Chenpi Mixture) in RAW 264.7 Macrophages and Mice Splenocytes along with Component Analysis

Kyunghwa Lee,[1] Yanni Pan,[2] YeonJun Lee,[3] WoonSeo Song,[4] and Kun-Young Park[5]

[1]*Mitosbio Inc., Chuncheon, Gangwon-do 24232, Republic of Korea*
[2]*Collaborative Innovation Center for Child Nutrition and Health Development, Chongqing Engineering Research Center of Functional Food, Chongqing Engineering Laboratory for Research and Development of Functional Food, Chongqing University of Education, Chongqing 400067, China*
[3]*Department of Food Science and Biotechnology, CHA University, Seongnam, Gyeonggi-do 13488, Republic of Korea*
[4]*Life Together Co., Ltd, Chuncheon, Gangwon-do 24232, Republic of Korea*
[5]*Graduate School of Integrative Medicine, CHA University, Seongnam, Gyeonggi-do 13488, Republic of Korea*

Correspondence should be addressed to Kun-Young Park; kypark9004@gmail.com

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Inflammation and the immune system are intricately linked, with the immune system serving as a vital defense mechanism in the human body. Consequently, there is great emphasis placed on the regulation of both the body's inflammatory response and the immune system. This study investigated the anti-inflammatory properties and immunomodulatory effects of Unitein (fermented *Glycine max*, *Panax ginseng*, and chenpi mixture) in both RAW 264.7 macrophages and mice splenocytes while also comprehensively analyzing its components. To this end, the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay was employed to evaluate the cytotoxicity of the samples to RAW 264.7 cells. The Griess assay was utilized to determine nitrite concentrations, while the enzyme-linked immunosorbent assay (ELISA) was employed to assess the levels of inflammation-related cytokines in RAW 264.7 cells and isolated mouse splenocytes. Additionally, quantitative polymerase chain reaction was employed to quantify the mRNA expression of inflammation-related genes in the cells, while the lactate dehydrogenase assay was used to analyze natural killer (NK) cell activity. Our results revealed that Unitein exhibited no toxicity to RAW 264.7 cells at concentrations below 1.5 mg/mL. However, Unitein effectively suppressed the release of proinflammatory cytokines tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, and interferon (IFN)-γ, as well as enzymes COX-2 and iNOS from RAW 264.7 cells, while concurrently promoting the release of anti-inflammatory cytokines IκB-α, IL-10, and IL-4. Furthermore, Unitein inhibited the increase in TNF-α, IL-1β, IFN-γ, IL-12, nuclear factor (NF)-κB p50, NF-κB p65, and iNOS induced by lipopolysaccharides (LPSs) in isolated mouse splenocytes. Notably, Unitein also exhibited the potential to stimulate NK cell activity. Metabolite analysis indicated that Unitein contained more active compounds with anti-inflammatory and immunity-enhancing properties than did unfermented Unitein. This study highlights the potential therapeutic role of Unitein in mitigating inflammation and enhancing immune responses, thereby deepening our understanding of its biological activities and underlying mechanisms. These findings underscore the significance of Unitein as a potential candidate for the development of novel anti-inflammatory and immunomodulatory interventions.

1. Introduction

Inflammation and immunity are essential physiological responses exhibited by organisms when faced with stressors such as infection, injury, and foreign body invasion; these two intricately interrelated processes maintain homeostasis and provide resistance against pathogenic assaults through complex cellular and molecular interactions [1]. Inflammation can be characterized as a nonspecific biological reaction, encompassing a cascade of intricate responses to injury, infection, or stimulation. The key components of the inflammatory process include localized vascular dilation, increased vascular permeability, leukocyte infiltration, and the release of cytokines and chemical mediators; these coordinated reactions aim to eliminate pathogens and facilitate tissue repair [2]. Immunity constitutes a defensive mechanism employed by organisms to resist exogenous pathogens. The immune system safeguards the body against infections by identifying and eliminating pathogens [3]. RAW 264.7 cells and splenocytes are cellular models commonly utilized for investigating the cellular and molecular mechanisms associated with immunity and inflammation [4, 5]. RAW 264.7 cells are a widely employed murine macrophage cell line, are derived from peritoneal macrophages of BALB/c mice, and are favored for their ease of cultivation and maintenance of stable immunological characteristics, which has been extensively utilized to explore immune and inflammatory responses [6]. RAW 264.7 cells are capable of producing various cytokines and chemical mediators, including tumor necrosis factor-alpha (TNF-α), interleukin-1 (IL-1), and nitric oxide (NO), which play crucial regulatory roles in immune and inflammatory responses [7]. Meanwhile, splenocytes comprise a population of nucleated cells within the spleen, encompassing lymphocytes, macrophages, and dendritic cells, among others, while the spleen is a vital lymphoid organ within the immune system that plays a crucial role during immune and inflammatory processes [8]. Research on splenocytes provides insights into lymphocyte immune responses and macrophage phagocytic activity, which furthers our understanding on immune function [8]. By simulating inflammatory stimuli in RAW 264.7 cells and splenocytes, processes such as cytokine secretion, cell proliferation, apoptosis, and cellular signal transduction can be observed and quantified. This enhances our comprehension of immune-inflammatory regulation, immune cell interactions, and potential therapeutic targets.

Chronic inflammation influences the onset and progression of tumors [9]. Therefore, the treatment of chronic inflammation holds significant importance in modern medicine. Steroidal and nonsteroidal anti-inflammatory drugs have been widely employed in contemporary society; however, they come with various side effects affecting the digestive tract, kidneys, and central nervous system [10]. These adverse effects have prompted further research aimed at uncovering novel, safe, and effective anti-inflammatory drugs or functional foods. In recent years, the heightened attention to health has driven individuals to seek the maintenance of physiological equilibrium through the consumption of stabilizing natural ingredients. Unitein is a product obtained through the fermentation of a mixture comprising soybeans, red ginseng, and chenpi. Fermentation is a process wherein microorganisms utilize macromolecular nutrients such as carbohydrates, proteins, or fats to produce small-molecule organic acids, alcohol, CO_2, and other fermentation byproducts [11]. This process aids in digestion, inhibits the growth of pathogens, prolongs product shelf life, enhances flavor and texture, and mostly employed promotes health-beneficial effects. The primary microorganisms contributing to fermentation are probiotics, such as *Bacillus subtilis* and *Lactobacillus* species [12]. Research suggests that the use of microbial fermentation in food production increases the abundance of phytochemicals and metabolites, enhancing the bioactivity of the food [13]. Thus, this study employed *Bacillus subtilis* and *Lactobacillus* species for the mixed fermentation of soybeans, red ginseng, and chenpi.

Soybeans (*Glycine max*) possess elevated protein and fat content, a distinct fishy odor, and oligosaccharides, the elimination of which necessitates fermentation. Fermentation generates various bioactive substances such as peptides, myo-inositol, phenolic compounds, and isoflavones [13]. Compared to unfermented foods, these substances have enhanced health functionalities. Furthermore, evidence has shown that fermented soybean extracts tended to reduce inflammation within the bladder tissues and downregulate TNF-α expression in a mouse model of lipopolysaccharide (LPS)-induced cystitis [14]. Ginseng (*Panax ginseng*) is continuously being explored for its multifunctional properties, including anticancer effects and immune enhancement. The "nine steaming and nine drying" technique employed to process ginseng has resulted in the substantial accumulation of beneficial physiological compounds [15]. In fact, evidence shows that ginseng processed using this method possesses better antioxidant properties than does regular red ginseng [16]. This processed ginseng not only reduces allergic inflammatory responses but also demonstrates significant efficacy against allergic dermatitis [17]. Dried citrus peel, also known as chenpi, is rich in carotenoids, flavonoids, limonoids, and dietary fiber. Its pharmacological effects include enhancing fat-digesting enzyme activity, preventing allergic reactions, and averting conditions such as atherosclerosis and hypertension [18]. Research indicates that fermentation of chenpi converts glycoside-bound flavonoids into aglycones, thereby augmenting their functional properties [19]. Currently, limited research is available on mixed fermented products with anti-inflammatory and immunity-enhancing effects. Hence, this study employed a mixed fermentation preparation named Unitein, which is derived from soybeans, red ginseng processed using the nine steaming and nine drying technique, and chenpi, to investigate its anti-inflammatory and immunity-promoting effects in RAW 264.7 cells and C57BL/6 mice splenocytes.

Metabolomic analysis is a systematic approach to studying metabolites within an organism. By quantitatively and qualitatively analyzing the metabolites present, this method unveils the metabolic landscape of a biological entity under specific physiological conditions [20]. Metabolomic

analysis has also been employed to identify alterations in metabolites before and after fermentation, as well as to pinpoint specific metabolites responsible for anti-inflammatory and immunity-enhancing effects [21]. Ultra-performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF MS) serves as a robust technique in metabolomic analysis [22]. Hence, this study used UPLC-Q-TOF MS to analyze the mixture of soybeans, red ginseng, and chenpi, as well as the fermented Unitein, to acquire detailed information regarding changes in metabolites and identify key metabolites contributing to the observed differences. The results obtained from this analysis could serve as a scientific basis for fermentation and Unitein development.

Inflammation and the immune system are intricately linked, with the immune system serving as a vital defense mechanism in the human body. Consequently, there is great emphasis placed on the regulation of both the body's inflammatory response and the immune system. To develop a diet that supports overall body health, an assessment of fermented foods was conducted to determine their impact on inflammatory response and immune regulation. The current study aimed to assess the anti-inflammatory and immunity-enhancing effects of the fermented product Unitein, derived from a mixture of soybeans, red ginseng, and chenpi, utilizing an LPS-induced inflammatory model in RAW 264.7 cells and mice splenocytes. Our assessment involved determining the viability of RAW 264.7 cells, measuring the production of NO and levels of inflammatory cytokines released by both RAW 264.7 cells and mice splenocytes, quantifying the mRNA expression of inflammation-related genes in these cells, evaluating natural killer (NK) cell activity, and utilizing UPLC-Q-TOF MS to analyze changes in metabolites before and after fermentation. These approaches were employed to investigate the mechanisms underlying the anti-inflammatory and immunity-enhancing properties of Unitein, as well as its active components. The present study aimed to establish a theoretical foundation and provide evidence for the development of fermented products with health-promoting, anti-inflammatory, and immunity-enhancing properties.

2. Methods

2.1. Sample Preparation. For this study, both unfermented (raw material mixture) and fermented Unitein were provided by Mybarite Co., Ltd. (Seoul, Korea). The raw materials for Unitein included soybeans, red ginseng, and chenpi, with the same proportions as in the final Unitein product. The process of creating the powdered Unitein product involved boiling the soybeans, fermenting them at 42°C–45°C for 48 h, adding red ginseng powder at 5%–10% of the soybean weight, fermenting the mixture for an additional 25 h, combining the soybean–red ginseng mixture with chenpi powder at a 25 : 75 ratio, and then finally fermenting the mixture for 18 h. And the *Bacillus subtilis* and *Lactobacillus* species used in the mixed fermentation of soybeans, red ginseng, and chenpi in this study were derived from red ginseng doenjang fermented in Buk-gu, Pohang, Gyeongsangbuk-do, South Korea, and were provided by Mybarite Co., Ltd. Approximately, 30 g of each sample powder was mixed with 600 mL of methanol (1 : 20 ratio) and extracted using a stirrer at room temperature for 24 h. After repeating the mentioned step repeated twice, the supernatants were combined to obtain methanol extracts. The methanol extracts were concentrated using a vacuum concentrator (EYELA, Tokyo Rikakikai Co., Tokyo, Japan) to form a powdered substance. Dimethyl sulfoxide (DMSO) was then added to achieve a concentration of 250 mg/mL for subsequent experiments.

2.2. RAW 264.7 Cell Culture. RAW 264.7 cells were procured from the Korean Cell Line Bank (KCLB, Seoul, Korea). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, GIBCO) and 1% penicillin–streptomycin solution (Welgene) at 37°C in a 5% CO_2 incubator. The cells were passaged 3–4 times weekly.

2.3. Isolation and Culture of Murine Splenocytes. Ten male C57BL/6 mice, 6 weeks old with a weight of 20 ± 2 g (Orient Bio, Seongnam, Korea), were housed in a specific pathogen-free room maintained at room temperature with controlled lighting (12 : 12 h) and 55% ± 5% relative humidity. After 1 week of adaptation, the mice were aseptically dissected to isolate spleens and prepare splenocyte suspensions. The obtained splenocyte suspension was filtered, treated with red blood cell lysis buffer, and centrifuged at 1200 rpm for 3 min. Thereafter, the pellet was resuspended in Roswell Park Memorial Institute (RPMI) 1640 medium (Welgene Inc., Daegu, Korea) supplemented with 10% FBS and 1% penicillin-streptomycin solution for subsequent experiments.

