

Effect of *Lactobacillus* Fermentation on the Anti-Inflammatory Potential of Turmeric

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Curcumin, the major bioactive constituent of turmeric, has been reported to have a wide range of pharmacological benefits; however, the low solubility in water has restricted its systemic bioavailability and therapeutic potential. Therefore, in the current study, we aimed to investigate the effect of turmeric fermentation on its curcumin content and anti-inflammatory activity by using several lactic acid bacteria. Fermentation with *Lactobacillus fermentum* significantly increased the curcumin content by 9.76% while showing no cytotoxicity in RAW 246.7 cells, as compared to the unfermented turmeric, regardless of the concentration of *L. fermentum*-fermented turmeric. The *L. fermentum*-fermented turmeric also promoted cell survival; a significantly higher number of viable cells in lipopolysaccharide (LPS)-induced RAW 264.7 cells were observed as compared to those treated with unfermented turmeric. It also displayed promising DPPH scavenging ($7.88 \pm 3.36\%$) and anti-inflammatory activities by significantly reducing the nitrite level and suppressing the expression of the pro-apoptotic tumor necrosis factor-alpha and Toll-like receptor-4 in LPS-induced RAW 264.7 cells. Western blot analysis further revealed that the anti-inflammatory activity of the fermented turmeric was exerted through suppression of the c-Jun N-terminal kinase signal pathway, but not in unfermented turmeric. Taken together, the results suggested that fermentation with lactic acid bacteria increases the curcumin content of turmeric without increasing its cytotoxicity, while strengthening the specific pharmacological activity, thus, highlighting its potential application as a functional food ingredient.

Keywords: Turmeric, curcumin, *Lactobacillus*, RAW 264.7 macrophage cells, anti-inflammatory

Introduction

Curcuma longa, commonly known as turmeric, is a member of the Zingiberaceae family, widely cultivated in India, China, and other Southeast Asian countries [1]. In addition to its use as a spice, food additive and natural yellow food coloring, turmeric also has a long history of use in traditional Ayurvedic medicine for various diseases and acts as a “blood purifier” [2]. In addition to alleviating inflammatory conditions, numerous studies have reported the pharmacological properties and benefits of turmeric consumption, including anti-Alzheimer’s activity [3, 4], hypolipidemic effect [5, 6], and anti-mutagenic activity [7], attributed to the active ingredient curcumin.

Curcumin (diferuloylmethane), the major bioactive constituent of turmeric with a vibrant yellow appearance, was first identified in 1910 [8]. Many researchers have demonstrated the ability of curcumin to interact with numerous molecular targets in inflammation, including impairing the pro-inflammatory cytokines, tumor necrosis factor-alpha (TNF- α) and interleukin-1 (IL-1) production in *Escherichia coli* lipopolysaccharide (LPS)-induced human monocytic macrophages and L929 fibroblast. It also inhibited the activation of nuclear factor-kappa B (NF-kB), a regulator of numerous inflammation-related genes, and the production of nitric oxide [9, 10]. However, as a lipophilic polyphenol, curcumin is nearly insoluble in water, limiting its systemic bioavailability to the host.

Upon consumption, curcumin is rapidly metabolized, conjugated in the liver, and excreted from the body [11].

Recently, plant-based fermentation by probiotic strains has drawn the attention of both scientific researchers and agricultural industries as a way to improve the nutritional value, flavor, and aroma of food, and for application as a feed additive or adjuvant to enhance the performance of livestock [12]. Probiotics are well known for their beneficial effects on the host; hence, fermentation of plant resources with probiotics might synergistically amplify their beneficial properties. To date, several fermented turmeric products have been developed that not only enhance liver function but also improve palatability while having antioxidant, anti-obesity, and antimicrobial activities [13–16]. However, there are limited studies reporting the immuno-modulatory activity of *Lactobacillus*-fermented turmeric upon bacterial infections in the host [17]. Therefore, this study aimed to discover the effect of *Lactobacillus* fermentation on curcumin content in turmeric and the immuno-modulatory events occurring in LPS-induced macrophages upon treatment with *Lactobacillus*-fermented turmeric. To our best knowledge, this is the first report demonstrating the anti-inflammatory activity of *Lactobacillus*-fermented turmeric in LPS-induced macrophages through the JNK signal pathway.

Materials and Methods

Cell Culture and Chemicals

Jungnong Bio Inc. (Korea) provided turmeric and *Lactobacillus*-fermented turmeric powders, which were heat-sterilized prior to freeze-drying into the final product, turmeric powder. Murine macrophage RAW 264.7 cells were obtained from the Korea Cell Line Bank (Korea) and maintained in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, USA) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) antibiotic/antimycotic (Invitrogen) at 37°C in 5% CO₂ humidified atmosphere.

