

Article

Fermentation with Lactic Acid Bacteria Enhances the Anti-Inflammatory Activity of Ginseng Sprout Extract in RAW 264.7 Macrophages

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Abstract

Perennial ginseng (*Panax ginseng*) has long been valued for its medicinal properties. However, ginseng sprouts are gaining prominence as a versatile food source due to the high levels of bioactive compounds in their leaves and stems. To further enhance their functional value, this study investigated the effects of fermentation using lactic acid bacteria, specifically *Lactobacillus* and *Enterococcus* strains, on the antioxidant and anti-inflammatory potential of ginseng sprout extract (GSE). Chemical analyses revealed that fermentation significantly increased total phenolic content (TPC) and ginsenoside Rb₁ levels, which were associated with enhanced radical-scavenging activity and superoxide dismutase (SOD)-like activity. In lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages, fermented GSE (FGSE) exhibited significantly greater anti-inflammatory effects than non-fermented GSE. This enhancement was evidenced by marked downregulation of pro-inflammatory mediators, including nitric oxide (NO) and prostaglandin E₂ (PGE₂), along with their corresponding enzymes, inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2). Notably, the anti-inflammatory profile of FGSE was distinguished by its ability to suppress specific cytokines that were not significantly affected by GSE. Although both GSE and FGSE attenuated interleukin-1β (IL-1β) and interleukin-6 (IL-6), only FGSE achieved statistically significant inhibition of tumor necrosis factor-α (TNF-α) and monocyte chemoattractant protein-1 (MCP-1). These findings indicate that fermentation is a critical process for surpassing the efficacy threshold of GSE against key inflammatory signals. Overall, the enrichment of bioactive metabolites during fermentation suggests that FGSE can serve as a potent functional ingredient for modulating inflammatory responses, with considerable potential for the development of advanced functional foods.

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1. Introduction

Inflammatory immune responses are essential for protecting the host against injury, infection, or toxin exposure. However, chronic inflammation markedly increases the risk of developing various pathological conditions, including cancer and cardiovascular diseases [1,2]. Macrophages play a central role in these responses by mediating cytokine production, antigen presentation, and immune regulation [3,4]. Upon activation, macrophages produce pro-inflammatory cytokines, such as tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), and interleukin-6 (IL-6), and inflammatory mediators including nitric oxide (NO) and prostaglandin E₂ (PGE₂) [4,5]. Consequently, modulation of these mediators is considered an important strategy for controlling chronic inflammatory conditions. Although conventional anti-inflammatory drugs, such as aspirin and ibuprofen, are widely used to suppress excessive inflammation [6,7], their long-term use is frequently associated with adverse effects, including gastrointestinal damage and renal complications [8,9]. Therefore, increasing attention has been directed toward the identification of natural products with anti-inflammatory potential and minimal side effects [10].

Ginseng (*Panax ginseng*) is a medicinal plant with a long history of use in East Asian traditional medicine. Conventional ginseng cultivation typically requires approximately six years and often involves intensive pest and disease management, which limits its utilization primarily to the root [11,12]. However, recent advances in smart farming technologies have enabled the production of ginseng sprouts in controlled environments, such as plant factories [13]. Unlike perennial ginseng, ginseng sprouts are cultivated as fresh functional vegetables and can be harvested within a few weeks from one-year-old seedlings without the use of pesticides [13,14]. Recent studies have reported that ginseng sprouts exhibit notable antioxidant and anti-melanogenic activities [14–16]. However, their potential application as a substrate for functional bioprocessing, including fermentation, remains insufficiently explored.

Fermentation is an effective bioprocessing approach used to modulate and enhance the bioactive components of natural products. Microbial biotransformation is widely employed to improve the bioavailability of ginseng-derived compounds [17,18] (e.g., by facilitating digestion and absorption [19]) and to support diverse pharmacological activities, including antidiabetic and antioxidant effects [20,21]. In particular, lactic acid bacteria are commonly used in fermentation due to their reported health-promoting properties, such as regulation of intestinal metabolism and potential benefits related to aging and cholesterol metabolism [11,22,23].

To expand the utility and enhance the functional profile of ginseng sprouts, it is essential to investigate the effects of lactic acid bacteria fermentation on their bioactive composition and physiological properties. In a previous study [13], we characterized the fermentation profile of ginseng sprout extract (GSE) using *Lactobacillus* and *Enterococcus* species under defined conditions. The resulting fermented ginseng sprout extract (FGSE) exhibited reduced pH and °Brix values, along with increased titratable acidity, compared with the unfermented GSE. Notably, lactic acid concentrations were substantially higher in FGSE (2.20–13.58 mg/100 g) than in GSE (0.23 mg/100 g), indicating successful lactic acid fermentation under these conditions. Based on this prior process characterization, the present study applied the same fermentation protocol to prepare FGSE and subsequently examined its functional properties.

Therefore, the present study aimed to comprehensively evaluate changes in the functional properties of ginseng sprouts following fermentation with two lactic acid bacteria strains, *Lactobacillus* and *Enterococcus*. Total phenolic content (TPC) of GSE and FGSE was determined, and their antioxidant activities were evaluated using DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging and superoxide dismutase (SOD)-like activity assays to characterize their initial bioactivity profiles. To further investigate fermentation-associated chemical modifications, changes in selected ginsenoside profiles were quantified using high-performance liquid chromatography (HPLC). Additionally, the modulatory effects of GSE and FGSE on lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages were evaluated by assessing cell viability and the production of inflammatory mediators, including NO and PGE₂. Finally, given its enhanced bioactivity following fermentation, potential molecular mechanisms were examined primarily in FGSE by analyzing the mRNA and protein expression levels of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), and pro-inflammatory cytokines, to assess the anti-inflammatory potential of FGSE under *in vitro* conditions.

2. Materials and Methods

2.1. Preparation of GSE and FGSE

Dried whole ginseng sprout powder (Gwangyangegameyeon Agricultural Co., Gwangyang, Republic of Korea) was used in this study. To prepare the unfermented extract, 100 g of the powder was subjected to reflux extraction with 1 L of distilled water at 80 °C for 2 h. The resulting extract was concentrated using a rotary evaporator and subsequently lyophilized to obtain the GSE products.

Two bacterial strains, *Lactobacillus bulgaricus* (KCTC 3635) and *Enterococcus faecalis* (KCTC 3206), were obtained from the Korean Collection for Type Culture (Daejeon, Republic of Korea). Single colonies were initially isolated from each strain, and glycerol stocks were prepared during the protocol development phase. To minimize batch-to-batch biological variability, all fermentation processes in the present study were performed according to the previously established protocol [13] using cultures revived from the same single-colony-derived frozen stock. The strains were pre-activated by cultivation at 37 °C for 12 h in the appropriate media: De Man, Rogosa, and Sharpe (MRS) broth (Difco™ Lactobacilli MRS, Difco, Detroit, MI, USA) for *L. bulgaricus* and Brain Heart Infusion (BHI) broth (Bacto™ BHI, Difco, Detroit, MI, USA) for *E. faecalis*. The resulting cultures were used as seed cultures (inocula). The extract was inoculated with 1% (*v/v*) of the corresponding seed culture and incubated at 37 °C for 24 h in a shaking incubator. After incubation, the fermented mixtures were collected and centrifuged at 15,000 × *g* and 4 °C for 15 min to remove bacterial biomass. The resulting supernatants were filtered through a 0.22 µm syringe filter (Millipore, Billerica, MA, USA) to eliminate any residual microbial cells. The clarified extracts were then lyophilized to obtain the final fermented GSE products (FGSE), hereafter referred to as LB-FGSE for GSE fermented with *L. bulgaricus* and EF-FGSE for GSE fermented with *E. faecalis*.

2.2. Total Polyphenol Content (TPC) Assay

TPCs of GSE and FGSE were determined using the Folin–Denis assay with a UV–VIS spectrophotometer (Libra s22, Biochrom, Cambridge, UK) [24]. Polyphenols were first extracted from the dried samples by refluxing with 70% ethanol. A 1 mL aliquot of the extract was mixed with 2 mL of Folin–Ciocalteu reagent and 5 mL of 10% sodium carbonate (Na₂CO₃) solution. After incubation in the dark at room temperature for 1 h, the absorbance was measured at 660 nm. TPC values were calculated using a standard calibration

curve and expressed as milligrams of tannic acid equivalents per 100 g of dry sample (mg TAE/100 g).

2.3. DPPH Radical Scavenging Activity Assay

Antioxidant capacities of GSE and FGSE were evaluated by measuring their DPPH radical scavenging activity using a modified Blois method [25]. Briefly, 100 μ L of each sample was mixed with 150 μ L of a 0.3 mM DPPH solution prepared in ethanol. The reaction mixture was vortexed thoroughly and incubated in the dark at room temperature for 30 min to allow radical stabilization. Absorbance was then measured at 530 nm using a UV–VIS spectrophotometer (Biochrom, Libra s22, Cambridge, UK). Radical scavenging activity was calculated as a percentage relative to the control, in which ethanol was used in place of the sample:

$$\text{DPPH radical scavenging activity (\%)} = \left(1 - \frac{A_{\text{sample}}}{A_{\text{control}}}\right) \times 100 \quad (1)$$

where A_{sample} represents the absorbance of the reaction mixture containing the sample, and A_{control} denotes the absorbance of the control solution containing ethanol instead of the sample.