2.4. YAC-1 Cell Culture. YAC-1 cells used in this experiment were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in RPMI 1640 medium supplemented with 10% FBS and 1% penicillin-streptomycin solution for subsequent experiments.

2.5. Assessment of Sample Toxicity in RAW 264.7 Cells. The RAW 264.7 cell count was measured using a Cell Counter (Luna automated cell counter, Logos Biosystems, Gyunggi-do, Korea). The cells were seeded at a concentration of 1×10^5 cells/mL in 96-well plates and incubated at 37°C and 5% CO_2 for 24 h. Following medium removal, different concentrations of Unitein samples were prepared in culture medium (100 μL per well) and incubated for 48 h. After removing the medium, 100 μL of a 500 μg/mL MTT (3-(4,5-dimethylthiazol2-yl)-2,5-diphenyltetrazolium bromide, Thermo Fisher Scientific, Ward Hill, MA, USA,) solution was added. After incubating for 3.5–4 h, formazan crystals were dissolved using DMSO, and the absorbance at 550 nm was measured after 15–30 min using a Wallac Victor3 1420 Multilabel Counter (Perkin-Elmer, Wellesley, MA, USA).

2.6. Determination of Nitrite (NO) Production. For nitrite determination, the protocol of agreement was followed using the Griess method [4]. RAW 264.7 cells (1×10^5 cells/mL) and murine splenocytes (1×10^6 cells/mL) were separately seeded in 6-well plates and incubated in a 5% CO_2 and 37°C incubator for 24 h. The culture medium was then removed, after which the medium containing specified concentrations of the samples along with LPS (1 μg/mL) was added to the 6-well plates and incubated for 48 h. Afterward, the cell culture medium was collected, 100 μL was added to a 96-well plate and treated with an equal volume of Griess reagent (Enzo Life Sciences, Inc., Farmingdale, NY, USA), and absorbance was measured at 550 nm using a Wallac Victor3 1420 Multilabel Counter.

2.7. Measurement of Inflammatory Cytokines Using Enzyme-Linked Immunosorbent Assay (ELISA). RAW 264.7 cells were seeded at a concentration of 2×10^5 cells/mL and murine splenocytes at a concentration of 1×10^6 cells/mL in 6-well plates and incubated at 37°C with 5% CO_2 for 24 h. After removing the medium, specified concentrations of Unitein and LPS (1 μg/mL) were added to each well and incubated for 48 h. Using ELISA kits (BioLegend, San Diego, CA, USA), the concentrations of TNF-α, IL-1β, IL-6, and IL-10 in the cell culture medium were measured. The specific testing protocol provided by the manufacturer was followed.

2.8. Measurement of Inflammation-Related Gene mRNA Levels Using RT-qPCR. RAW 264.7 cells at a concentration of 2×10^5 cells/mL and murine splenocytes at a concentration of 1×10^6 cells/mL were both seeded in 6-well plates and incubated at 37°C with 5% CO_2 for 24 h. After removing the medium, the Unitein extract was diluted in culture medium to a concentration of 1.5 mg/mL, and LPS (1 μg/mL) was added to each well. After 48 h, the medium was removed, and RNA was separated using 1 mL Trizol (Invitrogen, Carlsbad, CA, USA) per plate. RNA quantification was performed using NanoDrop ND-1000 (NanoDrop Technologies Inc., Wilmington, DE, USA), and Superscript II Reverse Transcriptase (Invitrogen) was used to synthesize cDNA. The gene expression of synthesized cDNA was analyzed using the Bio-Rad CFX-96 Real-Time System (Bio-Rad, Hercules, CA, USA). The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative transcription levels of mRNA. The specific experimental protocol followed prior research schemes. The expression levels of IFN-γ, IL-6, IL-10, IL-4, COX-2, iNOS, eNOS, and GAPDH genes in RAW 264.7 cells were determined, with the primer sequences provided in Table 1.

2.9. Measurement of Natural Killer (NK) Cell Activity. Splenocytes were seeded at 1×10^6 cells per well in a 96-well plate to serve as effector cells. Using YAC-1 cells as target cells, the effector-to-target cell ratios in each well were adjusted to 25 : 1, 10 : 1, and 5 : 1, after which the samples were treated at specified concentrations. After incubating for 4 hs at 37°C and 5% CO_2, lactate dehydrogenase (LDH) released by YAC-1 cells was measured at 450 nm using the EZ-LDH assay kit (DoGenBio, Geumcheon-gu, Seoul, Korea) to calculate the cytotoxicity of the samples toYAC-1 cells.

Table 1: Primer sequences of RT qPCR assay of this study.

Gene name	Primer sequence
NF-κB p65	F: 5′-ATGGCAGACGATGATCCCTAC-3′ R: 5′-CGGAATCGAAATCCCCTCTGTT-3′
NF-κB p50	F: 5′-ATGGCAGACGATGATCCCTAC-3′ R: 5′-CGGAATCGAAATCCCCTCTGTT-3′
IL-10	F: 5′-CCAAGCCTTATCGGAAATGA-3′ R: 5′-TTTTCACAGGGGAGAAATCG-3′
IL-4	F: 5′-TCAACCCCCAGCTAGTTGTC-3′ R: 5′-TGTTCTTCGTTGCTGTGAGG-3′
IL-6	F: 5′-ATGAAGTTCCTCTCTGCAA-3′ R: 5′-AGTGGTATCCTCTGTGAAG-3′
IL-1β	F: 5′-AAGGGCTGCTTCCAAAC-3′ R: 5′-CTCCACAGCCACAATGA-3′
IFN-γ	F: 5′-GCTTTGCAGCTCTTCCTCAT-3′ R: 5′-GTCACCATCCTTTTGCCAGT-3′
COX-2	F: 5′-GGTGCCTGGTCTGATGATG-3′ R: 5′-TGCTGGTTTGGAATAGTTGCT-3′
iNOS	F: 5′-ATGGCTTGCCCCTGGAA-3′ R: 5′-TATTGTTGGGCTGAGAA-3′
GAPDH	F: 5′-AGGTCGGTGTGAACGGATTTG-3′ R: 5′-GGGGTCGTTGATGGCAACA-3′

2.10. Sample Preparation for Metabolomic Analysis. Dried unfermented Unitein (UU) and fermented Unitein (Uni) powders were mixed with 80% methanol containing terfenadine and zidovudine as internal standards. Extraction was performed using a bullet blender homogenizer (Next Advance, NY, USA). After centrifugation, the supernatant was analyzed using UPLC-Q-TOF MS (Waters Corp., Milford, MA, USA) [4].

2.11. Metabolite Analysis Using UPLC-Q-TOF MS. Metabolites of UU and Uni were analyzed using the UPLC-Q-TOF MS system (Xevo™ G2-S, Waters, Milford, MA, USA). Samples were injected into the Acquity UPLC BEH C18 column (2.1 mm × 100 mm, 1.7 μm; Waters Corp.) and equilibrated with water containing 0.1% formic acid. Elution was performed using acetonitrile containing 0.1% formic acid in a gradient. A flow rate of 0.35 mL/min and a column temperature of 40°C were employed. Q-TOF MS with electrospray ionization was used for metabolite analysis. Solvent removal rates and temperatures were set at 800 L/h and 400°C, respectively, with a source temperature of 100°C. TOF MS scanning ranged from 100 to 1500 m/z with a scan time of 0.2 s, and capillary voltage and sampling cone voltage were set at 3 kV and 40 V, respectively. L-tryptophan-bradykinin ([M + H] = 556.2771) was injected at a flow rate of 20 μL/min and a lock mass for mass accuracy confirmation. Quality control samples prepared by mixing all samples were analyzed three times before starting and after

every 10 analyses. A collision energy that gradually increased from 10 to 30 eV was used to obtain MS/MS spectra in the 50–1500 m/z range [23].

2.12. UPLC-Q-TOF MS Data Processing. Acquisition, normalization, and alignment of MS data obtained from UPLC-Q-TOF MS analysis were performed using UNIFI version 1.8.2 (Waters Corp.). Peaks were collected with a peak-to-peak baseline noise of 1, peak width at 5% height of 1 s, noise elimination of 6, and intensity threshold of 10,000. Collected data were aligned using a mass window of 0.05 Da and a retention time window of 0.2 min. Normalization of all LC/MS data was based on the average mass intensity of internal standards. Metabolite identification was performed using online databases including UNIFI's ChemSpider database, METLIN database (https://www.metlin.scripps.edu), and the Human Metabolome Database (https://www.hmdb.ca) [4].

2.13. Statistical Analysis. Results from three or more parallel experiments were averaged. GraphPad Prism (GraphPad Prism 9.3.1) and SPSS version 22 software (SPSS Inc., IL, USA) were used for plotting and analysis, respectively. Mean and standard deviation were used to present experimental results. Differences between group means were evaluated using the unpaired *T*-test, two-way analysis of variance (ANOVA), or one-way ANOVA with Duncan's multiple range test. Statistical significance was set at $p < 0.05$ or $p < 0.1$ was considered. Metabolomic data were analyzed using SIMCA-P+ 12.0.1 (Umetrics, Umeå, Sweden) for LC-MS data and visualized with partial least squares discriminant analysis (PLS-DA) to reveal differences between sample groups. PLS-DA model quality was assessed using three parameters (R_2X and R_2Y: goodness of fit, Q_2: predictability, p value) and validated through permutation testing ($n = 200$). Normalized intensity differences of identified metabolites were tested using Duncan's SPSS version 17.0 (SPSS Inc., IL, USA) ($p < 0.05$).

3. Results

3.1. Cytotoxicity of the Samples to RAW 264.7 Cells. Figure 1 illustrates the cytotoxic effects of UU and Uni on RAW 264.7 cells. At concentrations up to 1.5 mg/mL, both UU and Uni exhibited minimal cytotoxic effects on RAW 264.7 cells with no significant difference between both. However, when concentrations exceeded 1.75 mg/mL, the viability of RAW 264.7 cells decreased following treatment with UU and Uni. Notably, Uni exhibited lower cytotoxicity to RAW 264.7 cells than did UU, with this difference being statistically significant ($p < 0.0001$). Therefore, UU and Uni concentrations of 1.5 mg/mL were selected for further experiments.

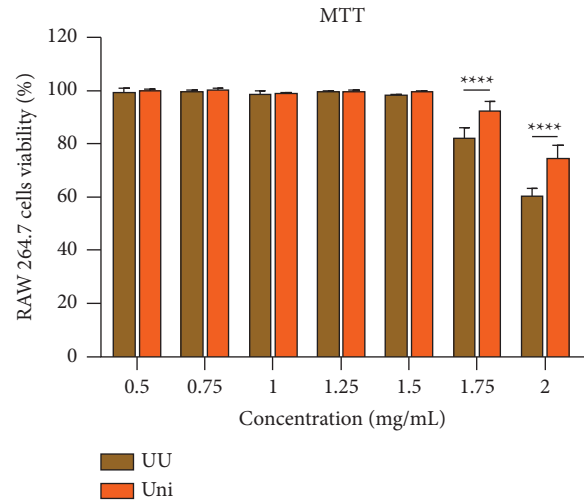


Figure 1: Effect of unfermented unitein (UU) and unitein (Uni) on raw 264.7 cell viability. **** $p < 0.0001$.

3.2. Effects of the Samples on NO Production in RAW 264.7 Cells and Mice Splenocytes. As shown in Figure 2, the lowest NO production was observed in the CON group for both RAW 264.7 cells and mouse spleen cells. Following LPS treatment, a significant increase in NO production was observed in both RAW 264.7 cells and mice splenocytes. However, under simultaneous treatment with UU and Uni, NO production significantly decreased. Notably, the reduction in NO production was more pronounced in the Uni group than in the UU group. These results indicated that both UU and Uni effectively inhibited LPS-induced NO production in both RAW 264.7 cells and mice splenocytes, with Uni exhibiting a more remarkable effect.

3.3. Effects of Samples on Cytokine Levels in RAW 264.7 Cells and Mouse Spleen Cells. The effects of the samples on cytokine secretion in RAW 264.7 cells are depicted in Figure 3(a). Accordingly, the LPS group exhibited the highest levels of inflammatory cytokines TNF-α, IL-1β, and IL-6. Following treatment with UU and Uni, a significant reduction in inflammatory cytokines was observed, with Uni demonstrating a superior inhibitory effect over UU. In the Uni group, the release of inflammatory cytokines by RAW 264.7 cells closely resembled that by the CON group. The LPS group had the lowest IL-10 levels, whereas the Uni group had the closest IL-10 levels to the CON group.