High-Performance Liquid Chromatography Analysis of *Lactobacillus*-Fermented Turmeric

The curcumin content of the *Lactobacillus*-fermented and unfermented turmeric was determined by high-performance liquid chromatography (HPLC) analysis according to the method of Tønnesen *et al.* [18]. The HPLC analysis was performed at the Advanced Analysis Center, Korea Institute of Science and Technology, Seoul, Korea.

In Vitro Cytotoxicity Assay

The RAW 264.7 cells were seeded in a 96-well plate at a density of 1×10^5 cells/well in DMEM medium and maintained under conditions mentioned previously until 70% confluency. The cells were treated with various concentrations of *Lactobacillus*-fermented

and unfermented turmeric samples (500.00, 250.00, 125.00, 62.50, and 31.25 µg/ml in 0.1% [v/v] dimethyl sulfoxide, DMSO) for 24 h at 37°C in 5% CO₂ humidified atmosphere. The cellular cytotoxicity of *Lactobacillus*-fermented and unfermented turmeric samples was quantified by the MTT assay [19].

Lipopolysaccharide-Induced Cell Viability Assay

For cell viability assay, RAW 264.7 cells were seeded in a 96-well plate at a density of 1×10^5 cells/well in DMEM medium and maintained under conditions mentioned previously until 70% confluency. The cells were treated with 1 µg/ml of lipopolysaccharide (LPS; *Escherichia coli* O111:B4; Sigma-Aldrich, USA) and various concentrations of *Lactobacillus*-fermented and unfermented turmeric samples for 24 h. The viability of LPS-induced RAW 264.7 cells was determined via MTT assay [19].

Nitric Oxide Determination

The nitric oxide (NO) production in LPS-induced RAW 264.7 cells, upon treatment with *Lactobacillus*-fermented and unfermented turmeric samples, was determined by measuring the nitrite accumulated in the culture media with Griess reagent (1.0% [w/v] sulfanilamide in 2.5% [v/v] phosphoric acid and 0.1% [w/v] naphthylethylenediamine-dihydrochloride). Briefly, the culture supernatant was mixed with an equal volume of Griess reagent (ratio 1:1) in a 96-well plate and incubated at room temperature for 15 min. The absorbance was measured at 540 nm, and fresh culture medium was used as blank. A standard curve was constructed using 0–100 µM sodium nitrite [19].

2,2-Diphenyl-1-Picrylhydrazyl (DPPH) Radical Scavenging Assay

The *Lactobacillus*-fermented and unfermented turmeric samples were dissolved in 95% (v/v) ethanol and mixed with 0.1 mM DPPH in 3:1 ratio in a 96-well plate. The mixtures were allowed to react in the dark for 30 min. The absorbance was measured at 517 nm and ethanol was used as a control. L-ascorbic acid was used as a positive control. The DPPH radical scavenging activity was calculated using the following equation [20]:

$$\text{DPPH radical scavenging activity (\%)} = [1 - (\text{OD}_{517\text{sample}} / \text{OD}_{517\text{control}})] \times 100$$

Determination of the Level of Pro-Inflammatory Factors

RAW 264.7 cells (3×10^5 cells/well in 6-well plate) were cultured with 1 µg/ml of LPS and *Lactobacillus*-fermented turmeric samples for 24 h. Total cellular RNA was isolated using GeneAll Ribospin (GeneAll Biotechnology Co., Ltd, Korea) and reverse-transcribed using Maxime RT Premix Kit (iNtRON Biotechnology, Korea), according to the manufacturer's instructions. A quantitative polymerase chain reaction (qPCR) was performed to determine the gene expression of TNF-α and Toll-like receptor 4 (TLR4) in LPS-induced RAW 264.7 cells using Kapa SYBR qPCR Universal Master Mix (Kapa Biosystems, USA) and a thermal cycler (BioRad, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. The primers used and the annealing

Table 1. Oligonucleotide primers used in this study.