2.4. Superoxide Dismutase (SOD) Assay

SOD-like activities of GSE and FGSE were determined by measuring the inhibition of pyrogallol autoxidation according to the method of Marklund and Marklund, with minor modifications [26]. Briefly, 0.2 mL of each sample was mixed with 2.6 mL of Tris–HCl buffer (50 mM Tris and 10 mM EDTA, pH 8.5) and 0.2 mL of 7.2 mM pyrogallol dissolved in 10 mM HCl. The reaction mixture was incubated at 25 °C for 10 min, and the reaction was terminated by adding 0.1 mL of 1 M HCl. The absorbance of oxidized pyrogallol was measured at 420 nm using a spectrophotometer (Biochrom, Libra s22, Cambridge, UK). The increase in absorbance was linear over the incubation period. SOD-like activity was calculated as the percentage inhibition of pyrogallol autoxidation using the following equation:

$$\text{SOD – like activity (inhibition rate, \%)} = \left(1 - \frac{S_{\text{sample}} - S_{\text{blank}}}{S_{\text{control}}}\right) \times 100 \quad (2)$$

where S_{control} is the absorbance of the control (without sample), S_{sample} is the absorbance of the reaction mixture containing the sample, and S_{blank} is the absorbance of the sample without pyrogallol.

2.5. HPLC Analysis

Quantitative determination of ginsenoside Rb₁ in GSE and FGSE was performed using an HPLC system (Shimadzu LC-20AD, Shimadzu Corp., Kyoto, Japan) equipped with a diode array detector. Chromatographic separation was achieved using an Agilent Eclipse Plus C18 column (4.6 mm $\times$ 250 mm, 5 μ m; Agilent Technologies, Santa Clara, CA, USA) maintained at 35 °C. The mobile phase consisted of water (solvent A) and acetonitrile (solvent B) at a flow rate of 1.0 mL/min. The gradient elution program, based on the Korean Food Code, was as follows: 0–5 min, 20% B; 5–20 min, 20–23% B; 20–25 min, 23–30% B; 25–45 min, 30–40% B; and 45–55 min, 40–50% B. The column was then re-equilibrated under the following conditions: 55–70 min, 50–20% B; and 70–75 min, 20% B.

Prior to analysis, samples were extracted with 70% methanol to obtain a final concentration of 10 mg/mL. The extracts were centrifuged at 9500 $\times$ g at 4 °C for 5 min to remove insoluble materials. The supernatants were collected and filtered through a 0.45 μ m syringe filter to prevent column blockage during HPLC injection. Standard stock solutions

of ginsenosides Rb₁ and Rg₃ (Sigma-Aldrich, St. Louis, MO, USA) were prepared using the same solvent. Calibration curves were generated over concentration ranges of 50–1000 µg/mL for ginsenoside Rb₁ and 10–500 µg/mL for ginsenoside Rg₃, showing good linearity with correlation coefficients (R²) greater than 0.99. The injection volume was 20 µL, and detection was performed at 203 nm.

2.6. Cell Culture and Cell Viability Assay

The RAW 264.7 murine macrophage cell line was obtained from the Korea Cell Line Bank (Seoul, Republic of Korea) and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum and 100 U/mL penicillin–streptomycin. Cells were maintained at 37 °C in a humidified incubator with 5% CO₂.

Cell viability was evaluated using a WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium, monosodium salt] assay kit (Biomax, Seoul, Republic of Korea) to assess the cytotoxic effects of GSE and FGSE. Briefly, RAW 264.7 cells were seeded into 96-well plates at a density of 2×10^4 cells/well and incubated for 24 h to allow cell attachment. To maintain consistency with the anti-inflammatory assay conditions, cells were pretreated with various concentrations of GSE or FGSE (0, 5, 10, 20, 30, or 40 µg/mL) for 4 h, followed by an additional 18 h incubation. Subsequently, 10 µL of WST-8 reagent was added to each well containing 100 µL of culture medium, and the cells were incubated for 1 h at 37 °C. Absorbance was measured at 450 nm using a spectrophotometer (TECAN, Infinite® F50, Grödig, Austria). Cell viability was expressed as a percentage relative to the untreated control group.

2.7. Measurement of Nitric Oxide (NO) Production

To quantify NO production, RAW 264.7 cells were seeded in a 96-well plate at a density of 3×10^4 cells/well and incubated for 24 h to allow cell attachment. To assess inhibitory effects, cells were pretreated with various concentrations of GSE or FGSE (0, 5, 10, 20, 30, and 40 µg/mL) for 4 h. Inflammation was then induced by stimulating the cells with 0.1 µg/mL LPS (Sigma-Aldrich, St. Louis, MO, USA) for an additional 18 h at 37 °C. NO production was indirectly quantified by measuring nitrite accumulation in the culture supernatant using the Griess assay (Sigma-Aldrich, St. Louis, MO, USA). Briefly, 100 µL of culture supernatant was mixed with an equal volume (100 µL) of Griess reagent and incubated for 15 min at room temperature in the dark. A standard curve was generated using known concentrations of sodium nitrite (NaNO₂). The absorbance of the resulting chromophore was measured at 540 nm using a microplate reader (BioTek Instruments, Synergy HTX multimode, Winooski, VT, USA).

2.8. Measurement of Prostaglandin E₂ (PGE₂) Production

PGE₂ levels were determined in RAW 264.7 cells cultured and treated under the same experimental conditions as described for the NO assay (Section 2.7). Following treatment, culture supernatants were collected and centrifuged at 160× g for 5 min to remove cellular debris. The concentration of PGE₂ in the supernatants was quantified using a PGE₂ ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. After the addition of the substrate solution and subsequent stop solution, absorbance was measured at 450 nm using a spectrophotometer (TECAN, Infinite® F50, Grödig, Austria). Final PGE₂ concentrations were calculated from a standard curve generated using known concentrations of the PGE₂ standard.

2.9. Quantitative Real-Time PCR (RT-qPCR) Analysis

To assess transcriptional changes in inflammatory mediators, mRNA expression levels of iNOS, COX-2, and pro-inflammatory cytokines were quantified by RT-qPCR. RAW 264.7 cells were seeded in 6-well plates (6×10^5 cells/well) for enzyme analysis and in 24-well plates (1×10^5 cells/well) for cytokine analysis. After 24 h of incubation to allow cell attachment, cells were pretreated with various concentrations of GSE or FGSE (0, 5, 10, 20, 30, and 40 $\mu\text{g/mL}$) for 4 h, followed by stimulation with 0.1 $\mu\text{g/mL}$ LPS for an additional 18 h. For cytokine modulation studies, a fixed non-cytotoxic concentration of 40 $\mu\text{g/mL}$ was used.

Total RNA was extracted using TRIzol reagent (Thermo Scientific, Waltham, MA, USA). RNA concentration and purity were assessed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). For complementary DNA (cDNA) synthesis, 1 μg of total RNA was reverse-transcribed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions.

RT-qPCR was performed using a real-time PCR detection system (Bio-Rad, CFX96™, Hercules, CA, USA). Each 10 μL reaction mixture contained Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and gene-specific primers (Cosmo Genetech, Seoul, Republic of Korea), as listed in Table 1. Relative gene expression levels were calculated using the comparative Ct method ($2^{-\Delta\Delta\text{Ct}}$) [27]. All target genes were normalized to the endogenous reference gene GAPDH. The results are presented as fold changes relative to the normal control group, which was set to 1.0.

Table 1. Primers used in quantitative real-time polymerase chain reaction.

Gene	Primer	Sequence (5'-3')
TNF- α	Forward	TGGGACAGTGACCTGGACTGT
	Reverse	TTCGAAAGCCCATTTGAGT
IL-1 β	Forward	AAGGGCTGCTTCCAAACCTTTGAC
	Reverse	ATACTGCCTGCCTGAAGCTCTTGT
IL-6	Forward	ATCCAGTTGCCTTCTTGGGACTGA
	Reverse	TAAGCCTCCGACTTGTGAAGTGGT
MCP-1	Forward	TGATCCCAATGAGTAGGCTGG
	Reverse	ATGTCTGGACCCATTCCTTCT
iNOS	Forward	ACGAGACGGATAGGCAGAGAT
	Reverse	AGTAGTAGCGGGGCTTCAAGA
COX-2	Forward	TCCCTAAAGGAAAAGTGGGACC
	Reverse	GAGCGCATTAACCTCAGGACC
GAPDH	Forward	CATGGCCTTCCGTGTTCTTA
	Reverse	GCGGCACGTCAGATCCA

TNF- α : tumor necrosis factor-alpha; IL-1 β : interleukin-1beta; IL-6: interleukin-6; MCP-1: monocyte chemoattractant protein-1; iNOS: inducible nitric oxide synthase; COX-2: cyclooxygenase-2; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

2.10. Western Blot Analysis

Protein expression levels of iNOS and COX-2 were evaluated by Western blot analysis. Cells were lysed using ice-cold radioimmunoprecipitation assay buffer (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with a protease inhibitor cocktail (Cell Signaling Technology, Danvers, MA, USA). Lysates were centrifuged, and protein concentrations in the supernatants were determined using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA).