Figure 3(b) presents the effects of samples on cytokine levels in mouse spleen cells. Similar to RAW 264.7 cells, a comparable trend in cytokine levels was observed. Although TNF-α, IL-1β, IFN-γ, and IL-12 levels were elevated in the LPS group, treatment with the sample promoted varying degrees of reduction in inflammatory cytokine levels. Notably, the Uni group exhibited lower levels of inflammatory cytokines compared to UU, with statistical significance ($p < 0.05$). These findings balanced that Unitein effectively balances the release of inflammatory cytokines induced by LPS.

3.4. Effects of the Samples on the mRNA Expression of Inflammation-Related Genes in RAW 264.7 Cells and Mice Splenocytes. In RAW 264.7 cells, under the condition where

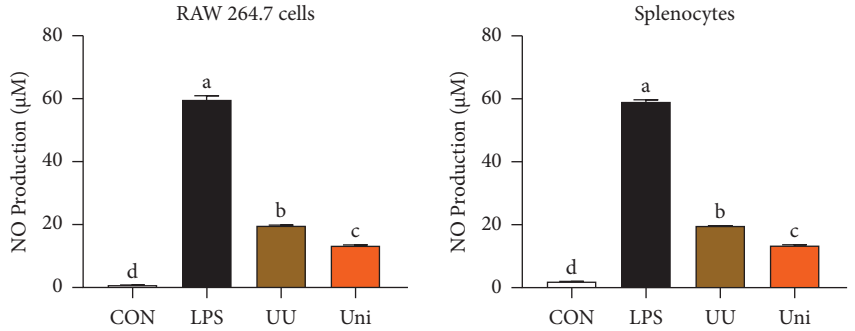


Figure 2: Nitric oxide (NO) production in RAW 264.7 cell and mice splenocyte after exposure to LPS, unfermented unitein (UU) and unitein (Uni). CON: no treatment; LPS: 1 μg/mL lipopolysaccharide (LPS); UU: UU 1 mg/mL + 1 μg/mL LPS; Uni: Uni 1 mg/mL + 1 μg/mL LPS. Means with the different letters (a–d) on the bars are significantly different ($p < 0.05$) by Duncan's multiple range test. The differences between groups are compared using letter labels, with letter "a" representing the highest significant difference between groups and letter "b" representing the second highest significant difference, and so on. When the letters are the same, it indicates that there is no significant difference between the corresponding groups.

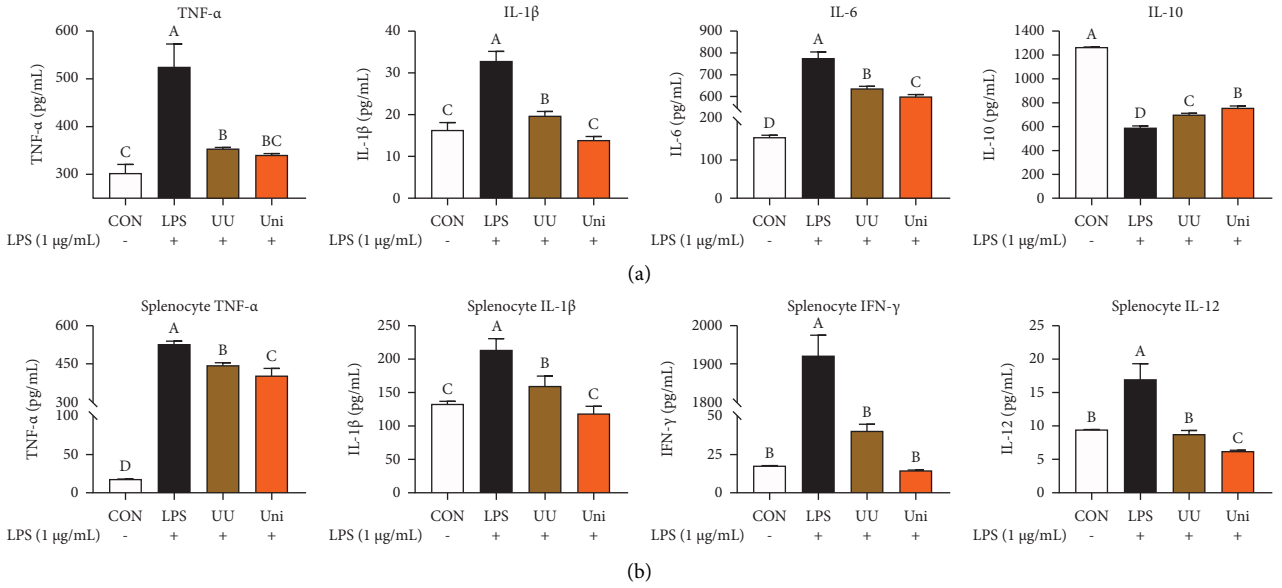


Figure 3: Concentrations of cytokines TNF-α, IL-6, IL-1β, and IL-10 in RAW 264.7 cell media and TNF-α, IL-1β, IFN-γ, and IL-12 in mice splenocyte media after exposure to LPS, unfermented unitein (UU) and unitein (Uni). CON: no treatment; LPS: 1 μg/mL Lipopolysaccharide (LPS); UU: UU 1 mg/mL + 1 μg/mL LPS; Uni: Uni 1 mg/mL + 1 μg/mL LPS. Means with the different letters (A–D) on the bars are significantly different ($p < 0.05$) by Duncan's multiple range test. The differences between groups are compared using letter labels, with letter "A" representing the highest significant difference between groups and letter "B" representing the second highest significant difference, and so on. When the letters are the same, it indicates that there is no significant difference between the corresponding groups.

the relative expression levels of IL-6, IFN-γ, COX-2, and iNOS mRNA in the CON group were considered as 1.00, treatment with LPS significantly increased the relative expression levels of IL-6, IFN-γ, COX-2, and iNOS mRNA to 3.51 ± 0.24, 3.70 ± 0.44, 7.81 ± 0.53, and 1.62 ± 0.10, respectively. At the same time, it decreased the relative expression levels of IkB-α, IL-10, and IL-4 to 0.39 ± 0.02, 0.52 ± 0.03, and 0.65 ± 0.05, respectively (Figure 4). Furthermore, compared to LPS, treatment with UU or Uni significantly downregulated the relative expression levels of IL-6, IFN-γ, COX-2, and iNOS mRNA, but upregulated the relative expression levels of IκB-α, IL-10, and IL-4 mRNA. After Uni treatment, the relative expression levels of IL-6, IFN-γ, COX-2, iNOS, IκB-α, IL-10, and IL-4 mRNA decreased to 2.46 ± 0.06, 1.93 ± 0.05, 3.39 ± 0.25, 1.12 ± 0.01, 1.87 ± 0.03, 1.27 ± 0.13, and 1.24 ± 0.11, respectively. Unitein treatment brought the mRNA expression levels of these inflammation-related genes closest to those in the CON group.

In mice splenocytes (Figure 5), compared to the CON group, LPS induction significantly ($p < 0.05$) upregulated the mRNA expression levels of these inflammation-related genes, including NF-κB p65, NF-κB p50, IL-1β, IFN-γ, and iNOS. Their relative expression levels were upregulated to 1.83 ± 0.02, 1.89 ± 0.06, 5.24 ± 0.16, 429.64 ± 47.18, and 49.13 ± 7.08, respectively. Among the relative expression

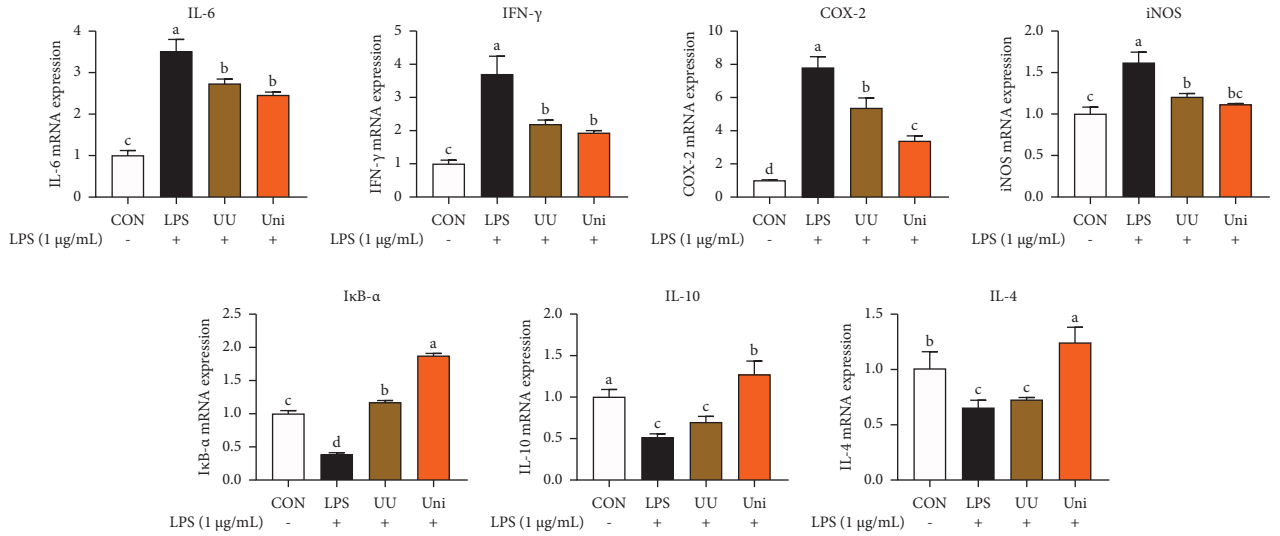


Figure 4: Effects of unfermented unitein (UU), and unitein (Uni) on mRNA expression of IκB-α, IL-10, IL-4, IL-6, IFN-γ, COX-2, and iNOS in RAW 264.7 cell. CON: no treatment; LPS: 1 μg/mL Lipopolysaccharide (LPS); UU: UU 1 mg/mL + 1 μg/mL LPS; Uni: Uni 1 mg/mL + 1 μg/mL LPS. Means with the different letters (a–d) on the bars are significantly different ($p < 0.05$) by Duncan's multiple range test. The differences between groups are compared using letter labels, with letter "a" representing the highest significant difference between groups and letter "b" representing the second highest significant difference, and so on. When the letters are the same, it indicates that there is no significant difference between the corresponding groups.

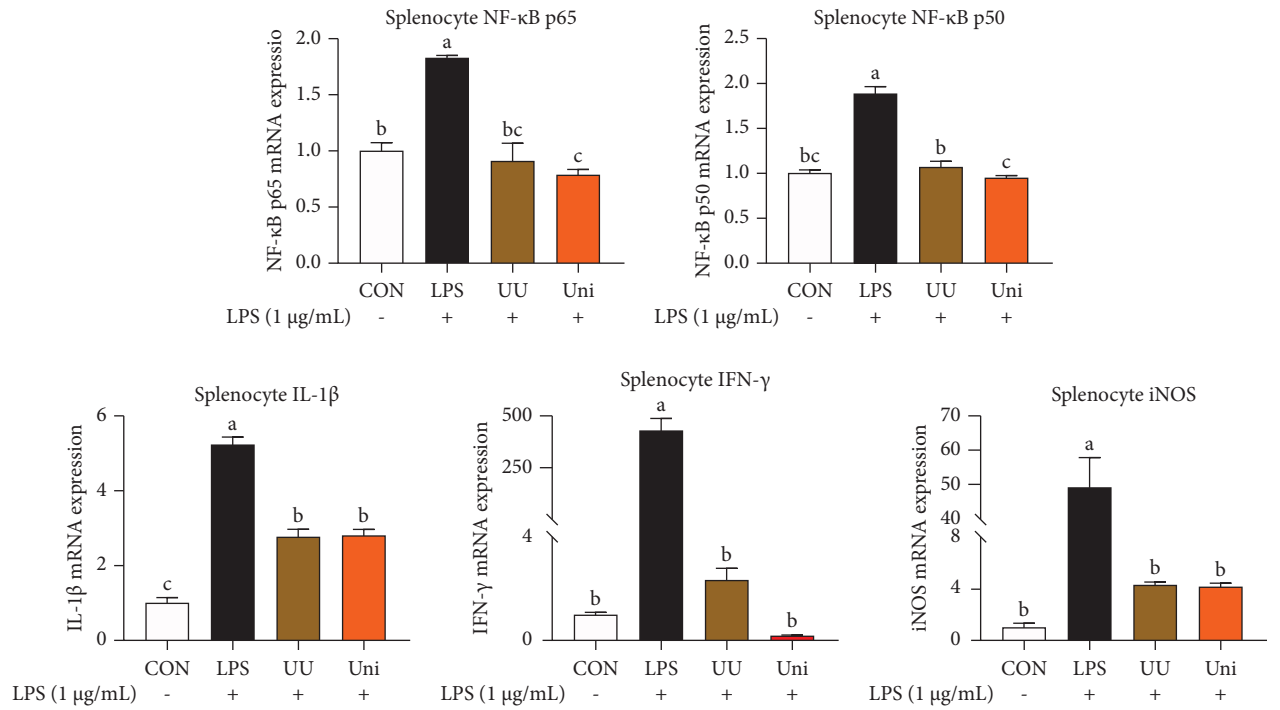


Figure 5: Effects of unfermented unitein (UU), and unitein (Uni) on mRNA expression of NF-κB p65, NF-κB p50, IL-1β, IFN-γ, and iNOS in mice splenocyte. CON: no treatment; LPS: 1 μg/mL lipopolysaccharide (LPS); UU: UU 1 mg/mL + 1 μg/mL LPS; Uni: Uni 1 mg/mL + 1 μg/mL LPS. Means with the different letters (a–d) on the bars are significantly different ($p < 0.05$) by Duncan's multiple range test. The differences between groups are compared using letter labels, with letter "a" representing the highest significant difference between groups and letter "b" representing the second highest significant difference, and so on. When the letters are the same, it indicates that there is no significant difference between the corresponding groups.