Genes	Oligonucleotide sequences	Annealing temperature	References
<i>TNF-α</i> F	ACTGAACTTCGGGGTGAT	60°C	[43]
<i>TNF-α</i> R	ACTTGGTGGTTTGCTACG		
<i>TLR4</i> F	TTCAGAACTTCAGTGGCTGGATTTA	60°C	[43]
<i>TLR4</i> R	GTCTCCACAGCCACCAGATTCTC		
<i>GAPDH</i> F	CATGACCACAGTCCATGCCATCAC	60°C	[17]
<i>GAPDH</i> R	TGAGGTCCACCACCCTGTTGCTGT		

temperature are tabulated in Table 1. The amplification reaction involved denaturation for 5 min at 95°C, followed by 45 cycles of denaturation for 15 sec at 95°C, annealing for 30 sec at 60°C, and extension for 30 sec at 72°C. The expression of each gene was determined via comparative analysis based on Ct value as follows:

$$\text{Relative expression} = 2^{-\Delta Ct} \text{ with } \Delta Ct = Ct_{\text{gene}} - Ct_{\text{GAPDH}}$$

Western Blot Analysis

RAW 264.7 cells (3×10^5 cells/well in 6-well plate) were cultured with 1 µg/ml of LPS and *Lactobacillus*-fermented turmeric samples for 24 h. After incubation, the supernatants were discarded, and the total protein was extracted using PRO-PREP Protein Extraction Solution (iNtRON Biotechnology), according to the manufacturer's protocol. The protein concentration was determined using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, USA) according to the manufacturer's protocol and the extracted protein was electroblotted onto a nitrocellulose membrane following separation using 12% SDS-polyacrylamide gel electrophoresis. Membranes were blocked overnight with primary antibodies for p-JNK, TNF-α, and β-actin at 4°C in Tween 20-Tris-buffered saline (TTBS), supplemented with 5% (w/v) skim milk. Membranes were then washed three times with TTBS, followed by incubation with a horseradish peroxidase-conjugated secondary antibody, and again washed thrice with TTBS prior to development through enhanced chemiluminescence.

Statistical Analyses

All values were expressed as mean ± standard deviation from triplicate runs ($n = 3$) and analyzed using SPSS version 20 (IBM SPSS Statistics, USA). Paired T-test analysis was used to determine the difference between two means with a significance level of $\rho < 0.05$, while Tukey's multiple range analysis was used to

determine the difference between samples with a significance level of $\rho < 0.05$.

Results

Curcumin Content of Unfermented and *Lactobacillus*-Fermented Turmeric

HPLC analysis revealed that fermentation with *Lactobacillus* improved the curcumin content (Table 2). Upon fermentation, the curcumin content was significantly increased by $9.62 \pm 1.17\%$ ($\rho < 0.05$) in *L. fermentum*-fermented turmeric and $6.26 \pm 0.95\%$ in *L. plantarum*-fermented turmeric, as compared to the unfermented turmeric. Meanwhile, the curcumin content of *L. brevis*-fermented turmeric was similar to unfermented turmeric, with $1.35 \pm 0.72\%$ increase only. Overall, the curcumin content in turmeric was markedly enhanced through fermentation with *Lactobacillus* strains.

Cytotoxicity of *Lactobacillus*-Fermented Turmeric

Upon fermentation, the *Lactobacillus*-fermented turmeric samples did not show cytotoxicity towards RAW 264.7 cells, regardless of the concentration of the fermented turmeric (31.25–500.00 µg/ml), when compared to the control (Fig. 1). Moreover, high concentrations of unfermented turmeric at 500 µg/ml and 250 µg/ml significantly decreased ($\rho < 0.05$) the cell viability of RAW 264.7 cells by $55.86 \pm 3.41\%$ and $27.18 \pm 8.96\%$, respectively, compared to the control. The cell viability of RAW264.7 cells was further increased upon treatment with the decreasing concentration of *Lactobacillus*-fermented turmeric samples. This indicates that fermentation

Table 2. HPLC analysis of the curcumin content of *Lactobacillus* fermented turmeric.

Samples	Curcumin content before fermentation, mg/g	Curcumin content after fermentation, mg/g
<i>L. fermentum</i> -fermented	1.855 ± 0.026	2.03 ± 0.012 ***
<i>L. plantarum</i> -fermented		1.971 ± 0.010 **
<i>L. brevis</i> -fermented		1.880 ± 0.013

Results are expressed as mean ± standard deviation of triplicate runs ($n = 3$). Paired T-test analysis was performed with asterisks (*) indicating that curcumin contents before and after fermentation differ significantly (** $\rho < 0.01$; *** $\rho < 0.001$).