Protein samples (40 µg for iNOS and 5 µg for COX-2) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (0.45 µm pore size; Millipore, Bedford, MA, USA). Membranes were blocked with 5% skim milk in Tris-buffered saline containing 0.1% Tween 20 for 1 h at room temperature to reduce non-specific binding. The membranes were then incubated overnight at 4 °C with primary antibodies against iNOS (1:2500; BD Biosciences, Wokingham, UK) and COX-2 (1:10,000; Cayman Chemical, Ann Arbor, MI, USA). After washing, membranes were incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (Enzo Life Sciences, Farmingdale, NY, USA) for 1 h at room temperature. Immunoreactive bands were detected using West-Q Pico ECL solution (GenDEPOT, Katy, TX, USA), and band intensities were quantified using ImageJ (version 1.53e, National Institutes of Health, Bethesda, MD, USA). Target protein expression levels were normalized to β-actin (Abcam, Cambridge, UK) to ensure equal protein loading.

2.11. Statistical Analysis

All data are presented as the mean ± standard error of the mean (SEM). Antioxidant assays and HPLC analyses were performed in triplicate across three independent experiments. Cell-based assays were conducted in five independent experiments, each performed in triplicate.

Statistical differences among groups were analyzed using two approaches based on the experimental design. For experiments involving two independent variables (e.g., fermentation condition and treatment concentration), a two-way mixed-effects model was applied. For single-factor experiments (e.g., comparison of fermentation conditions), the Brown–Forsythe one-way analysis of variance (ANOVA) was used. These robust statistical methods were selected to account for potential heteroscedasticity and inter-sample variability, thereby providing more conservative and reliable *p*-values. To maintain statistical consistency, the same analytical model was applied across all datasets within each figure. Post hoc analyses were conducted as follows: for the two-way model, Dunnett’s multiple comparisons test was used to compare each group with its corresponding control (untreated or LPS-only treated); for the one-way model, Dunnett’s T3 test was used for comparisons with the normal control and for pairwise comparisons among GSE, LB-FGSE, and EF-FGSE. Statistical significance was defined as *p* < 0.05. All analyses were performed using GraphPad Prism (version 10.3, GraphPad Software, Boston, MA, USA).

3. Results and Discussion

Ginseng is a widely recognized medicinal plant with diverse pharmacological properties, including antioxidant and anti-inflammatory activities [11,12,28]. Recently, microbial fermentation has gained increasing attention as an effective strategy to enhance the functional value of natural products by promoting the bioconversion of complex glycosides into more readily absorbable low-molecular-weight metabolites [15,21,29–32]. In the present study, we systematically investigated how fermentation with *Lactobacillus* and *Enterococcus* modulates the biological potential of ginseng sprouts. To this end, we first examined changes in the overall phytochemical profile using TPC measurements and standard antioxidant assays. In addition, HPLC was employed to quantify changes in the representative protopanaxadiol-type ginsenoside Rb₁ to assess fermentation-associated chemical variations. Finally, the inhibitory effects of these extracts on pro-inflammatory mediators, including nitric oxide (NO), prostaglandin E₂ (PGE₂), inflammatory enzymes (iNOS and COX-2), and pro-inflammatory cytokines and chemokines (TNF-α, IL-6, IL-1β, and MCP-1), were evaluated in RAW 264.7 macrophages. This sequential approach was designed to provide a comprehensive understanding of the relationship between fermentation-induced alterations in chemical composition and potential physiological benefits.

3.1. Enhanced Antioxidant Capacity of FGSE

Consistent with previous reports [31], fermentation of ginseng sprouts with *Lactobacillus* and *Enterococcus* resulted in notable changes in phytochemical-related characteristics. As shown in Figure 1a, the TPC was higher in FGSEs than in GSE, with EF-FGSE exhibiting the highest value and being significantly higher than both GSE and LB-FGSE. This increase in phenolic content was accompanied by enhanced antioxidant activities, as evidenced by significantly increased DPPH radical scavenging and SOD-like activities in the FGSE groups compared with the non-fermented GSE (Figure 1b,c). Notably, EF-FGSE showed greater radical scavenging and SOD-like activities than both GSE and LB-FGSE, suggesting that differences in microbial strains may contribute to variations in the functional properties of the fermented extracts.

Microbial fermentation by lactic acid bacteria can modify the structural characteristics of polyphenols through enzymatic reactions, particularly via glycosidases (e.g., β -glucosidase) or decarboxylation-related processes [33,34]. Because the antioxidant efficacy of phenolic compounds is highly dependent on their molecular structure and the availability of free hydroxyl groups [35], the improvements observed in FGSE are likely associated with the bioconversion of glycosylated phenolics into aglycones or the generation of lower-molecular-weight phenolic metabolites during fermentation. These structural modifications enhance hydrogen-donating capacity, thereby improving overall radical-scavenging efficiency. Moreover, the increased SOD-like activity suggests that fermentation promotes the formation or release of bioactive compounds capable of neutralizing superoxide radicals, further contributing to the integrated antioxidant defense system.

Although these cell-free assays effectively demonstrate the chemical radical-scavenging potential of FGSE, they represent a preliminary biochemical evaluation. In biological systems, intracellular reactive oxygen species (ROS) function as key secondary messengers that activate inflammatory signaling pathways, including nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) and mitogen-activated protein kinase (MAPK) pathways [36]. Therefore, the enhanced antioxidant capacity of FGSE, as shown by our TPC and DPPH results, suggests its potential to mitigate excessive oxidative stress within the cellular environment. This effect may, in turn, contribute to the attenuation of downstream inflammatory cascades, providing a plausible explanation for the observed anti-inflammatory effects. Nevertheless, future studies incorporating more comprehensive cell-based oxidative stress assessments, such as intracellular ROS quantification, lipid peroxidation analysis, and evaluation of antioxidant response markers, will be necessary to more clearly define the biological relevance of the antioxidant activity and its mechanistic linkage to the anti-inflammatory properties of FGSE within complex cellular environments.

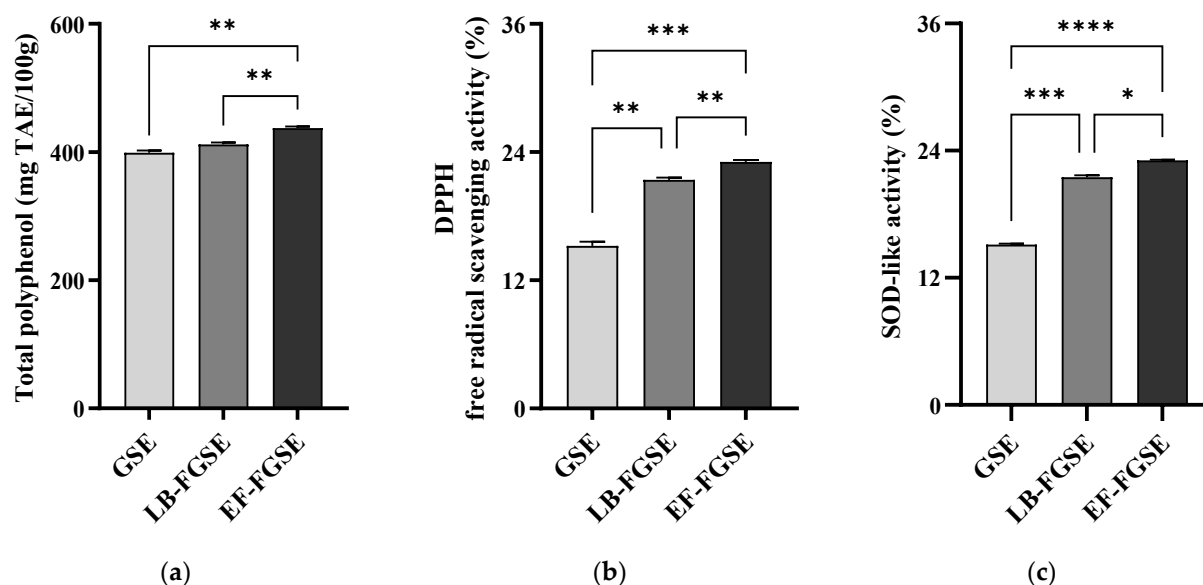


Figure 1. Antioxidant capacity of ginseng sprout extract (GSE) and fermented GSE (FGSE): (a) total polyphenol content (TPC), (b) DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity, and (c) superoxide dismutase (SOD)-like activity of GSE, FGSE fermented with *Lactobacillus bulgaricus* (LB-FGSE), and FGSE fermented with *Enterococcus faecalis* (EF-FGSE). Data represent the mean $\pm$ SEM of three independent experiments. The group means were compared using the Brown–Forsythe ANOVA followed by Dunnett’s T3 multiple comparisons test. Asterisks indicate significant differences: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

3.2. Fermentation-Induced Shifts in Ginsenoside Rb₁ and Chromatographic Fingerprints

The pharmacological effects of ginseng are largely attributed to its diverse secondary metabolites, with ginsenosides commonly used as quality-control markers [12,14]. Among these, protopanaxadiol (PPD)-type ginsenosides Rb₁ (major) and Rg₃ (minor), which are known for their anti-inflammatory and immunomodulatory activities [28,37], were quantified by HPLC to compare chromatographic fingerprints before and after fermentation (Figure 2).