results of NF-κB p50, IFN-γ, and iNOS mRNA, it was found that after Uni treatment, the mRNA expression levels of these genes were closest to the CON group, with values of 0.95 ± 0.02, 0.19 ± 0.02, and 4.20 ± 0.23, respectively. In the UU group, the mRNA expression levels of these genes were: 1.07 ± 0.05, 2.36 ± 0.37, and 4.34 ± 0.18. Regarding the

expression results of NF-κB p65 and IL-1β, although the mRNA expression levels in the UU group were closer to the CON group, Uni treatment also reached expression levels that were not significantly different from the UU group ($p > 0.05$). These results indicate that both Uni and UU can effectively control the inflammatory response induced by LPS, with Uni showing superiority in controlling the expression levels of NF-κB p50, IFN-γ, and iNOS.

3.5. Effects of Samples on NK Cell Activity in Spleen Cells. The effects of UU and Uni on NK cell activity are shown in Figure 6. As the ratio of spleen cells to YAC-1 cells increased, NK cell activity also increased. Uni exhibited higher NK cell activity than did UU at any experimental ratio, with this difference being significant ($p < 0.05$). These findings suggested that the immunity-enhancing effects of Uni surpass that of UU.

3.6. Metabolomic Analysis and PLS-DA Score Plot. Metabolomic profiling of UU and Uni samples was conducted using UPLC-Q-TOF MS, and differences between both samples were visualized through PLS-DA. Positive and negative (Figure 7) PLS-DA score plots of LC-MS data clearly demonstrated distinct separation among all sample groups, with statistically acceptable quality parameters (Positive mode: $R_2X = 0.861$, $R_2Y = 0.997$, $Q_2 = 0.989$, p value $= 3.56 \times 10^{-5}$; Negative mode: $R_2X = 0.843$, $R_2Y = 0.999$, $Q_2 = 0.989$).

Furthermore, the cross-validation values determined by permutation tests (R_2 and Q_2 intercepts <0.05) suggested the statistical validity of the PLS-DA model. The results indicated significant separation between UU and Uni, and that the changes in metabolomic profiles contributed to the distinct separation in the PLS-DA score plot.

3.7. Identification of Major Metabolites. Through LC/MS data analysis in positive and negative modes, major metabolites from UU and Uni were identified using multistage tandem mass spectrometry in combination with retention time, accurate mass, MS fragments, VIP, and p values. These identifications were based on online databases such as ChemSpider, METLIN, and Human Metabolome Database using UNIFI software. A total of 39 metabolites were identified in positive mode (Figure 8(a) and Table 2), whereas 32 metabolites were identified in negative mode (Figure 8(b), Table 3).

3.8. Heatmap Analysis of UU and Uni Metabolites. A heatmap was used to visualize the differences in metabolite levels between UU and Uni. The results of the positive mode heatmap (Figure 9(a)) showed that glutarylcarnitine, tryptophan, daidzin/puerarin, galangin/genistein/apigenin/hydroxydaidzein, LPC (18 : 3), LPE (18 : 2), LPC (18 : 2), linolenic acid, LPC (16 : 0), LPC (18 : 1), linolenelaidic acid/gamma-linolenic acid/alpha-linolenic acid, and LPC (18 : 0) were more abundant in Uni than in UU.

In the negative mode (Figure 9(b)), shanzhiside, secoxyloganin/6 beta-hydroxygeniposide/gardenoside, pisumoside B, chrysophanic acid/dihydroxyflavone/rubiadin/daidzein,

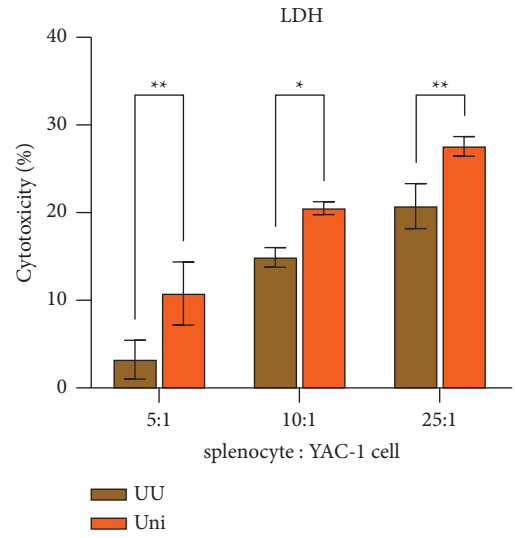


Figure 6: Activity of NK cell in C57BL/6 mice splenocytes after unfermented Unitein (UU) and Unitein (Uni). UU: UU 1 mg/mL; Uni: Uni 1 mg/mL. 5–25:1means splenocyte: YAC 1 cell. $^{*}p < 0.05$; $^{**}p < 0.01$.

crocin 3, genistein/apigenin/archin/galangin, pinellic acid, soyasaponin Ba/asiaticoside, soyasaponin I, soyasaponin II, LPE (18 : 2), hydroxyoctadecadienoic acid, LPE (18 : 0), LPC (17 : 1), and LPC (17 : 0) had higher concentrations in Uni than in UU.

4. Discussion

Inflammation, as a biological response, typically serves as the immune system's first line of defense against infection and injury [1]. Inflammation triggers the activation and migration of immune cells such as leukocytes and macrophages, to clear sources of infection and dead cells. Immune cells also secrete cytokines, such as interleukins, to regulate the intensity and duration of the inflammatory response [24]. Inflammation can also modulate the type and strength of immune responses, with immune cells adaptively activating or suppressing immune reactions based on the degree and type of inflammation to maintain immune balance [1]. Therefore, the close interaction between inflammation and immunity enables them to collaborate in protecting the body from infection, maintaining immune equilibrium, and promoting tissue health. In other words, controlling inflammatory responses is an effective approach to immune regulation [25].

NO plays crucial physiological and immunoregulatory roles within the biological system [26]. NO is a small gas molecule synthesized by NO synthase (NOS) from L-arginine to L-citrulline [27]. NOS comprises endogenous NOS (eNOS and nNOS) and inducible NOS (iNOS). Although moderate NO production assists in pathogen clearance, excessive NO production might induce tissue damage and exacerbate inflammatory reactions [26]. Research findings suggest that under immune stimulation, such as exposure to LPS, the expression and activation of iNOS in RAW 264.7 cells and mice splenocytes increase,

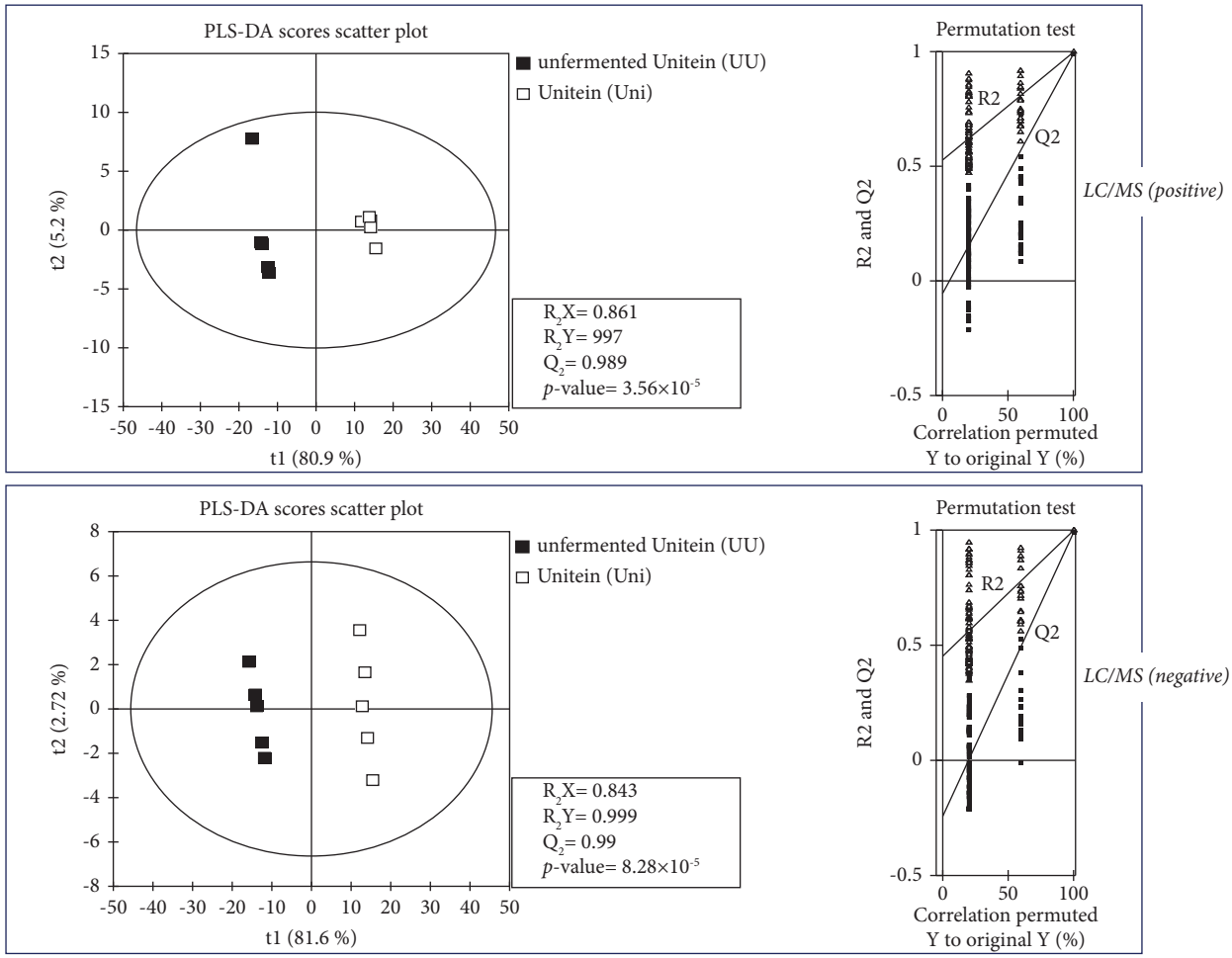


Figure 7: Partial least squares discriminant analysis (PLS-DA) score plots of positive mode and negative mode in unfermented unitein (UU) and unitein (Uni).

resulting in elevated NO production that participates in immune and inflammatory regulation [4, 5]. Our evaluation of NO generation after LPS treatment revealed significantly lower NO production in the sample-treated groups than in the LPS-treated group. Moreover, Uni promoted a more pronounced reduction in NO generation than did UU. Consistently, Unitein treatment similarly attenuated NO production in C57BL/6 mice splenocytes, reflecting its effective control of LPS-induced inflammatory responses.