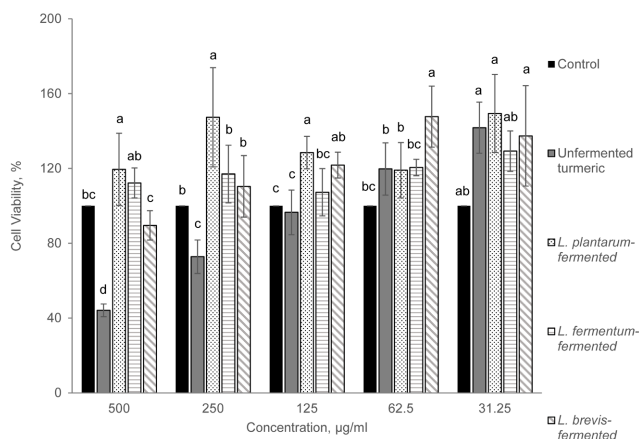


Fig. 1. Cell viability of murine macrophages, RAW 264.7 cells upon treatment with different concentrations of *Lactobacillus*-fermented turmeric (500.00, 250.00, 125.00, 62.50, and 31.25 µg/ml in 0.1% [v/v] DMSO).

Untreated RAW 264.7 cells were used as naive control. Results are expressed as means \pm standard deviations ($n = 3$). Tukey's multiple range analysis was performed with different lowercase letters (a, b, c, and d) demonstrating the significant differences between samples ($\rho < 0.05$).

of turmeric with *Lactobacillus* reduced the cytotoxicity of turmeric. Considering the toxicity of the unfermented turmeric sample on RAW 264.7 cells at a concentration higher than 250 µg/ml, all samples at 500 µg/ml and 250 µg/ml were excluded from subsequent analyses.

Cell Viability of LPS-Induced RAW 264.7 Cells

In this study, an enhanced protective effect was observed on *Lactobacillus*-fermented turmeric-treated RAW 264.7 cells upon LPS induction, compared to unfermented turmeric. Upon stimulation with LPS, the cell viability of untreated LPS-induced RAW 264.7 cells was significantly reduced by $18.25 \pm 4.46\%$ ($\rho < 0.05$), as compared to the naive control (Fig. 2). The unfermented turmeric at 125 µg/ml was unable to protect RAW 264.7 cells from the LPS challenge, and the cell viability was reduced by $25.92 \pm 2.36\%$ ($\rho < 0.05$). Among *Lactobacillus*-fermented turmeric samples, only *L. fermentum*-fermented turmeric at the concentration of 31.25 µg/ml significantly increased the cell viability of LPS-induced RAW 264.7 cells by $11.61 \pm 2.87\%$ ($\rho < 0.05$) compared to untreated LPS-induced RAW 264.7 cells. Therefore, 31.25 µg/ml *L. fermentum*-fermented turmeric was selected for subsequent analyses.

Nitric Oxide Assay

In the current study, the accumulated nitrite in the

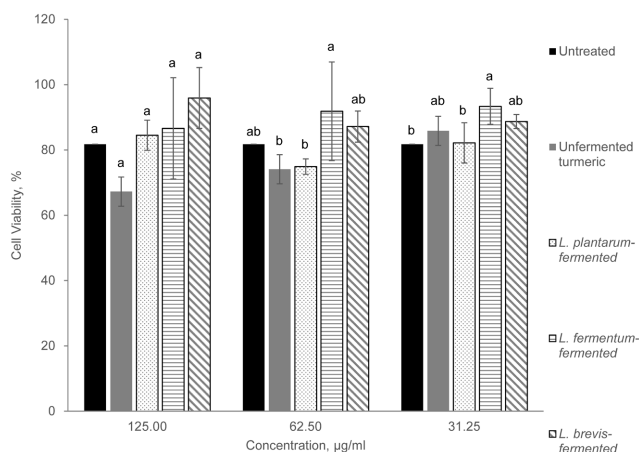


Fig. 2. Cell viability of murine macrophages, RAW 264.7 cells upon stimulation with lipopolysaccharides (LPS) from *Escherichia coli* and treatment with different concentrations of *Lactobacillus*-fermented turmeric.

Non-LPS-induced RAW 264.7 cells were used as a naive control. Results are expressed as means \pm standard deviations ($n = 3$). Tukey's multiple range analysis was performed with different lowercase letters (a and b) demonstrating the significant differences between samples ($\rho < 0.05$).

culture supernatant was determined via the Griess method as an index for NO produced by the LPS-induced RAW 264.7 cells. As shown in Fig. 3, the production of nitrite was reduced upon treatment with *L. fermentum*-fermented turmeric samples. The naive control RAW 264.7 cells (control) generate only a small amount (16.326 ± 0.424 µM) of nitrite. Upon stimulation with LPS, the concentration of nitrite sharply increased to 36.843 ± 0.448 µM in untreated LPS-induced RAW 264.7 cells. Both *L. fermentum*-fermented turmeric and unfermented turmeric at the concentration of 31.25 µg/ml significantly reduced ($\rho < 0.05$) the production of nitrite by 5.257 ± 0.462 µM and 7.859 ± 0.417 µM, respectively, with a slightly higher nitrite reduction effect observed in unfermented turmeric (28.984 ± 0.282 µM) as compared to *L. fermentum*-fermented turmeric (31.585 ± 0.163 µM).