Under the current chromatographic conditions, ginsenoside Rb₁ eluted at approximately 37.5 min and was detected in all the samples. Its content increased after fermentation (LB-FGSE: 3.48 ± 0.40 mg/g; EF-FGSE: 3.21 ± 0.34 mg/g) compared with unfermented GSE (2.52 ± 0.18 mg/g). This increase was statistically significant for LB-FGSE ($p < 0.05$), whereas EF-FGSE showed a non-significant upward trend ($p = 0.086$). The observed increase may reflect improved recovery of Rb₁ during microbial processing and/or broader shifts in saponin composition rather than changes in Rb₁ alone [38]. However, because other major ginsenosides and potential conversion products were not quantitatively tracked, these data cannot distinguish between improved recovery and true biotransformation. Meanwhile, ginsenoside Rg₃ was below the limit of quantification in all the samples, suggesting that under the current fermentation conditions, the conversion pathway from Rb₁ to minor saponins such as Rg₃ was not predominant.

Importantly, the chromatogram suggested broader compositional changes beyond Rb₁. Several peaks changed in the 25–31 min and 40–42 min regions, with a prominent peak at ~27.5 min appearing specifically in EF-FGSE. These fingerprint-level differences indicate that *Enterococcus* and *Lactobacillus* may drive distinct transformations, potentially affecting ginsenosides and other glycosylated constituents. Microbial enzymes, such as β -glucosidases, may hydrolyze glycosides, generating fermentation-derived metabolites

with altered polarity and bioactivity, including phenolic derivatives and minor saponin-related compounds [34].

Given that ginseng sprouts contain substantial phenolics and saponins in their aerial parts (leaves and stems), the observed fermentation patterns may differ from those reported for root-derived ginseng [14,39]. While Rb₁ served as a representative marker in this study, the observed bioactivities cannot be attributed to Rb₁ alone, and the identities of the altered peaks remain unresolved. Thus, the present data support fermentation-associated changes at the level of chromatographic fingerprints and total phenolics (Figure 1a). A more comprehensive analysis of ginsenoside and metabolite profiles (e.g., Rc, Rd, and other major/minor ginsenosides, and potential conversion products such as compound K) will be necessary to enable a more detailed mechanistic interpretation [32,38]. Future targeted and/or untargeted LC–MS/MS profiling, including quantitative tracking of precursor ginsenosides and putative conversion products, will be required to identify the metabolites responsible and to elucidate the underlying biotransformation pathways.

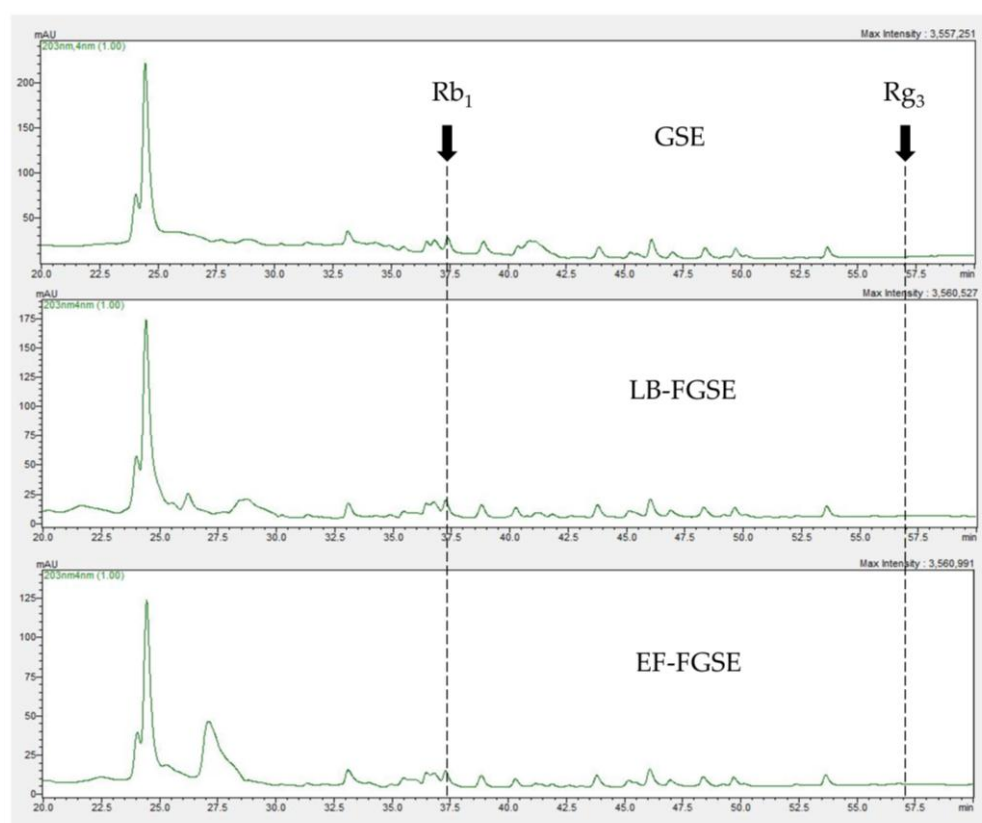


Figure 2. HPLC chromatograms of GSE and FGSE before and after fermentation. GSE and FGSE were analyzed by HPLC at 203 nm. The peak corresponding to ginsenoside Rb₁ (retention time ~37.5 min) is indicated by an arrow. The peak areas of Rb₁ were increased in both LB-FGSE and EF-FGSE compared with GSE, consistent with the quantitative Rb₁ results. The retention time of ginsenoside Rg₃ (~57 min) is indicated for reference. Under the present conditions, Rg₃ was below the limit of quantification.

3.3. Non-Cytotoxic Effect on Cell Viability

To assess the potential cytotoxicity of the samples, cell viability was measured in RAW 264.7 macrophages using the WST assay and normalized to the untreated control, which was set at 100%. Cell viability remained high across all treatment groups, indicating that the tested samples were generally comparable to the normal control (Figure 3). Statistical analysis showed that both treatment concentration and fermentation condition

significantly influenced cell viability ($p < 0.0001$), with a significant interaction observed between the two factors ($p < 0.05$). These results suggest that each fermentation condition exerts a distinct biological effect on cellular metabolic activity.

The absence of significant cytotoxicity, even at the maximum tested concentration (40 $\mu\text{g/mL}$), confirms the biocompatibility of the treatment conditions, while the observed variations in viability highlight the differential potency of the evaluated fermentation conditions. Notably, at low treatment concentrations (e.g., 5 $\mu\text{g/mL}$), viability slightly exceeded 100% with statistical significance ($p < 0.01$), suggesting that the samples are not only safe but may also stimulate cellular metabolic activity without inducing cell damage. At the highest treatment concentration (40 $\mu\text{g/mL}$), viability was maintained at $90 \pm 4\%$ for LB-FGSE and $91 \pm 3\%$ for EF-FGSE, showing no statistically significant reduction compared with the normal control. In general, cell viability values above 90% of the control are considered indicative of minimal cytotoxic effects in *in vitro* assays using colorimetric methods such as MTT or WST [29]. Therefore, all treatment concentrations used in the present study were regarded as non-toxic to RAW 264.7 cells, ensuring that the subsequent evaluation of anti-inflammatory effects was not confounded by cellular depletion.

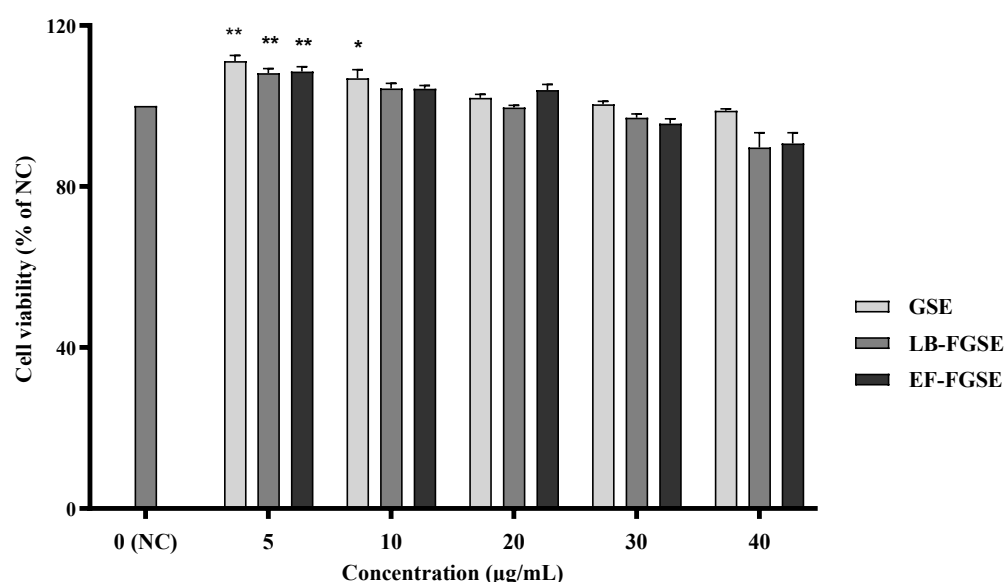


Figure 3. Effects of GSE and FGSE on RAW 264.7 cell viability. RAW 264.7 cells were treated with various concentrations of GSE, LB-FGSE, and EF-FGSE for 4 h, and cell viability was assessed using the WST assay. Data represent the mean $\pm$ SEM of five independent experiments. The group means were compared using a mixed-effects model (two-way), followed by Dunnett's multiple comparisons test. Asterisks indicate significant differences compared with the normal control (NC) group (* $p < 0.05$, ** $p < 0.01$).