Cytokines, which play pivotal roles in both inflammation and immune regulation, serve as signaling molecules within the immune system that transmit information as well as coordinate and modulate immune and inflammatory responses [28]. Inflammatory cytokines, such as TNF-α, IL-1β, and IL-6, play crucial roles in inflammatory reactions by recruiting immune cells, enhancing inflammatory cell activity, and promoting the inflammatory process [28]. TNF-α, an essential proinflammatory cytokine in immune cells, induces fever and apoptosis through the production of IL-1β and IL-6, subsequently triggering inflammation. Additionally, TNF-α, which serves as both an activator and a product of NF-κB, positively regulates NF-κB to induce inflammation [29]. At low concentrations, TNF-α regulates cell growth and differentiation, whereas at high concentrations, it can induce apoptosis and functions as a key factor in inflammatory damage, thus TNF-α expression has been closely associated with the occurrence of numerous diseases [30]. IL-1β, a proinflammatory cytokine, increases in response to infection, injury, and immune stimulation, thereby eliciting inflammatory reactions [31]. IL-6, a multifunctional cytokine, plays diverse roles in immune responses, including inflammation regulation, cell proliferation, and differentiation. During infection, trauma, and tissue damage, IL-6 production increases, promoting immune cell activation and inflammatory responses [32]. Based on multiple research findings, it has been shown that LPS has an impact on RAW 264.7 cells and splenocytes by increasing the levels of inflammatory cytokines, including TNF-α, IL-1β and IL-6 [4, 5, 33–35].

Furthermore, several cytokines, such as IL-10, IL-4, interferon-gamma (IFN-γ), and IL-12, play critical roles in immune regulation [36]. IL-10 and IL-4 are considered anti-inflammatory cytokines that can inhibit the production of inflammatory cytokines, regulate immune cell activity, and thus maintain immune balance [28, 37]. IFN-γ, the production of which is induced by immune cell activation

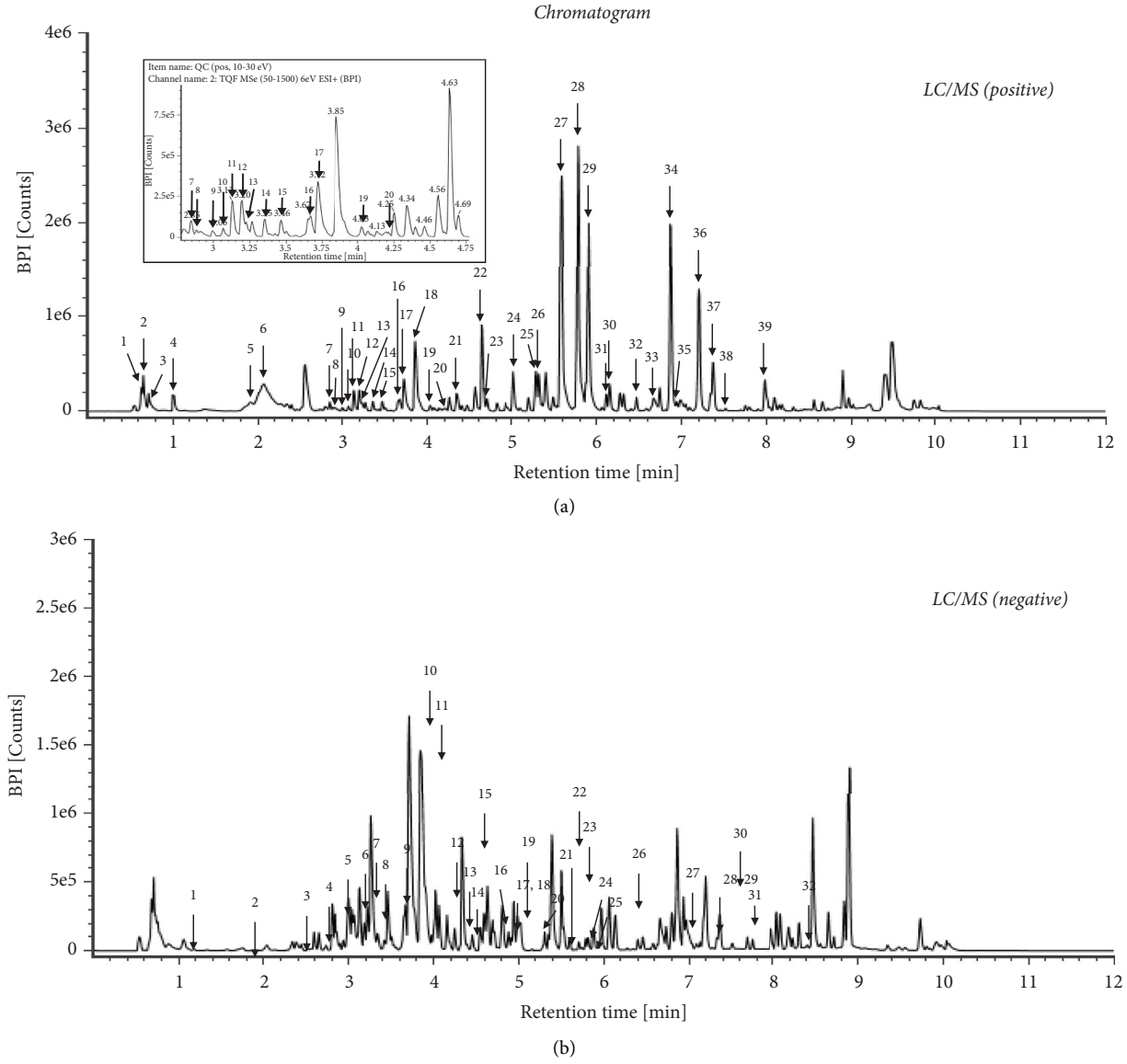


Figure 8: Representative chromatogram of metabolite profiles in positive mode (a) and negative mode (b).

(e.g., T and NK cells), participates in the regulation of immune cell activation and proliferation, as well as antiviral responses in murine splenic cells. Simultaneously, IFN-γ enhances IL-12 production by activating dendritic cells and macrophages [38]. IL-12 promotes Th1 cell differentiation and IFN-γ production. IFN-γ and IL-12 mutually regulate each other in murine splenic cells, forming a positive feedback loop that amplifies immune responses and inflammatory reactions [39]. Studies have found that LPS leads to a reduction in IL-10 levels in RAW 264.7 cells and an increase in IFN-γ and IL-12 levels in splenocytes [35, 40]. The current study found that the administration of Unitein effectively suppressed the LPS-induced increase in TNF-α, IL-1β, IL-6, IFN-γ, and IL-12, in both RAW 264.7 cells and mice splenocytes while also significantly increasing IL-10 and IL-4 levels, thereby restoring immune balance.

The NF-κB signaling pathway is a crucial route for LPS-induced inflammation and has been closely associated with the expression of inflammatory enzymes such as COX-2 and iNOS [41]. NF-κB, a family of transcription factors comprising various subunits, with p65 and p50 being common subunits, plays a pivotal role in inflammation and immune responses [42]. In the non-activated state, NF-κB is sequestered within IκB (inhibitor protein) [43]. However, when immune cells are stimulated through, for instance, inflammatory factors or infection, IκB proteins undergo degradation and promoted the release of NF-κB, which translocates to the nucleus and initiates the transcription of genes involved in inflammation and immune regulation, including inflammatory cytokines (e.g., IL-1β, IL-6) and inflammatory enzymes (COX-2 and iNOS) [44]. COX-2 participates in prostaglandin synthesis and constitutes an

Table 2: Identification of major metabolites contributing the separation among sample groups in positive mode.

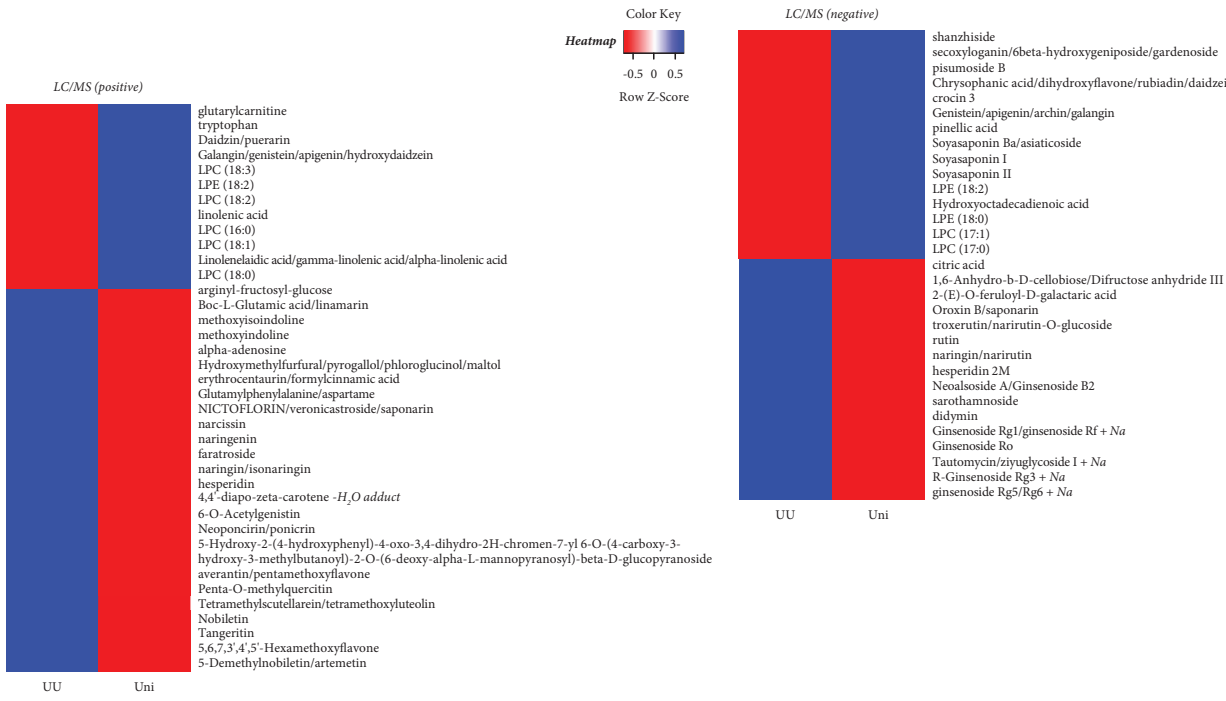
No.	RT (min)	Compound	Exact mass (M + H)	MS fragments	VIP	p value
1	0.64	Arginyl-fructosyl-glucose	499.2238	114, 319, 337, 463	1.09	$1.71E-06$
2	0.68	Boc-L-glutamic acid/linamarin	248.1137	128, 160, 230	0.91	$3.89E-03$
3	0.78	Methoxyisoindoline	150.0923	107, 119, 134, 135	1.09	$9.41E-07$
4	1.03	Methoxyindoline	150.0927	107, 119, 135	1.08	$5.74E-06$
5	1.96	Alpha-adenosine	268.1039	136	1.08	$3.40E-04$
6	2.17	Glutarylcarnitine	276.1475	143, 212, 230	1.08	$3.18E-06$
7	2.85	Tryptophan	205.0987	188	0.84	0.012
8	2.90	Hydroxymethylfurfural/pyrogallol/phloroglucinol/maltol	127.0402	109	1.09	$5.70E-07$
9	3.04	Erythrocentaurin/formylcinnamic acid	177.0575	117, 145, 149	0.85	0.013
10	3.07	Glutamylphenylalanine/aspartame	295.1293	120, 149, 166, 278	1.10	$1.16E-05$
11	3.13	NICTOFLORIN/veronicastroside/saponarin	595.1646	559, 577	1.02	$2.38E-04$
12	3.19	Narcissin	625.1827	433, 595	0.87	$9.89E-03$
13	3.22	Naringenin	273.0732	119, 147, 153	1.08	$5.48E-06$
14	3.35	Daidzin/puerarin	417.1178	255	0.86	$9.51E-03$
15	3.46	Rutin	611.1627	303, 333	0.70	0.065
16	3.66	Faratroside	815.2205	271, 347, 377	1.03	$2.13E-03$
17	3.72	Naringin/isonaringin	581.1899	177, 273, 419, 435	1.04	$8.36E-05$
18	3.85	Hesperidin	611.1992	177, 303, 449	0.81	0.026
19	4.03	4,4′-diapo-zeta-carotene -H_2O *adduct*	423.3610	109, 147, 187, 203	1.08	$1.95E-04$
20	4.21	6-O-Acetylgenistin	475.1218	271	1.10	$1.55E-05$
21	4.34	Neoponcirin/ponicrin	595.2024	287, 419	0.89	0.021
22	4.63	5-Hydroxy-2-(4-hydroxyphenyl)-4-oxo-3,4-dihydro-2H-chromen-7-yl 6-O-(4-carboxy-3-hydroxy-3-methylbutanoyl)-2-O-(6-deoxy-alpha-L-mannopyranosyl)-beta-D-glucopyranoside	725.2277	419	0.94	$2.24E-03$
23	4.69	Galangin/genistein/apigenin/hydroxydaidzein	271.0619	153, 215	1.10	$6.86E-05$
24	5.01	Averantin/pentamethoxyflavone	373.1303	315, 343	1.01	$3.45E-04$
25	5.28	Penta-O-methylquercitin	373.1310	151, 181, 312	1.01	$3.24E-04$
26	5.32	Tetramethylscutellarein/tetramethoxyluteolin	343.1183	313	1.05	$4.20E-05$
27	5.58	Nobiletin	403.1384	241, 355, 373	1.05	$5.11E-05$
28	5.77	3,3′,4′,5,6,7,8-heptamethoxyflavone	433.1495	385, 403	0.37	0.351
29	5.89	Tangeritin	373.1283	325, 343, 358	1.07	$1.00E-05$
30	6.14	5,6,7,3′,4′,5′-hexamethoxyflavone	403.1440	355, 373	0.88	$7.73E-03$
31	6.18	5-Demethylnobiletin/artemetin	389.1246	227, 341, 359	1.03	$1.12E-04$
32	6.46	LPC (18 : 3)	518.3226	104, 184	1.08	$9.93E-05$
33	6.67	LPE (18 : 2)	640.3483	188, 293, 309, 490, 604	1.09	$1.42E-06$
34	6.84	LPC (18 : 2)	520.3402	104, 184	1.06	$1.74E-05$
35	6.95	Linolenic acid	279.2301	95, 109	1.09	$5.37E-05$
36	7.19	LPC (16 : 0)	496.3418	104, 184	1.11	$1.33E-08$
37	7.35	LPC (18 : 1)	522.3560	104, 184	1.08	$5.38E-06$
38	7.50	Linolenelaidic acid/gamma-linolenic acid/alpha-linolenic acid	279.2312	67, 95, 109, 135, 161	1.09	$4.51E-07$
39	7.97	LPC (18 : 0)	524.3719	104, 184	1.09	$3.17E-07$