2,2-Diphenyl-1-Picrylhydrazyl (DPPH) Radical Scavenging Activity

In this study, the antioxidant activity of *L. fermentum*-fermented turmeric was determined by reducing the DPPH radicals. Fig. 4 illustrates a significant DPPH radical scavenging effect ($\rho < 0.05$) of both *L. fermentum*-fermented turmeric ($7.88 \pm 3.36\%$) and unfermented turmeric ($9.36 \pm 4.24\%$) at the concentration of 31.25 µg/ml. Notably, the current findings indicated that both *L. fermentum*-fermented

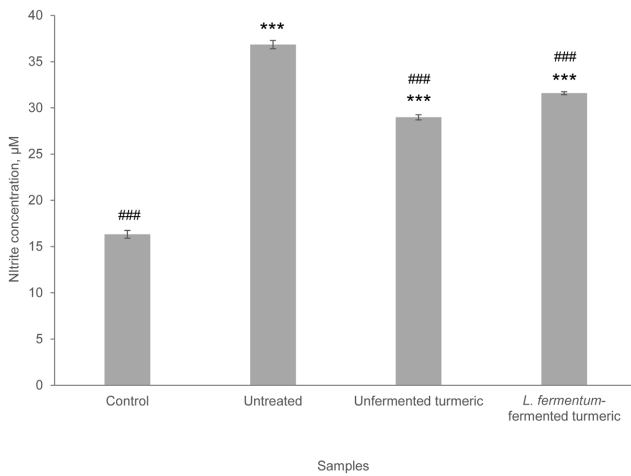


Fig. 3. Effect of *L. fermentum*-fermented turmeric (31.25 µg/ml) on nitrite production in LPS-induced RAW 264.7 cells. Results are expressed as means \pm standard deviations ($n = 3$). Paired T-test analysis was performed to determine the group differences. Control group vs. samples (** $\rho < 0.001$); untreated group vs. samples (** $\rho < 0.001$).

turmeric and unfermented turmeric show a significantly lower DPPH radical scavenging activity ($\rho < 0.05$) as compared to the positive control.

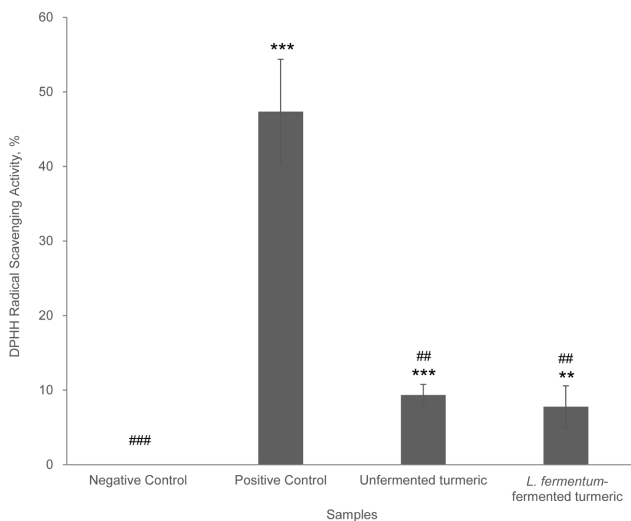


Fig. 4. DPPH radical scavenging activity of *L. fermentum*-fermented (31.25 µg/ml) and unfermented turmeric (31.25 µg/ml). Results are expressed as means \pm standard deviations ($n = 3$). Positive control consists of 500 µg/ml L-ascorbic acid. Paired T-test analysis was performed to determine the group differences. Negative control group vs. samples (** $\rho < 0.01$; *** $\rho < 0.001$); Positive control group vs. samples (** $\rho < 0.01$; *** $\rho < 0.001$).

Gene Expression of Pro-Inflammatory Factors

The gene expressions of TNF- α and TLR4 in LPS-induced RAW 264.7 cells upon treatment with *L. fermentum*-fermented turmeric are shown in Fig. 5. In the present study, both unfermented and *L. fermentum*-fermented turmeric displayed a profound anti-inflammatory activity by significantly reducing the gene expression level of the pro-inflammatory cytokine, TNF- α in LPS-induced RAW 264.7 cells to the basal level after 24 h. The gene expression level of TNF- α in the untreated control remained at an elevated level (2.72 ± 0.46) after 24 h. Moreover, only *L. fermentum*-fermented turmeric significantly suppressed ($\rho < 0.05$) the gene expression of the TLR4 to the basal level. The unfermented turmeric was unable to exert a profound effect on suppression of the gene expression of TLR4 upon challenge by LPS induction and remained activated as in the untreated control. Thus, the findings showed that *L. fermentum*-fermented turmeric exhibited a profound anti-inflammatory activity than unfermented turmeric by suppressing the prolonged production of both TNF- α and TLR4 upon LPS stimulation.