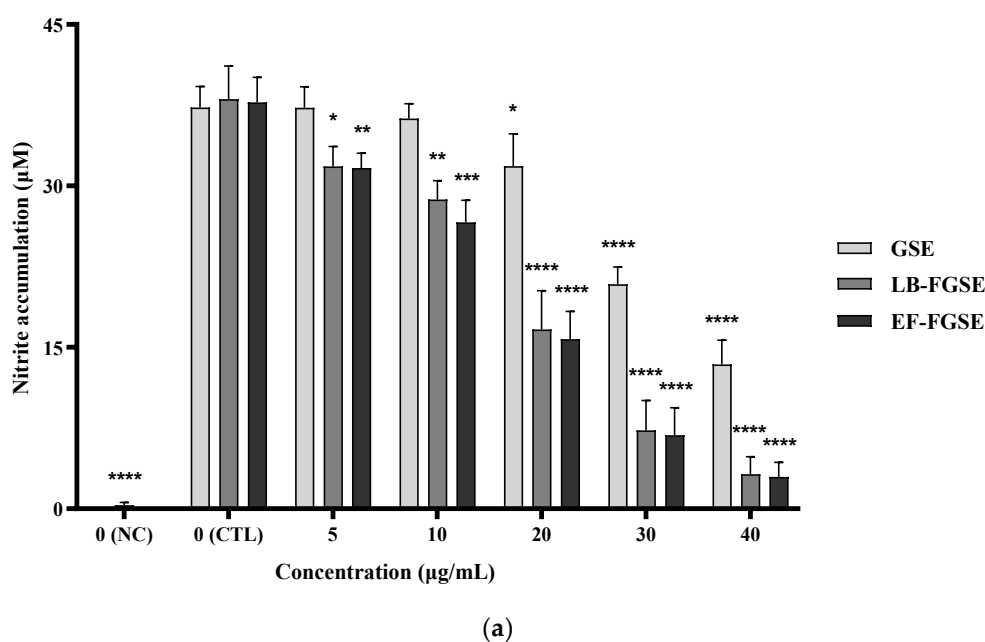
3.4. Inhibition of LPS-Induced NO and PGE₂ Production

To evaluate the anti-inflammatory efficacy of the tested samples, we measured the production of NO and PGE₂, which are key mediators of macrophage-driven inflammatory responses. LPS stimulation caused a marked increase in nitrite accumulation (a stable oxidation product of NO) reaching approximately $37.7 \pm 2.3 \mu\text{M}$, representing a 116-fold increase compared with the unstimulated control (Figure 4a). Pretreatment with the tested samples suppressed LPS-induced nitrite production in a concentration-dependent manner. Statistical analysis indicated that both treatment concentration and fermentation condition significantly influenced nitrite levels ($p < 0.0001$), with a significant interaction between the two factors ($p < 0.0001$). Notably, at the highest treatment concentration (40

$\mu\text{g/mL}$), LB-FGSE and EF-FGSE reduced nitrite production by approximately 92%, whereas GSE exhibited a significantly lower inhibitory effect of 64%.

A similar inhibitory trend was observed for PGE₂ secretion (Figure 4b). LPS stimulation increased PGE₂ levels to approximately 280 pg/mL, and this elevation was attenuated by the tested samples in a concentration-dependent manner. Statistical analysis confirmed significant effects of treatment concentration, fermentation condition, and their interaction on PGE₂ production ($p < 0.0001$). At 40 $\mu\text{g/mL}$, EF-FGSE and LB-FGSE suppressed PGE₂ levels by 71% and 58%, respectively, while GSE showed limited inhibition (20% reduction). Interestingly, EF-FGSE exhibited a lower effective threshold than the other samples: a significant reduction compared with the LPS-only-treated control (CTL) was first observed at 20 $\mu\text{g/mL}$ ($p < 0.05$), whereas GSE and LB-FGSE did not reach statistical significance at this concentration. This pattern suggests that fermentation with *Enterococcus* may generate metabolites that enhance anti-inflammatory activity more effectively than non-fermented GSE or *Lactobacillus*-fermented GSE.

The concentration-dependent inhibition of both NO and PGE₂ production by FGSE provides biochemical evidence for its anti-inflammatory potential. NO is a radical species synthesized from L-arginine by inducible nitric oxide synthase (iNOS) in activated macrophages, and its excessive production is a hallmark of both acute and chronic inflammation [5,28]. Similarly, PGE₂ is a potent pro-inflammatory mediator produced from arachidonic acid primarily via cyclooxygenase-2 (COX-2) in response to inflammatory stimuli such as LPS [28,36].



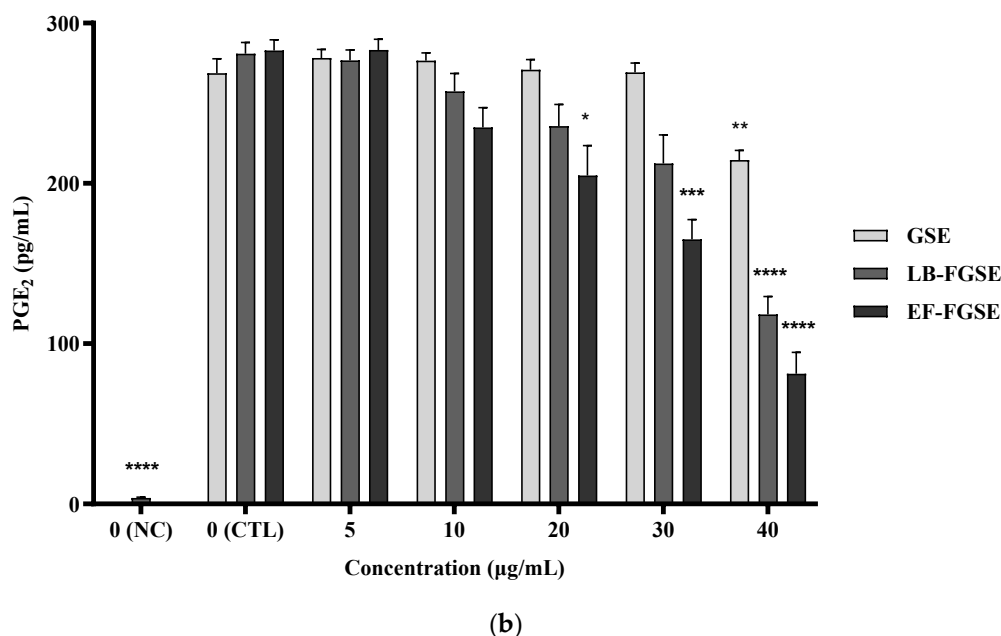


Figure 4. Effects of GSE and FGSE on LPS-induced production of nitrite and PGE₂. RAW 264.7 cells were pretreated with various concentrations of GSE, LB-FGSE, and EF-FGSE for 4 h, followed by stimulation with 0.1 µg/mL LPS for 18 h. Nitrite levels in the culture medium were determined using the Griess reaction (a), while PGE₂ levels were measured using an enzyme immunoassay (b). Data represent the mean ± SEM of five independent experiments. The group means were compared using a mixed-effects model (two-way), followed by Dunnett's multiple comparisons test. Asterisks indicate significant differences compared with the LPS-only-treated control (CTL) group (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

The enhanced anti-inflammatory activity of FGSE, particularly EF-FGSE, may be linked to fermentation-induced changes in the phytochemical profile observed in Sections 3.1 and 3.2. As shown in Figures 1 and 2, fermentation not only significantly increased the TPC and antioxidant capacity but also altered the ginsenoside profile, including an increase in Rb₁. While Rb₁ is reported to modulate inflammatory signaling pathways [28,37], the enhanced efficacy of EF-FGSE is likely due to a combined effect of enriched saponins and other fermentation-derived metabolites (e.g., the peak observed at 27.5 min in Figure 2). Although our antioxidant assays were cell-free and cannot directly infer the involvement of redox-sensitive signaling in macrophages, the tested samples may influence the modulation of redox-sensitive molecular switches required for the synthesis of these inflammatory mediators, such as NO and PGE₂. Accordingly, to further investigate the underlying mechanisms, we next examined whether FGSE modulates the expression of iNOS and COX-2.

3.5. Downregulation of LPS-Induced iNOS and COX-2 mRNA and Protein Expression

To elucidate the mechanisms underlying the attenuation of NO and PGE₂ production, the expression levels of their respective biosynthetic enzymes, iNOS and COX-2, were examined at both the transcriptional and translational levels. Based on the highest non-cytotoxic concentration that produced the strongest inhibitory effects, 40 µg/mL of both GSE and FGSE was initially selected to screen for transcriptional modulation prior to comprehensive protein quantification. Upon LPS stimulation (LPS-only-treated control, CTL), mRNA expression of both iNOS and COX-2 was markedly upregulated relative to the normal control (expression level set to 1) (Figure 5). Pretreatment with the tested samples effectively downregulated these transcriptional levels. Notably, both LB-FGSE and EF-

FGSE exhibited significantly greater attenuation of LPS-induced mRNA expression than non-fermented GSE. These results suggest that the inhibitory effects of FGSE on NO and PGE₂ secretion are mediated, at least in part, through the transcriptional downregulation of these key biosynthetic enzymes.