Table 3: Identification of major metabolites contributing the separation among sample groups in negative mode.

No.	RT (min)	Compound	Exact mass (M + H)	MS fragments	VIP	p value
1	1.13	Citric acid	191.0117	173	0.90	$5.05E-03$
2	1.81	1,6-Anhydro-b-D-cellobiose/difructose anhydride III	323.0922	87, 235	1.09	$8.88E-05$
3	2.61	Shanzhiside	391.1200	85, 315	1.11	$2.71E-06$
4	2.82	Secoxyloganin/6beta-hydroxygeniposide/gardenoside	403.1200	101, 241, 355	1.10	$4.20E-06$
5	2.85	Pisumoside B	691.3222	345, 477	1.10	$2.23E-05$
6	3.02	2-(E)-O-feruloyl-D-galactaric acid	385.0724	147, 191, 209	0.80	$2.06E-02$
7	3.12	Oroxin B/saponarin	593.1518	383, 473, 503, 533	0.99	$5.85E-04$
8	3.22	Troxerutin/narirutin-O-glucoside	741.2315	367, 433	0.93	$2.23E-03$
9	3.46	Rutin	609.1476	59, 300, 493	0.97	$8.90E-04$
10	3.71	Naringin/narirutin	579.1721	271, 459	1.06	$1.03E-05$
11	3.85	Hesperidin	609.1834	286, 301, 549	0.33	$5.38E-01$
12	4.01	Neoalsoside A/ginsenoside B2	991.5646	637, 799, 845	1.04	$5.85E-05$
13	4.20	Sarothamnoside	857.2483	695, 797	1.07	$1.71E-04$
14	4.25	Chrysophanic acid/dihydroxyflavone/rubiadin/daidzein	253.0432	223	1.11	$4.85E-11$
15	4.34	Didymin	593.1880	270, 285, 417	0.91	$3.87E-03$
16	4.60	Crocin 3	651.2700	179, 283, 327, 369	1.08	$3.08E-04$
17	4.70	Genistein/apigenin/archin/galangin	269.0385	133	1.11	$2.06E-06$
18	4.70	Ginsenoside Rg1/ginsenoside Rf + Na	845.4994	475, 637, 783	1.09	$4.22E-07$
19	4.81	Ginsenoside ro	955.5051	161, 569, 731, 793, 851	1.08	$1.67E-06$
20	5.00	Pinellic acid	329.2270	171, 211, 255	1.10	$2.49E-06$
21	5.33	Soyasaponin ba/asiaticoside	957.5186	221, 597	0.90	$1.37E-02$
22	5.40	Soyasaponin I	941.5261	615, 733, 795, 927	0.72	$4.35E-02$
23	5.52	Soyasaponin II	911.5145	205, 441, 457	0.75	$3.07E-02$
24	5.54	Soyasaponin III	795.4611	615	0.61	$1.03E-01$
25	5.61	Tautomycin/ziyuglycoside I	765.4497	281	0.50	$2.30E-01$
26	6.07	R-Ginsenoside Rg3 + Na	829.5041	459, 603, 621	1.03	$2.45E-03$
27	6.66	LPE (18 : 2)	638.3345	152, 279, 476	1.08	$9.96E-07$
28	6.95	Ginsenoside Rg5/Rg6 + Na	811.4947	603, 614	1.10	$2.57E-05$
29	6.95	Hydroxyoctadecadienoic acid	295.2210	71, 195, 233	1.05	$1.56E-03$
30	7.20	LPE (18 : 0)	480.3072	168, 255, 409, 452	1.09	$3.89E-07$
31	7.36	LPC (17 : 1)	506.3236	224, 279, 281	1.04	$7.10E-05$
32	7.96	LPC (17 : 0)	568.3599	508.34 + CH3COO	1.09	$1.26E-07$

integral part of inflammatory reactions and immune regulation, with its expression being significantly upregulated under inflammatory and injury conditions [45]. Inflammation and immune responses promote increased NO production by inducing the expression of iNOS, which is responsible for NO production. Excessive NO production might cause oxidative stress and cellular damage, which exert detrimental effects on the organism [46]. The research results by Wei-Chuan Chen et al. suggest that NF-κB activation is essential for LPS-induced COX-2 expression in RAW264.7 macrophages [47]. In a study on the anti-inflammatory properties of LPS-stimulated macrophages conducted by Shi-yun Du et al., it was found that LPS stimulation led to the expression of COX-2 and iNOS in RAW264.7 cells [34]. In RAW 264.7 cells, the sample-treated group exhibited significantly lower gene expression of the inflammatory enzymes COX-2 and iNOS than did the LPS group, particularly in the case of COX-2. Additionally, Uni promoted a greater decrease in the expression of these enzymes than did UU. These results confirm the enhanced anti-inflammatory effects of Unitein produced through the fermentation process.

NK cells are essential immune cells that play diverse roles within murine splenic cells. They possess the ability to lyse tumor cells, infected cells, and cells infected with viruses [48]. These cells are capable of recognizing and eliminating cancer cells, including metastatic tumor cells. Upon detecting aberrant cell surface markers, NK cells release cytotoxic agents, inducing tumor cell apoptosis [49]. Furthermore, NK cells help maintain immune response equilibrium within the spleen by modulating the function of other immune cells through the secretion of cytokines such as IFN-γ and IL-12 [50]. Concurrently, NK cells regulate the degree and duration of inflammatory responses. In some instances, they can mitigate inflammation by producing anti-inflammatory factors, thereby protecting tissues from excessive inflammatory damage [51]. NK cell activity refers to its ability to kill target cells, and there is a close relationship between NK cell activity and enhanced immune function [52]. High activity of NK cells can increase their cytotoxicity against tumor cells and infected cells, and produce cytokines to regulate immune responses [53]. This helps the body effectively eliminate abnormal cells and pathogens, thereby improving overall immune function. By enhancing NK cell activity, it is possible to increase the ability of immune cells to attack pathogens and promote immune system response [54]. This is of great significance in the prevention and treatment of infectious diseases and tumors. In the current study, we observed that Unitein



(a) (b)

Figure 9: Heatmap of LC/MS positive mode (a) and negative mode (b) in unfermented unitein (UU) and unitein (Uni).

significantly enhanced NK cell activity more significantly than did UU. This could be one of the factors contributing to Unitein's regulation of inflammatory responses.

Soybean (*Glycine max* L.), a protein-rich food with a protein content of over 40%, contains various bioactive substances such as saponins, lecithin, phytic acid, and isoflavones [55]. Notably, isoflavones present in soybean structurally and functionally resemble to the female hormone estrogen, hence being referred to as phytoestrogens [56]. Within the isoflavone category, substances such as daidzein and genistein possess antiobesity, anticancer, and cholesterol-lowering effects [57]. However, these isoflavones are predominantly present as daidzin and genistin, which exist as large molecular weight glycosides, leading to low absorption rates [58]. The conversion of glycosides into their nonglycoside form, which has faster digestion and absorption rates, is achieved through the fermentation process with *Bacillus subtilis*. Fermentation transforms glycoside substances, such as daidzin, into their nonglycoside form, such as daidzein, facilitated by the enhanced activity of β-glucosidase enzyme of the fermenting lactic acid bacteria. This process also applies to substances such as daidzein and genistin, consequently enhancing the bioavailability and functional efficacy of these bioactive compounds [59]. There have been studies showing that fermented soybean paste (douchi) can inhibit the production of NO, IL-1β, and TNF-α in LPS-induced RAW 264.7 cells. It is considered a nutritional food with potential anti-inflammatory and analgesic effects [60]. Red ginseng, which is prepared by drying steamed ginseng, has been known to have better medicinal effects than non-steamed ginseng, with fewer side effects. This phenomenon is attributed to the influence of heat-induced hydrolysis during steaming, causing changes in the types and contents of polyphenols, flavonoids, and ginsenosides in ginseng [61]. In recent times, the "nine steaming and nine drying" method has gained attention for its ability to enhance the active ingredients in red ginseng. This method involves nine cycles of steaming and drying, resulting in "black red ginseng" [62]. The composition and content of the ginsenosides and saponins in black red ginseng differ from those in regular red ginseng. Black red Ginseng, which exhibits greater antioxidant effects than does regular red ginseng has sparked interest given its enhanced physiological activities [17]. Korean red ginseng has been shown to have anti-inflammatory and anti-fatigue effects in patients with non-alcoholic fatty liver disease by reducing the expression of the pro-inflammatory cytokine TNF-α [63]. Chenpi is the dried peel of citrus fruit and is commonly used as a traditional Chinese herb [64]. Citrus contains plant constituents such as flavonoids and carotenoids. The primary flavonoid components of Chenpi include naringin and hesperidin, which impart bitterness and demonstrate various functions such as antibacterial, anticancer, hepatoprotective, and cholesterol-lowering effects [65]. Research has confirmed that fermentation of Chenpi using *Aureobasidium pullulans* converts flavonoids from their glycoside form to their aglycon form, which is more readily absorbed by the body, thereby enhancing their functions such as their antidiabetic effects [19].

Unitein, a product resulting from the mixed fermentation of soybean, red ginseng, and Chenpi, has been studied for its anti-inflammatory and immunity-enhancing effects

through metabolomics analysis. Metabolomics analysis enables the identification of changes in metabolites before and after fermentation, pinpointing specific substances responsible for anti-inflammatory and immunity-enhancing effects [21]. This study employed LC/MS analysis to investigate alterations in Unitein components before and after fermentation. LC/MS operates in two polarity modes, positive and negative, with the primary distinction being that the negative mode involves the deprotonation of analytes, whereas positive mode involves protonation [66].

In the positive ionization mode, the analysis of substances between UU and Uni showed that various metabolites were more abundant in Uni than in UU. Tryptophan is an essential amino acid for human nutrition that serves as a building block for protein synthesis, and it also exhibits antidepressant effects [67]. Daidzin, is a prevalent isoflavone in soybean that has significant effects in preventing osteoporosis and regulating glucose homeostasis [68]. Puerarin plays a role in glucose homeostasis regulation [68]. Genistein, which exists in its nonglycoside form after fermentation, is one of the isoflavones present in soybean, has been proven anticancer activity [69]. Apigenin, a common aglycone natural product in various plants, has been recognized for its anti-inflammatory, antioxidant, and anticancer effects [70, 71]. Among the lysophosphatidylcholines (LPC) substances, LPC (18 : 3/18 : 2/18 : 1/18 : 0/16 : 0) exhibited the most significant variation in content. LPCs are naturally found in various foods and exhibit low selectivity. In particular, they are reduced in patients with colorectal cancer and obesity, rendering them potential biomarkers for these conditions [72]. LPE (18 : 2) possesses anticancer properties [73], whereas linoleic acid is an essential fatty acid found in skin and soybean that is known for its anticancer, anti-inflammatory, antioxidant, and antiobesity effects [74]. Gamma-linolenic acid is used to treat chronic diseases, and alpha-linolenic acid plays a cardioprotective role, and both have anti-inflammatory effects [75]. These components in Uni appear to be related to its enhanced anti-inflammatory effects.