Western Blot Analysis

The results obtained from the western blot analysis further justified the anti-inflammatory activity of *L. fermentum*-

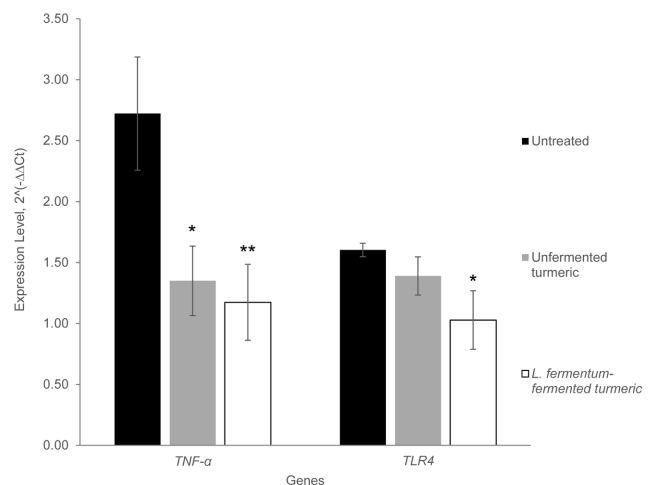


Fig. 5. Effects of *L. fermentum*-fermented turmeric (31.25 µg/ml) on the gene expression of (a) tumor necrosis factor-alpha (TNF- α) and (b) Toll-like receptor 4 (TLR4) in LPS-induced RAW 264.7 cells.

Results are expressed as means \pm standard deviations ($n = 3$). Paired T-test analysis was performed, with asterisks (*) indicating that the differences between untreated control and samples within the same group are significant (* $\rho < 0.05$; ** $\rho < 0.01$).

fermented turmeric through the suppression of the phosphorylated-JNK (p-JNK) in RAW 264.7 cells. Treatment with *L. fermentum*-fermented turmeric caused a significant decrease ($p < 0.05$) in the level of phosphorylation of JNK in LPS-induced RAW 264.7 cells, while the unfermented turmeric-treated and untreated LPS-induced RAW 264.7 cells demonstrated strong phosphorylation of JNK after 24 h treatment (Fig. 6). In addition, the western blot analysis further justified the gene expression analysis, where the production of TNF- α was inhibited upon treatment with *L. fermentum*-fermented turmeric, while the level of TNF- α in untreated LPS-induced RAW 264.7 cells remained at an elevated level (Fig. 6).

Discussion

Turmeric has been long used as a food additive and herbal medicine owing to its wide range of biological activity, including anti-inflammatory [9, 10], anti-diabetes [21, 22], and anti-Alzheimer's disease activities [3, 4]. The wide range of pharmacological benefits is attributed to the presence of curcumin, the major bioactive constituent of turmeric. Curcumin only makes up 2-5% of turmeric [23], hence, probiotics fermentation has been used to improve the curcumin content and pharmacological activities of turmeric. In this study, HPLC analysis revealed that fermentation with *Lactobacillus* improved the curcumin

content. This increment could be attributed to the enzymatic conversion of other curcumin analogs, including bisdemethoxycurcumin or demethoxycurcumin by *Lactobacillus* in the fermentation process.

Although the curcumin content has been improved via fermentation, it is of utmost importance to validate the cytotoxicity of the *Lactobacillus*-fermented turmeric. Past studies have shown that the toxicity of unfermented turmeric was directly correlated with its concentration, where a high concentration of unfermented turmeric resulted in a high cellular level of reactive oxygen species (ROS) and cell death [24, 25]. In accordance with the past studies, the results obtained from this study showed that a high concentration of unfermented turmeric reduced the cell viability of RAW 264.7 cells. However, this is the first study that demonstrated that regardless of the concentration used, the *Lactobacillus*-fermented turmeric samples showed no cytotoxicity towards RAW 264.7 cells. This indicates that fermentation of turmeric with *Lactobacillus* helps reduce the cytotoxicity of curcumin, which might be attributed to the inhibition of the apoptosis pathway and generation of oxidative stress that leads to cell apoptosis.