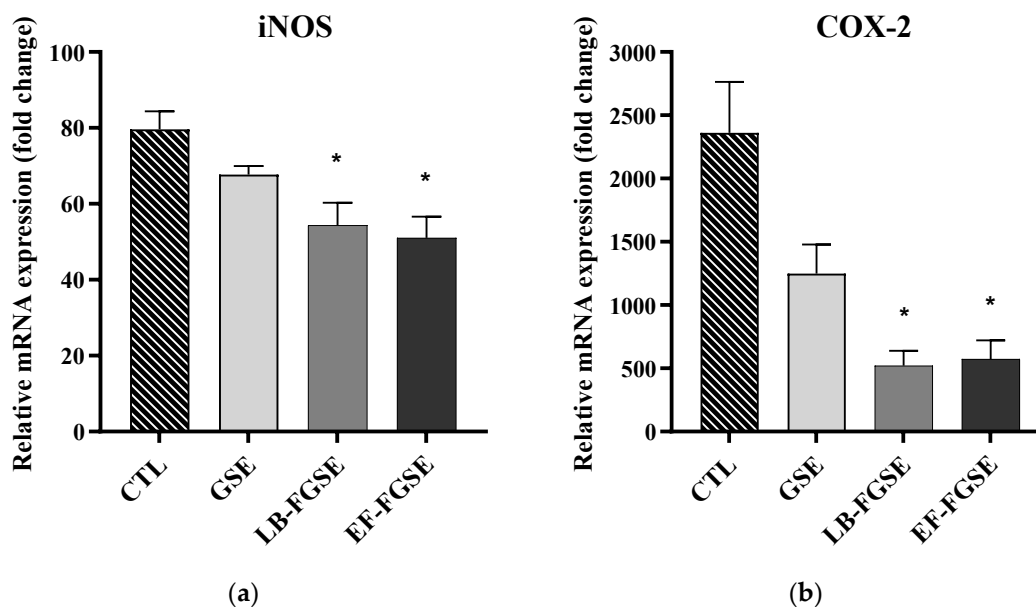
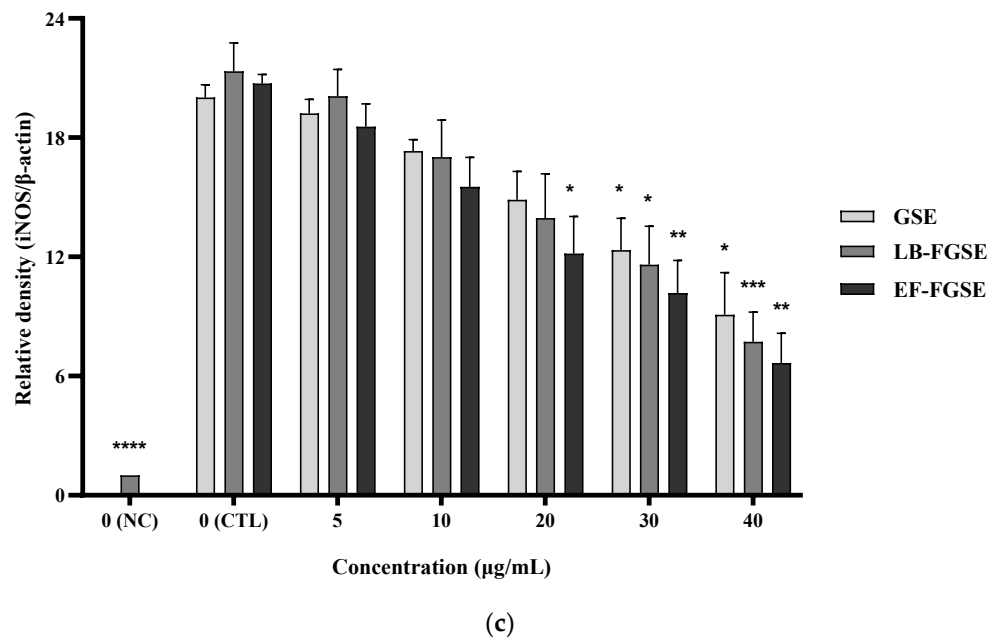
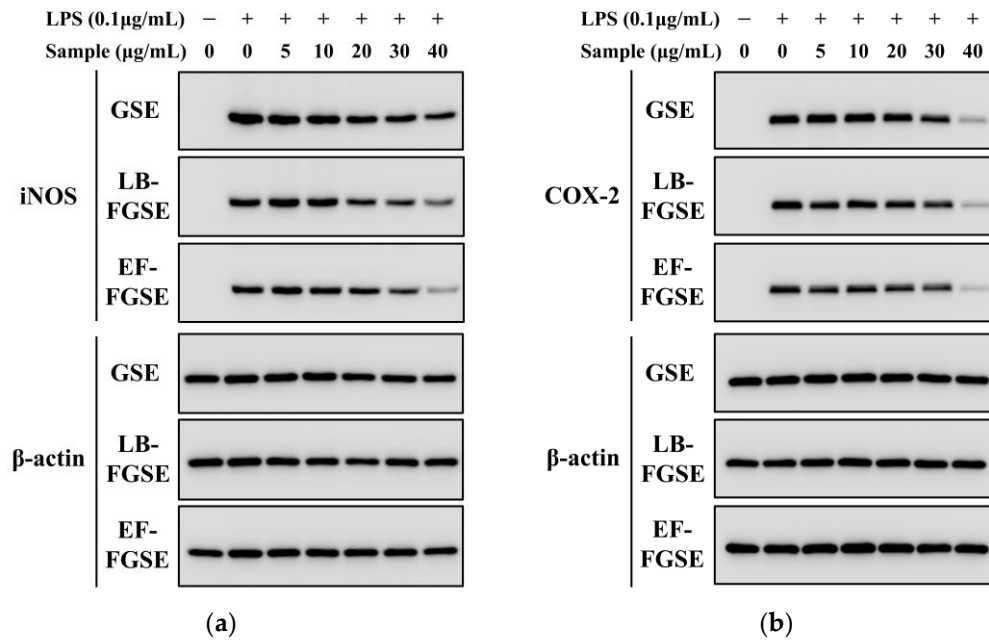


Figure 5. Effects of GSE and FGSE on LPS-induced mRNA expression of iNOS and COX-2. RAW 264.7 cells were pretreated with various concentrations of GSE, LB-FGSE, and EF-FGSE for 4 h, followed by stimulation with 0.1 μ g/mL LPS for 18 h. The mRNA levels of iNOS (a) and COX-2 (b) at 40 μ g/mL were determined using quantitative real-time polymerase chain reaction. Expression levels were normalized to GAPDH and expressed as fold change relative to the normal control (expression level set to 1). Data represent the mean $\pm$ SEM of five independent experiments. The group means were compared using Brown–Forsythe ANOVA, followed by Dunnett’s T3 multiple comparisons test. Asterisks indicate significant differences compared with the LPS-only-treated control (CTL) group (* $p < 0.05$).

To further confirm these findings across a range of treatment concentrations, protein expression was analyzed via Western blot (Figure 6). Consistent with the mRNA results, LPS stimulation caused a pronounced increase in iNOS and COX-2 protein levels. Pretreatment with the tested samples attenuated this LPS-induced upregulation in a concentration-dependent manner, although the sensitivity varied by enzyme and fermentation condition.



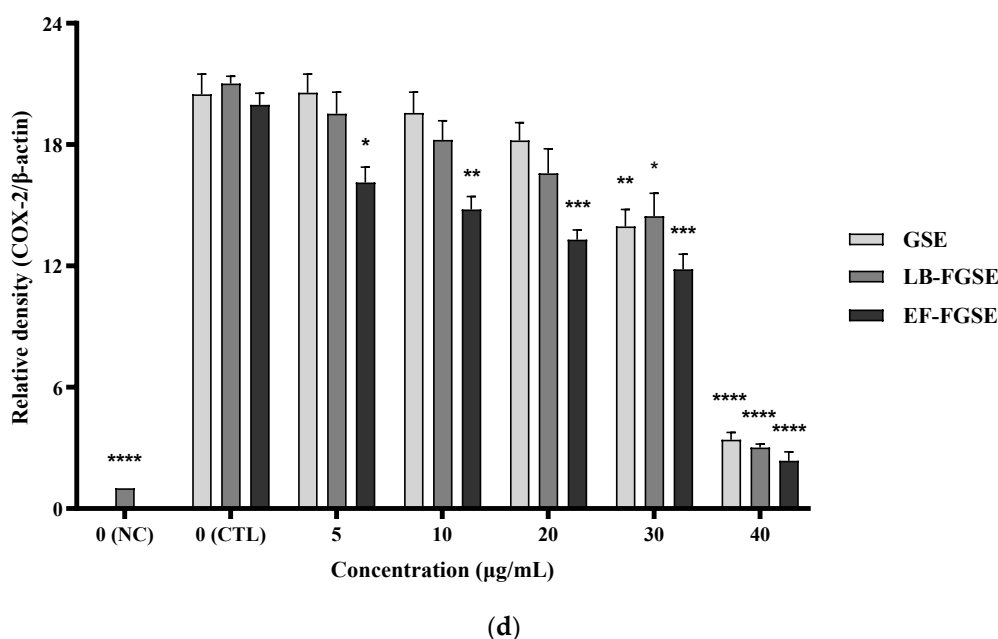


Figure 6. Effects of GSE and FGSE on LPS-induced iNOS and COX-2 protein levels. RAW 264.7 cells were pretreated with various concentrations of GSE, LB-FGSE, and EF-FGSE for 4 h, followed by stimulation with 0.1 μg/mL LPS for 18 h. Protein levels of iNOS and COX-2 were determined by Western blot analysis, with β-actin used as a loading control (a,b). Protein band intensities were quantified using ImageJ software (c,d). Data represent the mean ± SEM of five independent experiments. The group means were compared using a mixed-effects model (two-way), followed by Dunnett's multiple comparisons test. Asterisks indicate significant differences compared with the LPS-only-treated control (CTL) group (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

For COX-2, all groups showed a significant reduction at 40 μg/mL compared with the LPS-only control ($p < 0.0001$). Notably, EF-FGSE demonstrated a significantly lower effective threshold, initiating suppression at 5 μg/mL ($p < 0.05$) with progressively stronger effects at 10–20 μg/mL. In contrast, LB-FGSE and GSE did not show significant inhibitory effects at concentrations up to 20 μg/mL. Two-way statistical analysis revealed a significant interaction between treatment concentration and fermentation condition ($p < 0.05$), indicating that COX-2 suppression across different concentrations is highly dependent on the fermentation method used.