In the negative ionization mode, the analysis of metabolites revealed various substances with potential effects: shanzhiside are iridoid glucoside compounds studied for their robust anti-inflammatory properties and neuroprotective effects [76]. Secoxyloganin exhibits antioxidant and antiallergic characteristics, while gardenoside, known for its anti-inflammatory and anti-obesity effects, has been observed [77]. Chrysophanic acid is a naturally occurring compound with anticancer and anti-inflammatory properties in various plants, animals, and microbes [78]. Dihydroxyflavone, found in root plants, interacts with soil bacterial communities and can be used to treat central nervous system disorders [79]. Rubiadin demonstrates antioxidant and hepatoprotective effects [80]. Daidzein, after soybean fermentation, converts to its nonglycoside form (daidzin), which can alleviate menopausal symptoms and lower blood cholesterol [81]. Crocin 3 possesses antioxidant and neuroprotective properties [82]. Pinellic acid, primarily acting as an adjuvant, exhibits anti-inflammatory properties and is commonly found in root plants. Soyasaponin is known for its cholesterol-lowering, antioxidant, antiviral, liver detoxification, anticancer, and immunity-boosting effects [83]. Asiaticoside promotes collagen synthesis and wound healing. Hydroxyoctadecadienoic acid, an oxidized derivative of linoleic acid, is believed to regulate macrophage differentiation and atherosclerosis [84]. These findings suggest that the substances generated through Unitein fermentation appear to be associated with its enhanced anti-inflammatory effects. Therefore, during the fermentation of Unitein by *Bacillus subtilis*, the active components from soybean, red ginseng, and chenpi undergo fermentation and are transformed into substances with higher activity, thereby increasing the anti-inflammatory activity of Unitein. However, further experimental validation is required to confirm the targeted substances responsible for the anti-inflammatory and immunity-enhancing effects in Unitein.

In summary, this study demonstrated that Uni exhibited lower cytotoxicity to RAW 264.7 cells than did UU. Uni treatment inhibited the production of NO induced by LPS in RAW 264.7 cells and splenocytes. This process also reduced the release of pro-inflammatory cytokines TNF-α, IL-1β, IL-6, and IFN-γ and enzymes COX-2 and iNOS in RAW 264.7 cells while promoting the release of IκB-α and anti-inflammatory cytokines IL-10 and IL-4. Additionally, Uni inhibited the LPS-induced increase in TNF-α, IL-1β, IFN-γ, IL-12, NF-κB p50, NF-κB p65, and iNOS in C57BL/6 mice splenocytes. Furthermore, Uni efficiently enhanced the activity of NK cells. Metabolomics analysis also indicated that Uni contained substances with more active anti-inflammatory and immunity-enhancing properties compared to UU. Overall, these results suggest that compared to UU, Uni had a higher potential for immune enhancement and could be developed as a natural fermented product with anti-inflammatory and immunity-enhancing properties. However, further research and validation are needed for the specific targeted substances.

Data Availability

The research data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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References

[1] L. Chen, H. Deng, H. Cui et al., "Inflammatory responses and inflammation-associated diseases in organs," *Oncotarget*, vol. 9, no. 6, pp. 7204–7218, 2018.

[2] K. B. Megha, X. Joseph, V. Akhil, and P. V. Mohanan, "Cascade of immune mechanism and consequences of inflammatory disorders," *Phytomedicine*, vol. 91, Article ID 153712, 2021.

[3] J. S. Marshall, R. Warrington, W. Watson, and H. L. Kim, "An introduction to immunology and immunopathology," *Allergy, Asthma and Clinical Immunology*, vol. 14, no. S2, p. 49, 2018.

[4] Y. Pan, X. Zhao, S. H. Kim, S. A. Kang, Y. G. Kim, and K. Y. Park, "Anti-inflammatory effects of Beopje curly dock (Rumex crispus L.) in LPS-induced RAW 264.7 cells and its active compounds," *Journal of Food Biochemistry*, vol. 44, no. 7, Article ID e13291, 2020.

[5] Y. Pan, Y.-J. Lee, S.-I. Sin, S.-H. Park, and K.-Y. Park, "Anticancer activity of mineral-supplemented organically cultivated carrot on HT-29 cells and its anti-inflammatory effect on mice splenocytes," *Applied Sciences*, vol. 13, no. 16, p. 9209, 2023.

[6] L. Merly and S. L. Smith, "Murine RAW 264.7 cell line as an immune target: are we missing something?" *Immunopharmacology and Immunotoxicology*, vol. 39, no. 2, pp. 55–58, 2017.

[7] K. Muniandy, S. Gothai, K. M. H. Badran, S. Suresh Kumar, N. M. Esa, and P. Arulselvan, "Suppression of proinflammatory cytokines and mediators in LPS-induced RAW 264.7 macrophages by stem extract of *Alternanthera sessilis* via the inhibition of the NF-κB pathway," *Journal of Immunology Research*, vol. 2018, Article ID 3430684, 12 pages, 2018.

[8] S. M. Lewis, A. Williams, and S. C. Eisenbarth, "Structure and function of the immune system in the spleen," *Science Immunology*, vol. 4, no. 33, Article ID eaau6085, 2019.

[9] C. B. Ahn, Y. S. Cho, and J. Y. Je, "Purification and anti-inflammatory action of tripeptide from salmon pectoral fin byproduct protein hydrolysate," *Food Chemistry*, vol. 168, pp. 151–156, 2015.

[10] M. N. Islam, I. J. Ishita, S. E. Jin et al., "Anti-inflammatory activity of edible brown alga Saccharina japonica and its constituents pheophorbide a and pheophytin a in LPS-stimulated RAW 264.7 macrophage cells," *Food and Chemical Toxicology*, vol. 55, pp. 541–548, 2013.

[11] F. An, J. Wu, Y. Feng et al., "A systematic review on the flavor of soy-based fermented foods: core fermentation microbiome, multisensory flavor substances, key enzymes, and metabolic pathways," *Comprehensive Reviews in Food Science and Food Safety*, vol. 22, no. 4, pp. 2773–2801, 2023.

[12] K.-Y. Park, "Increased health functionality of fermented foods," *The Food Industry and Nutrition*, vol. 17, pp. 1–8, 2012.

[13] J. R. Lee, Y. W. Kim, S. H. Byun, S. C. Kim, and S. J. Park, "Anti-inflammatory effects of the fermentation extracts consisting of soybean, red ginseng and Citrus Unshiu Peel," *The Korea Journal of Herbology*, vol. 30, no. 5, pp. 59–65, 2015.

[14] H. Yoon, *The Effect of Soybean Fermentation Extracts on Inflammatory Reaction and Tumor Necrosis Factor-Alpha in a Mouse Model of Lipopolysaccharide-Induced Cystitis*, Hanyang University, Seoul, South Korea, 2015.

[15] H.-J. Park, A.-J. Kim, Y.-P. Cheon, and M. Lee, "Anti-obesity effects of water and ethanol extracts of black ginseng," *Journal of the Korean Society of Food Science and Nutrition*, vol. 44, no. 3, pp. 314–323, 2015.

[16] H.-J. Kim, J.-Y. Lee, B.-R. You et al., "Antioxidant activities of ethanol extracts from black ginseng prepared by steaming-drying cycles," *Journal of the Korean Society of Food Science and Nutrition*, vol. 40, no. 2, pp. 156–162, 2011.

[17] M. Yeom, J.-Y. Oh, B. Lee, D.-H. Hahm, and H.-J. Park, "Inhibitory effects of black-red ginseng extracts on allergic inflammation in vitro and in vivo," *Korean Journal of Pharmacognosy*, vol. 48, pp. 38–45, 2017.

[18] J.-S. Hyon, S.-M. Kang, M. Senevirathne et al., "Antioxidative activities of dried and fresh citrus peels in jeju," *Korean Journal of Food and Cookery Science*, vol. 26, pp. 88–94, 2010.

[19] C.-D. Park, H.-K. Jung, C.-H. Park et al., "Isolation of citrus peel flavonoid bioconversion microorganism and inhibitory effect on the oxidative damage in pancreatic beta cells," *KSBB Journal*, vol. 27, no. 1, pp. 67–74, 2012.

[20] J. R. Idle and F. J. Gonzalez, "Metabolomics," *Cell Metabolism*, vol. 6, no. 5, pp. 348–351, 2007.

[21] D. S. Wishart, "Metabolomics: applications to food science and nutrition research," *Trends in Food Science and Technology*, vol. 19, no. 9, pp. 482–493, 2008.

[22] Q. Zhang, Y. Shi, L. Ma, X. Yi, and J. Ruan, "Metabolomic analysis using ultra-performance liquid chromatography-quadrupole-time of flight mass spectrometry (UPLC-Q-TOF MS) uncovers the effects of light intensity and temperature under shading treatments on the metabolites in tea," *PLoS One*, vol. 9, no. 11, Article ID e112572, 2014.

[23] H. Kim, O. W. Kim, J. H. Ahn, B. M. Kim, J. Oh, and H. J. Kim, "Metabolomic analysis of germinated Brown rice at different germination stages," *Foods*, vol. 9, no. 8, p. 1130, 2020.

[24] C.-H. Lee and E. Y. Choi, "Macrophages and inflammation," *Journal of Rheumatic Diseases*, vol. 25, no. 1, p. 11, 2018.

[25] J. H. Woo, J. H. Lee, H. Kim, S. J. Park, E. H. Joe, and I. Jou, "Control of inflammatory responses: a new paradigm for the treatment of chronic neuronal diseases," *Experimental Neurobiology*, vol. 24, no. 2, pp. 95–102, 2015.

[26] S. M. Andrabi, N. S. Sharma, A. Karan et al., "Nitric oxide: physiological functions, delivery, and biomedical applications," *Advanced Science*, vol. 10, no. 30, Article ID e2303259, 2023.

[27] U. Forstermann and W. C. Sessa, "Nitric oxide synthases: regulation and function," *European Heart Journal*, vol. 33, no. 7, pp. 829–837, 2012.

[28] J. M. Zhang and J. An, "Cytokines, inflammation, and pain," *International Anesthesiology Clinics*, vol. 45, no. 2, pp. 27–37, 2007.

[29] S. Erkasap, N. Erkasap, B. Bradford et al., "The effect of leptin and resveratrol on JAK/STAT pathways and Sirt-1 gene expression in the renal tissue of ischemia/reperfusion induced rats," *Bratislava Medical Journal*, vol. 118, no. 08, pp. 443–448, 2017.

[30] S. K. Manna, A. Mukhopadhyay, and B. B. Aggarwal, "Resveratrol suppresses TNF-induced activation of nuclear transcription factors NF-kappa B, activator protein-1, and apoptosis: potential role of reactive oxygen intermediates and lipid peroxidation," *The Journal of Immunology*, vol. 164, no. 12, pp. 6509–6519, 2000.

[31] G. Lopez-Castejon and D. Brough, "Understanding the mechanism of IL-1β secretion," *Cytokine and Growth Factor Reviews*, vol. 22, no. 4, pp. 189–195, 2011.

[32] M. Aliyu, F. T. Zohora, A. U. Anka et al., "Interleukin-6 cytokine: an overview of the immune regulation, immune dysregulation, and therapeutic approach," *International Immunopharmacology*, vol. 111, Article ID 109130, 2022.

[33] Y. Pan, Y. Lee, J. H. Chung, K. Kwack, X. Zhao, and K.-Y. Park, "The anti-oxidative capacity of fermented lemon peel and its inhibitory effects on Lipopolysaccharide (LPS)-induced RAW 264.7 cell inflammatory response and cell apoptosis," *Food Science and Technology*, vol. 43, p. 43, 2023.

[34] S. Y. Du, H. F. Huang, X. Q. Li et al., "Anti-inflammatory properties of uvaol on DSS-induced colitis and LPS-stimulated macrophages," *Chinese Medicine*, vol. 15, no. 1, p. 43, 2020.

[35] H. Tanaka, Y. Nishikawa, T. Fukushima et al., "Lipopolysaccharide inhibits hepatic gluconeogenesis in rats: the role of immune cells," *Journal of Diabetes Investigation*, vol. 9, no. 3, pp. 494–504, 2017.

[36] G. Trinchieri, "Cytokines acting on or secreted by macrophages during intracellular infection (IL-10, IL-12, IFN-γ)," *Current Opinion in Immunology*, vol. 9, no. 1, pp. 17–23, 1997.