Subsequently, efforts were made to determine the protective effect of *Lactobacillus*-fermented turmeric upon bacterial infections. Previous studies reported that turmeric could enhance phagocytic activity, anti-inflammatory and antioxidant activities of macrophages to prevent cell death

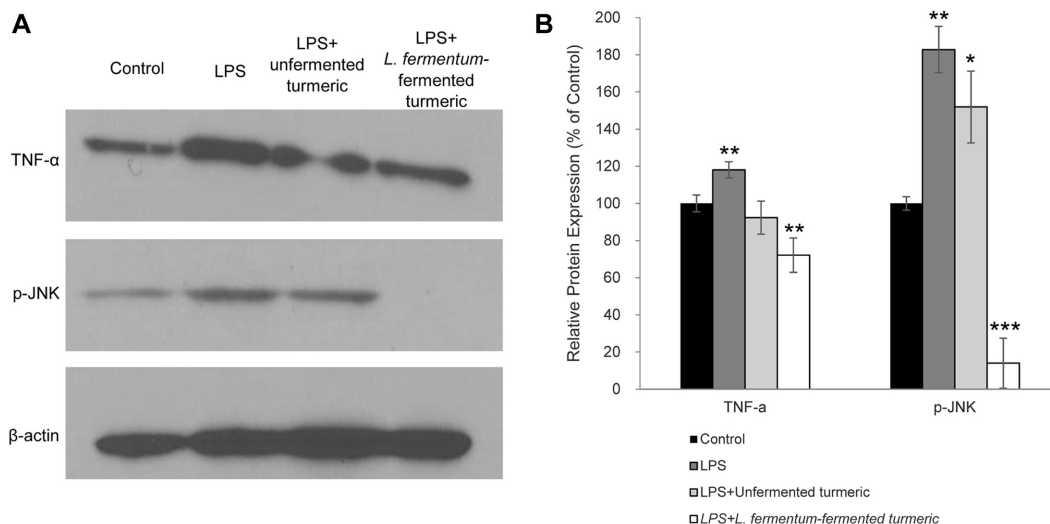


Fig. 6. Effects of *L. fermentum*-fermented turmeric (31.25 $\mu\text{g/ml}$) on the activation of phosphorylated-JNK (p-JNK) and TNF- α of LPS-induced RAW 264.7 cells.

(A) Protein levels of p-JNK and TNF- α were detected by western blotting using p-JNK and TNF- α specific antibodies, where β -actin was used as an internal control. Data shown are representative of three independent experiments ($n = 3$). (B) Relative protein expression of p-JNK and TNF- α was quantified via densitometry analysis. Results are expressed as means \pm standard deviations ($n = 3$). Paired T-test analysis was performed, with asterisks (*) indicating that the differences between control and samples within the same antibody group are significant (** $p < 0.01$; *** $p < 0.001$).

and apoptosis upon bacterial infection [17, 26]. In the current study, *Lactobacillus*-fermented turmeric demonstrated an enhanced protective effect on RAW 264.7 cells upon LPS induction when compared to unfermented turmeric. This was likely attributed to the synergistic protective effect of both the curcumin within the turmeric and the *Lactobacillus* to reduce the generation of oxidative stress and prevent the prolonged production of pro-inflammatory cytokines, resulting in a greater survival rate upon bacterial infection.

Pro-inflammatory cytokines are produced by the activated macrophages and are essential to elicit the inflammatory responses. The TLR4, a transmembrane protein on the surface of macrophages, monocytes, and dendritic cells, belongs to the pattern recognition receptor family and is essential for the initiation of the innate immune response [27]. Hence, the presence of LPS is detected by the immune cells, recognized by TLR4 to form a TLR4-myeloid differentiation protein 2 (MD2)-LPS complex, resulting in the activation of intracellular signaling pathway NF- κ B and innate immune system to promote inflammation and drive pro-inflammatory cascades [27–29].

As a pro-inflammatory cytokine, TNF- α initiates the pro-inflammatory cascades, recruiting and activating various inflammatory cells for recovery purposes [30, 31]. However, the over-production of TNF- α could lead to chronic inflammation and cell apoptosis [31]. In the present study, *L. fermentum*-fermented turmeric suppressed the production of TNF- α of LPS-induced infection, as evidenced by the results of western blotting. Furthermore, the suppression of TNF- α and TLR4 genes to the basal level (base expression level = 1.0) by *L. fermentum*-fermented turmeric, was showed in gene expression analysis, as compared to the untreated and unfermented group. Thus, the findings demonstrated that the protective effect of *L. fermentum*-fermented turmeric was attributed to its profound anti-inflammatory activity by suppressing the prolonged activation of TLR4 and pro-inflammation cascade, for instance, the over-production of the pro-inflammatory cytokine, TNF- α upon LPS stimulation.