Similarly, iNOS protein levels decreased as the treatment concentration increased. EF-FGSE showed significant suppression starting at 20 μg/mL ($p < 0.05$), while all groups reached significance within the 30–40 μg/mL range. Interestingly, for iNOS, although significant main effects were observed for treatment concentration ($p < 0.0001$) and fermentation condition ($p < 0.05$), the interaction was not significant. This suggests that, while FGSE overall suppressed iNOS more effectively than GSE (main effect of fermentation condition), the concentration–response trend for iNOS was broadly similar across all groups, unlike the strain-specific sensitivity observed for COX-2.

Collectively, these findings demonstrate that both transcriptional and translational regulation of iNOS and COX-2 contribute to the suppression of LPS-induced inflammatory mediator production, with distinct sensitivity patterns depending on the enzyme and fermentation condition. The differential responsiveness of COX-2 and iNOS to FGSE suggests that these enzymes may be regulated through partially distinct upstream signaling mechanisms. COX-2 expression is tightly controlled by multiple signaling pathways downstream of Toll-like receptor 4 (TLR4) activation, including NF-κB and MAPK cascades, whereas iNOS induction is often governed by more conserved inflammatory

signaling responses in macrophages [3,4,40–43]. The earlier and more pronounced suppression of COX-2 by EF-FGSE, particularly at lower concentrations, therefore suggests that fermentation with *Enterococcus faecalis* may generate bioactive metabolites that preferentially interfere with COX-2-associated regulatory pathways rather than uniformly suppressing all inflammatory genes.

The significant interaction observed between treatment concentration and fermentation condition for COX-2 expression further emphasizes the importance of microbial strain selection in determining the functional outcome of fermentation. Lactic acid bacteria differ in their enzymatic repertoires, including glycosidases and esterases, which can influence the conversion of saponins and phenolic compounds into structurally and functionally distinct metabolites [17,34,38]. Accordingly, the greater potency of EF-FGSE may reflect qualitative changes in bioactive composition rather than a simple increase in total phytochemical content.

Although both iNOS and COX-2 mRNA levels were reduced by FGSE, transcriptional analysis was performed at a single concentration, whereas protein expression showed clear concentration-dependent regulation. Thus, while transcriptional suppression likely contributes to the observed effects, additional regulatory mechanisms, including post-transcriptional control, translational efficiency, or protein stability, cannot be excluded [3,4]. Future studies examining multiple concentrations, temporal expression patterns, and upstream signaling pathways will be necessary to clarify the relative contributions of these regulatory levels.

Importantly, the pronounced inhibition of COX-2 expression by EF-FGSE starting from lower treatment concentrations aligns with the significant reduction in PGE₂ production, supporting a functional link between molecular regulation and downstream inflammatory mediator output. Together, these findings suggest that fermentation enhances not only the magnitude but also the efficiency of the anti-inflammatory response.

3.6. Suppression of LPS-Induced Pro-Inflammatory Cytokines

To further evaluate the anti-inflammatory potential of the tested samples, the mRNA expression levels of key pro-inflammatory cytokines and chemokines, including TNF- α , IL-1 β , IL-6, and MCP-1, were measured via RT-qPCR. As shown in Figure 7, LPS stimulation induced a significant upregulation of all examined cytokines and chemokines compared with the unstimulated (NC, expression level set to 1). The general anti-inflammatory efficacy of the groups was first assessed by comparisons with the CTL group (indicated by asterisks). The results showed that IL-1 β and IL-6 were significantly attenuated by all tested samples (Figure 7b,c), whereas TNF- α and MCP-1 were significantly suppressed only by the FGSE groups, with non-fermented GSE failing to reach statistical significance versus the CTL group (Figure 7a,d).

The enhancement of bioactivity through fermentation was further evaluated by comparing differences among the groups (indicated by letters A–C). This effect was both cytokine-specific and strain-dependent. For TNF- α , EF-FGSE (letter 'B') demonstrated a significant reduction compared with GSE (letter 'A'), while LB-FGSE (letter 'AB') showed an intermediate effect without statistical significance relative to either group (Figure 7a). Conversely, for IL-1 β and IL-6, LB-FGSE (letter 'C') exhibited significantly greater suppression than GSE (letter 'B'), whereas EF-FGSE (letter 'BC') showed an intermediate effect that was not statistically different from GSE (Figure 7b,c). Interestingly, for MCP-1, no statistically significant difference was observed among the tested samples (letters 'AB' and 'B'); however, significant inhibition relative to the CTL group was observed in the FGSE groups, while GSE remained ineffective (Figure 7d). These findings indicate that fermentation is critical for surpassing the efficacy threshold of GSE against specific chemokines such as MCP-1 at the tested concentration. Overall, fermentation generally enhances the

inhibitory profile of GSE, but the degree of improvement varies depending on the cytokine target and the microbial strain employed.

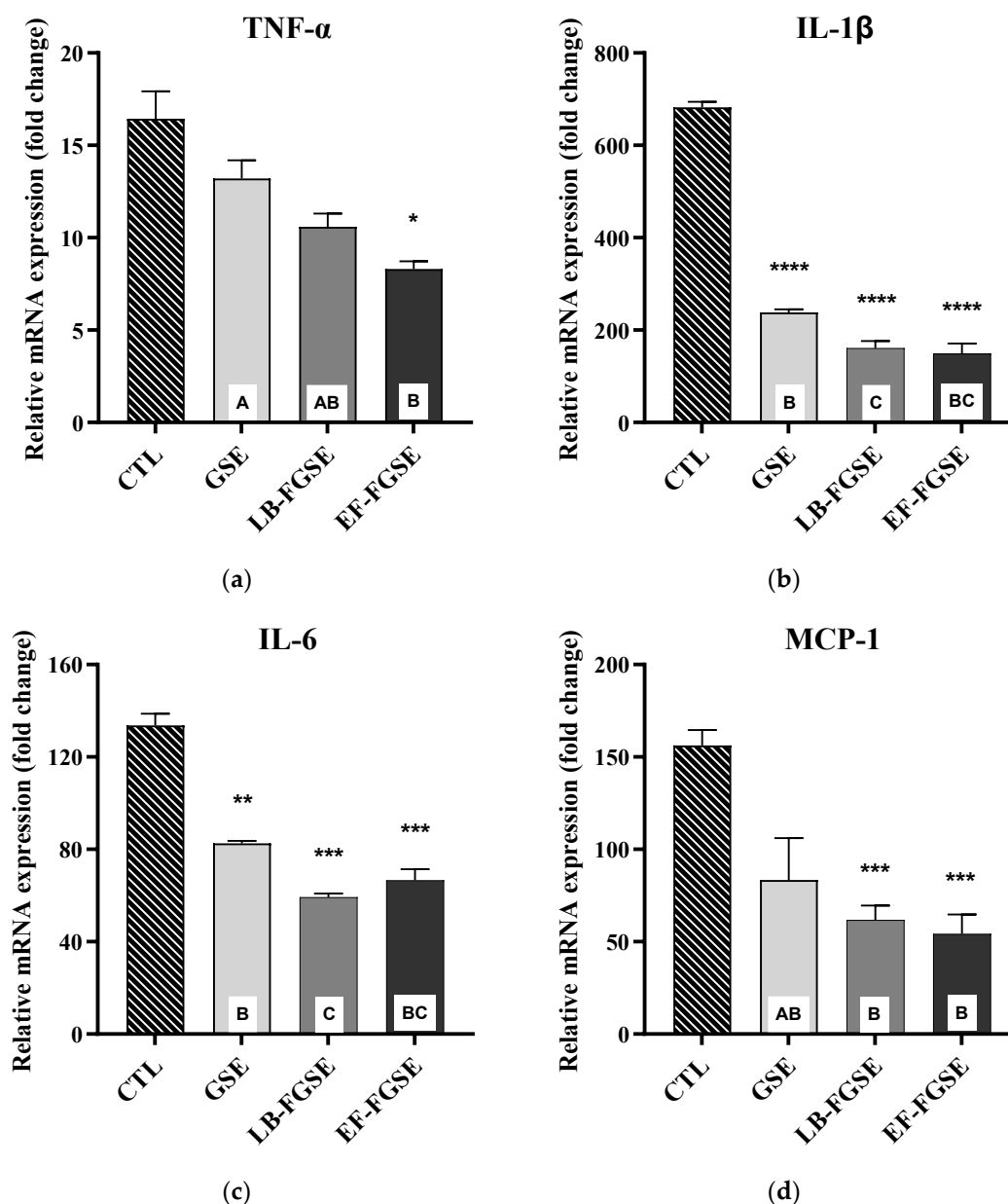


Figure 7. Effects of GSE and FGSE on LPS-induced mRNA expression of pro-inflammatory cytokines. RAW 264.7 cells were pretreated with 40 $\mu\text{g/mL}$ of GSE, LB-FGSE, or EF-FGSE for 4 h, followed by stimulation with 0.1 $\mu\text{g/mL}$ LPS for 18 h. Total RNA was extracted, and mRNA levels of TNF- α (a), IL-1 β (b), IL-6 (c), and MCP-1 (d) were analyzed using quantitative real-time PCR. Expression levels were normalized to GAPDH and expressed as fold change relative to the normal control (expression level set to 1). Data represent the mean $\pm$ SEM of five independent experiments. The group means were compared using Brown-Forsythe ANOVA, followed by Dunnett's T3 multiple comparisons test. Asterisks denote significant differences compared with the LPS-only-treated control (CTL) group (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$). Different letters indicate significant differences specifically among the treatment groups at $p < 0.05$.