[37] S. S. Iyer and G. Cheng, "Role of interleukin 10 transcriptional regulation in inflammation and autoimmune disease," *Critical Reviews in Immunology*, vol. 32, no. 1, pp. 23–63, 2012.

[38] D. Jorgovanovic, M. Song, L. Wang, and Y. Zhang, "Roles of IFN-gamma in tumor progression and regression: a review," *Biomarker research*, vol. 8, no. 1, p. 49, 2020.

[39] J. Liu, S. Cao, S. Kim et al., "Interleukin-12: an update on its immunological activities, signaling and regulation of gene expression," *Current Immunology Reviews*, vol. 1, no. 2, pp. 119–137, 2005.

[40] A. B. Hsouna, A. Boye, B. B. Ackacha et al., "Thiamine demonstrates bio-preservative and anti-microbial effects in minced beef meat storage and lipopolysaccharide (LPS)-Stimulated RAW 264.7 macrophages," *Animals*, vol. 12, no. 13, p. 1646, 2022.

[41] K. C. Lee, H. H. Chang, Y. H. Chung, and T. Y. Lee, "Andrographolide acts as an anti-inflammatory agent in LPS-stimulated RAW264.7 macrophages by inhibiting STAT3-mediated suppression of the NF-κB pathway," *Journal of Ethnopharmacology*, vol. 135, no. 3, pp. 678–684, 2011.

[42] M. Mussbacher, M. Salzmann, C. Brostjan et al., "Cell type-specific roles of NF-κB linking inflammation and thrombosis," *Frontiers in Immunology*, vol. 10, p. 85, 2019.

[43] T. Liu, L. Zhang, D. Joo, and S. C. Sun, "NF-κB signaling in inflammation," *Signal Transduction and Targeted Therapy*, vol. 2, no. 1, Article ID 17023, 2017.

[44] H. Yu, L. Lin, Z. Zhang, H. Zhang, and H. Hu, "Targeting NF-κB pathway for the therapy of diseases: mechanism and clinical study," *Signal Transduction and Targeted Therapy*, vol. 5, no. 1, p. 209, 2020.

[45] A. Kulesza, L. Paczek, and A. Burdzinska, "The role of COX-2 and PGE2 in the regulation of immunomodulation and other functions of mesenchymal stromal cells," *Biomedicines*, vol. 11, no. 2, p. 445, 2023.

[46] S. Ibiza and J. M. Serrador, "The role of nitric oxide in the regulation of adaptive immune responses," *Inmunología*, vol. 27, no. 3, pp. 103–117, 2008.

[47] W. C. Chen, C. S. Yen, W. J. Huang, Y. F. Hsu, G. Ou, and M. J. Hsu, "WMJ-S-001, a novel aliphatic hydroxamate derivative, exhibits anti-inflammatory properties via MKP-1 in LPS-stimulated RAW264.7 macrophages," *British Journal of Pharmacology*, vol. 172, no. 7, pp. 1894–1908, 2015.

[48] F. Pappalardo, E. Fichera, N. Paparone et al., "A computational model to predict the immune system activation by citrus-derived vaccine adjuvants," *Bioinformatics*, vol. 32, no. 17, pp. 2672–2680, 2016.

[49] S. Liu, V. Galat, Y. Galat, Y. K. A. Lee, D. Wainwright, and J. Wu, "NK cell-based cancer immunotherapy: from basic biology to clinical development," *Journal of Hematology and Oncology*, vol. 14, no. 1, p. 7, 2021.

[50] E. Alspach, D. M. Lussier, and R. D. Schreiber, "Interferon gamma and its important roles in promoting and inhibiting spontaneous and therapeutic cancer immunity," *Cold Spring Harbor Perspectives in Biology*, vol. 11, no. 3, Article ID a028480, 2019.

[51] P. Sun, Y. Kim, H. Lee et al., "Carrot pomace polysaccharide (CPP) improves influenza vaccine efficacy in immunosuppressed mice via dendritic cell activation," *Nutrients*, vol. 12, no. 9, p. 2740, 2020.

[52] C. Paniz, J. F. Bertinato, M. R. Lucena et al., "A daily dose of 5 mg folic acid for 90 Days is associated with increased serum unmetabolized folic acid and reduced natural killer cell cytotoxicity in healthy Brazilian adults," *The Journal of Nutrition*, vol. 147, no. 9, pp. 1677–1685, 2017.

[53] L. Luo, P. Feng, Q. Yang et al., "Transcription factor TOX maintains the expression of Mst1 in controlling the early mouse NK cell development," *Theranostics*, vol. 13, no. 7, pp. 2072–2087, 2023.

[54] Y. Ma, C. Zhang, B. Zhang, H. Yu, and Q. Yu, "circRNA of AR-suppressed PABPC1 91 bp enhances the cytotoxicity of natural killer cells against hepatocellular carcinoma via upregulating UL16 binding protein 1," *Oncology Letters*, vol. 17, pp. 388–397, 2019.

[55] I. S. Kim, C. H. Kim, and W. S. Yang, "Physiologically active molecules and functional properties of soybeans in human health-A current perspective," *International Journal of Molecular Sciences*, vol. 22, no. 8, p. 4054, 2021.

[56] H. Wang and P. A. Murphy, "Isoflavone composition of American and Japanese soybeans in Iowa: effects of variety, crop year, and location," *Journal of Agricultural and Food Chemistry*, vol. 42, no. 8, pp. 1674–1677, 2002.

[57] A. V. Sirotkin and A. H. Harrath, "Phytoestrogens and their effects," *European Journal of Pharmacology*, vol. 741, pp. 230–236, 2014.

[58] J.-W. Oh, J.-H. Lee, M.-L. Cho et al., "Development and validation of analytical method for pectolinarin and pectolinarigenin in fermented Cirsium setidens nakai by bioconversion," *Journal of the Korean Society of Food Science and Nutrition*, vol. 44, no. 10, pp. 1504–1509, 2015.

[59] I.-B. Kim, S. Shin, B.-L. Lim, G.-S. Seong, and Y.-E. Lee, "Bioconversion of soybean isoflavone by Lactobacillus plantarum and bifidobacterium longum," *Korean Journal of Food and Cookery Science*, vol. 26, pp. 214–219, 2010.

[60] H. M. Yusof, N. M. Ali, S. K. Yeap et al., "Anti-inflammatory, analgesic and acute toxicity effects of fermented soybean," *BMC Complementary and Alternative Medicine*, vol. 19, no. 1, p. 373, 2019.

[61] S. M. Lee, B. S. Bae, H. W. Park et al., "Characterization of Korean red ginseng (Panax ginseng meyer): history, preparation method, and chemical composition," *Journal of Ginseng Research*, vol. 39, no. 4, pp. 384–391, 2015.

[62] J.-H. Lee, G.-N. Shen, E.-K. Kim et al., "Preparation of black ginseng and its antitumor activity," *Journal of Physiology and Pathology in Korean Medicine*, vol. 20, pp. 951–956, 2006.

[63] M. Hong, Y. H. Lee, S. Kim et al., "Anti-inflammatory and antifatigue effect of Korean Red Ginseng in patients with nonalcoholic fatty liver disease," *Journal of Ginseng Research*, vol. 40, no. 3, pp. 203–210, 2016.

[64] X. Yu, S. Sun, Y. Guo et al., "Citri Reticulatae Pericarpium (Chenpi): botany, ethnopharmacology, phytochemistry, and pharmacology of a frequently used traditional Chinese medicine," *Journal of Ethnopharmacology*, vol. 220, pp. 265–282, 2018.

[65] R. K. Saini, A. Ranjit, K. Sharma et al., "Bioactive compounds of citrus fruits: a review of composition and health benefits of carotenoids, flavonoids, limonoids, and terpenes," *Antioxidants*, vol. 11, no. 2, p. 239, 2022.

[66] A. Feldman, D. Mukha, I. I. Maor et al., "Blimp1(+) cells generate functional mouse sebaceous gland organoids in vitro," *Nature Communications*, vol. 10, no. 1, p. 2348, 2019.

[67] K. A. Shaw, J. Turner, and C. Del Mar, "Tryptophan and 5-Hydroxytryptophan for depression," *Cochrane Database of Systematic Reviews*, vol. 2010, no. 1, Article ID CD003198, 2002.

[68] E. Meezan, E. M. Meezan, K. Jones, R. Moore, S. Barnes, and J. K. Prasain, "Contrasting effects of puerarin and daidzin on glucose homeostasis in mice," *Journal of Agricultural and Food Chemistry*, vol. 53, no. 22, pp. 8760–8767, 2005.
[69] R. Dixon, "Genistein," *Phytochemistry*, vol. 60, no. 3, pp. 205–211, 2002.
[70] F. Ali, Rahul, F. Naz, S. Jyoti, and Y. H. Siddique, "Health functionality of apigenin: a review," *International Journal of Food Properties*, vol. 20, no. 6, pp. 1197–1238, 2016.
[71] S. Shukla and S. Gupta, "Apigenin: a promising molecule for cancer prevention," *Pharmaceutical Research*, vol. 27, no. 6, pp. 962–978, 2010.
[72] M. N. Barber, S. Risis, C. Yang et al., "Plasma lysophosphatidylcholine levels are reduced in obesity and type 2 diabetes," *PLoS One*, vol. 7, Article ID e41456, 2012.
[73] A. Nishina, H. Kimura, A. Sekiguchi, R. H. Fukumoto, S. Nakajima, and S. Furukawa, "Lysophosphatidylethanolamine in Grifola frondosa as a neurotrophic activator via activation of MAPK," *Journal of Lipid Research*, vol. 47, no. 7, pp. 1434–1443, 2006.
[74] G. F. Yuan, X. E. Chen, and D. Li, "Conjugated linolenic acids and their bioactivities: a review," *Food and Function*, vol. 5, no. 7, pp. 1360–1368, 2014.
[75] N. Erdinest, O. Shmueli, Y. Grossman, H. Ovadia, and A. Solomon, "Anti-inflammatory effects of alpha linolenic acid on human corneal epithelial cells," *Investigative Ophthalmology and Visual Science*, vol. 53, no. 8, pp. 4396–4406, 2012.
[76] W. Jiang, S. Zhang, F. Fu, H. Zhu, and J. Hou, "Inhibition of nuclear factor-κB by 6-O-acetyl shanzhiside methyl ester protects brain against injury in a rat model of ischemia and reperfusion," *Journal of Neuroinflammation*, vol. 7, no. 1, p. 55, 2010.
[77] H. Liang, L. Zhang, H. Wang et al., "Inhibitory effect of gardenoside on free fatty acid-induced steatosis in HepG2 hepatocytes," *International Journal of Molecular Sciences*, vol. 16, no. 11, pp. 27749–27756, 2015.
[78] L. Xie, H. Tang, J. Song, J. Long, L. Zhang, and X. Li, "Chrysophanol: a review of its pharmacology, toxicity and pharmacokinetics," *Journal of Pharmacy and Pharmacology*, vol. 71, no. 10, pp. 1475–1487, 2019.
[79] Z. Zhang, X. Liu, J. P. Schroeder et al., "7,8-dihydroxyflavone prevents synaptic loss and memory deficits in a mouse model of Alzheimer's disease," *Neuropsychopharmacology*, vol. 39, no. 3, pp. 638–650, 2014.
[80] G. M. Rao, C. V. Rao, P. Pushpangadan, and A. Shirwaikar, "Hepatoprotective effects of rubiadin, a major constituent of Rubia cordifolia Linn," *Journal of Ethnopharmacology*, vol. 103, no. 3, pp. 484–490, 2006.
[81] Y. Wang, M. Yang, J. Qin, and W. Wa, "Interactions between puerarin/daidzein and micellar casein," *Journal of Food Biochemistry*, vol. 46, no. 2, Article ID e14048, 2022.
[82] M. Scuto, S. Modafferi, F. Rampulla et al., "Redox modulation of stress resilience by Crocus sativus L. for potential neuroprotective and anti-neuroinflammatory applications in brain disorders: from molecular basis to therapy," *Mechanism of Ageing and Development*, vol. 205, Article ID 111686, 2022.
[83] S.-L. Kim, J.-E. Lee, Y.-H. Kim et al., "Isolation of isoflavones and soyasaponins from the germ of soybean," *Korean Journal of Crop Science*, vol. 58, no. 2, pp. 149–160, 2013.
[84] V. Vangaveti, B. T. Baune, and R. L. Kennedy, "Review: hydroxyoctadecadienoic acids: novel regulators of macrophage differentiation and atherogenesis," *Therapeutic Advances in Endocrinology and Metabolism*, vol. 1, no. 2, pp. 51–60, 2010.