The suppression of pro-inflammatory cytokines observed in the present study suggests that FGSE exerts anti-inflammatory effects at the level of cytokine and chemokine expression in LPS-stimulated macrophages. TNF- α , IL-1 β , and IL-6 are central mediators

of macrophage-driven inflammatory responses and play key roles in amplifying and sustaining inflammatory signaling networks [3,4]. In particular, TNF- α occupies an upstream position in the cytokine cascade and can promote the induction of other cytokines and inflammatory mediators [32]. Accordingly, the fermentation-dependent reduction in TNF- α observed in this study suggests that FGSE may influence early stages of cytokine regulation, although the precise hierarchy was not directly examined. Similarly, IL-1 β is a potent pyrogenic cytokine that plays a central role in amplifying inflammatory responses and activating immune cells [41]. The significant reduction in IL-1 β expression in both GSE and FGSE suggests that this cytokine is highly responsive to ginseng sprout-derived components, whereas fermentation may not be essential for its modulation. In addition, the reduction in IL-6 expression by FGSE is notable, as IL-6 contributes to the persistence of inflammatory responses and the transition from acute to chronic inflammation [2,4]. In contrast, MCP-1 primarily functions in recruiting monocytes to inflammatory sites rather than amplifying cytokine signaling [3,44]. The finding that MCP-1 suppression occurred only after fermentation suggests that bioconversion may enhance the capacity of the extract to modulate chemokine expression, even when non-fermented extracts are ineffective at the same concentration.

Together with the findings in Section 3.5, which focused on enzyme-mediated inflammatory outputs (iNOS and COX-2), the present cytokine data indicate that fermentation influences inflammatory regulation at multiple levels, including cytokine and chemokine expression. The distinct response patterns among TNF- α , IL-1 β , IL-6, and MCP-1 further suggest that the anti-inflammatory effects of FGSE are target-selective rather than uniformly suppressive, likely reflecting qualitative differences in fermentation-derived bioactive constituents [15,29,32].

These inhibitory effects are frequently associated with modulation of upstream signaling pathways, such as TLR4-mediated NF- κ B and MAPK cascades [3,4,45]. In our study, FGSE effectively downregulated the expression of iNOS and COX-2, and multiple downstream cytokines. However, direct biochemical assessments of these pathways, including the phosphorylation of I κ B α , p65, or MAPKs (ERK, JNK, and p38), were not performed. Therefore, while the observed patterns are consistent with suppression of canonical inflammatory signaling downstream of TLR4, definitive involvement of these specific pathways cannot be established based on the current data. Previous reports have shown that protopanaxadiol-type ginsenosides, including Rb₁, can exert anti-inflammatory effects through modulation of LPS-induced signaling, including TLR4 and NF- κ B/MAPK pathways [37,46]. Given that fermentation increased Rb₁ content and shifted chromatographic fingerprints in our study (Section 3.2), these chemical changes may contribute to the observed bioactivity. Nevertheless, further studies using pathway-specific inhibitors or phosphorylation assays are required to elucidate the precise molecular mechanisms of FGSE.

3.7. Limitations

Several limitations should be considered when interpreting the outcomes of this study. First, the primary aim of this study was to assess fermentation-associated functional changes by directly comparing FGSE with matched unfermented GSE. Accordingly, unfermented GSE served as the baseline comparator throughout the study. While an increase in ginsenoside Rb₁ was observed after fermentation, the enhanced anti-inflammatory and antioxidant activities of FGSE should not be attributed solely to this marker. Rather, these effects likely reflect the combined influence of multiple fermentation-associated compositional changes, as indicated by altered chromatographic fingerprints and increased total phenolic content. However, individual metabolites were not comprehensively identified or quantified, and causal relationships between specific components and

bioactivities were not directly tested. Future work should include broader metabolite profiling, such as targeted/untargeted LC–MS/MS, and validation using purified standards and/or fractionation-based approaches to delineate the contributions of individual metabolites and better define fermentation-driven biotransformation pathways.

Second, although the fermentation process was previously characterized to confirm successful microbial processing [13], detailed process-level measurements were not collected in this study. The absence of high-resolution time-course data (e.g., microbial growth/viability, pH, organic acids, and titratable acidity) limits the kinetic interpretation of fermentation and its relationship to compositional changes. Future studies integrating real-time monitoring of fermentation parameters with profiling of biotransformation-related products (e.g., ginsenoside conversion metabolites) and microbial population dynamics would strengthen process–composition–function links.

Third, a reference anti-inflammatory compound was not included as a positive control, limiting direct benchmarking of inhibitory magnitude against a standard drug and hindering cross-study comparability (Figures 4–7). Inclusion of a standard comparator (e.g., dexamethasone or indomethacin) in future studies will allow clearer assessment of biological relevance and facilitate interpretation of relative effect sizes across endpoints. Additionally, a non-lactic acid bacterial fermentation control was not included, so strain- and process-specific advantages of lactic acid bacterial fermentation could not be directly compared with alternative systems. Comparative fermentations under matched conditions, integrated with chromatographic or metabolomic profiling, will help clarify whether the observed compositional and functional changes are specific to lactic acid bacteria or reflect more general fermentation effects. Finally, further validation in *in vivo* models, together with advanced metabolomic profiling, is necessary to assess efficacy under physiological conditions and refine multi-target mechanistic interpretation.

4. Conclusions

In conclusion, this study demonstrates that fermentation of GSE with *Lactobacillus* and *Enterococcus* strains enhances the anti-inflammatory effects. This functional improvement is associated with increased ginsenoside Rb₁ and total phenolic content, correlating with enhanced antioxidant capacity. FGSEs more effectively inhibited multiple inflammatory mediators, including NO and PGE₂, by downregulating their respective enzymes, iNOS and COX-2. Notably, FGSE significantly suppressed key pro-inflammatory cytokines and chemokines such as TNF- α and MCP-1, whereas non-fermented GSE did not reach statistical significance for these markers. This suggests that fermentation selectively enhances GSE activity against specific inflammatory endpoints. Although upstream signaling events were not directly characterized, the coordinated suppression of multiple downstream targets indicates that FGSE may exert broad, multi-target anti-inflammatory effects. Overall, these results support the potential of FGSE as a promising functional ingredient. Further studies employing pathway-focused mechanistic assays and *in vivo* models are warranted to fully validate the underlying mechanisms and evaluate efficacy under physiological conditions.

Author Contributions: Conceptualization, J.K., C.-K.H., H.-K.H. and I.O.; methodology, S.P., C.-K.H., H.S., D.H. and J.K.; validation, S.P., C.-K.H. and D.H.; formal analysis, S.P. and J.K.; investigation, S.P., Y.-R.C. and S.Y.; resources, H.S., I.O. and H.-K.H.; writing—original draft preparation, S.P. and S.Y.; writing—review and editing, J.K., C.-K.H. and D.H.; project administration, J.K., C.-K.H. and D.H.; funding acquisition, J.K. All authors have read and agreed to the published version of the manuscript.

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Abbreviations

The following abbreviations are used in this manuscript:

GSE	Ginseng sprout extract
LPS	Lipopolysaccharide
FGSE	Fermented ginseng sprout extract
TNF	Tumor necrosis factor- α
IL-1 β	interleukin-1 β
IL-6	interleukin-6
MCP-1	monocyte chemoattractant protein-1
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
NO	Nitric oxide
PGE ₂	Prostaglandin E ₂
HPLC	High-performance liquid chromatography
TPC	Total polyphenol content
DPPH	2,2-diphenyl-1-picrylhydrazyl
SOD	Superoxide dismutase
LB	<i>Lactobacillus bulgaricus</i>
EF	<i>Enterococcus faecalis</i>
MRS	De Man, Rogosa, and Sharpe
BHI	Brain Heart Infusion
TAE	Tannic acid equivalents
DMEM	Dulbecco's modified Eagle's medium
RT-qPCR	Quantitative reverse transcription polymerase chain reaction
cDNA	Complementary DNA
iNOS	Inducible nitric oxide synthase
COX	Cyclooxygenase
MAPK	Mitogen-activated protein kinase
TLR4	Toll-like receptor 4
JNK	c-Jun N-terminal kinase
SEM	Standard error of mean
ANOVA	One-way analysis of variance
NC	Normal control
CTL	LPS-only-treated control

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