In addition, LPS also induces oxidative stress by stimulating the activated macrophages for the production of NO and ROS. A high level of ROS produced upon LPS stimulation will alter host homeostasis condition, thereby causing oxidative damage and cell apoptosis [32]. Notably, *L. fermentum*-fermented turmeric also exhibits antioxidant activity by reducing the DPPH radicals. This radical scavenging ability was attributed to the H atom from the phenolic OH of curcumin, or the metal-chelating abilities, enzymatic antioxidants, and formation of certain antioxidant

biomolecules like exopolysaccharides from *Lactobacillus* [33–35]. Although fermentation of plant-based products commonly increases the bioactive phenolic compounds and enhances their antioxidant activity, in this study, *L. fermentum*-fermented turmeric did not augment the antioxidant activity when compared to the unfermented curcumin. A previous study suggested that the changes in antioxidant activity may be influenced by the fermentation conditions as well as the species of the microorganism employed [36].

Previous studies also reported that a high level of NO could induce apoptosis directly or indirectly by rendering many cells susceptible to apoptosis [37, 38]. The data showed that the protective effect of *L. fermentum*-fermented turmeric was also attributed to the reduction of NO. Such reduction was suggested to be associated with the suppression of TNF- α expression. The expression of TNF- α is essential for the synergistic production of NO in LPS-induced macrophages, as demonstrated by Jun *et al.* [39]. In this study, a lower NO and TNF- α production, and higher cell viability were observed in LPS-induced RAW 264.7 cells upon treatment with *L. fermentum*-fermented turmeric, suggesting that the NO reduction ability of *L. fermentum*-fermented turmeric helps to enhance the survival of LPS-induced RAW 264.7 cells.

Interestingly, the protective effect of *L. fermentum*-fermented turmeric was also shown to be involved with the suppression of the JNK signal transduction pathway. The JNK pathway is induced by activated macrophages in response to LPS and plays a key role in LPS-induced apoptosis [40–42]. The JNK pathway could be either directly activated by LPS, resulting in sustained phosphorylation of JNK or mediated by NO upon LPS-stimulation and ultimately lead to cell death [42]. Notably, treatment with *L. fermentum*-fermented turmeric caused a decrease in the level of phosphorylation of JNK in LPS-induced RAW 264.7 cells. This could be either attributed to direct inhibition by *L. fermentum*-fermented turmeric or indirectly through the suppression of NO production, or a combination of both. Thus, the suppression of the JNK signal pathway by *L. fermentum*-fermented turmeric resulted in a profound protective effect on the LPS-induced RAW 264.7, as demonstrated by a higher survival rate in the cell viability assay.

The present study clearly demonstrated that *Lactobacillus* fermentation improved the curcumin content and reduced the cytotoxicity of turmeric. Moreover, the protective effect of turmeric was enhanced upon fermentation, where the profound anti-inflammatory and antioxidant activities

were demonstrated via the suppression on TNF- α and NO production, the JNK signal pathway, and the reduction of DPPH radicals. The current findings implied that *Lactobacillus* fermentation could overcome the low curcumin content in turmeric while strengthening the specific pharmacological activities of turmeric, highlighting its potential as a functional food ingredient.

Acknowledgments

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

References

- Ammon HP, Wahl MA. 1991. Pharmacology of *Curcuma longa*. *Planta Med.* **57**: 1-7.
- Hatcher H, Planalp R, Cho J, Torti F, Torti S. 2008. Curcumin: from ancient medicine to current clinical trials. *Cell. Mol. Life Sci.* **65**: 1631-1652.
- Ishrat T, Hoda MN, Khan MB, Yousuf S, Ahmad M, Khan MM, et al. 2009. Amelioration of cognitive deficits and neurodegeneration by curcumin in rat model of sporadic dementia of Alzheimer's type (SDAT). *Eur. Neuropsychopharmacol.* **19**: 636-647.
- Ma Q-L, Yang F, Rosario ER, Ubeda OJ, Beech W, Gant DJ, et al. 2009. β -amyloid oligomers induce phosphorylation of tau and inactivation of insulin receptor substrate via c-Jun N-terminal kinase signaling: suppression by omega-3 fatty acids and curcumin. *J. Neurosci.* **29**: 9078-9089.
- Arafa HM. 2005. Curcumin attenuates diet-induced hypercholesterolemia in rats. *Med. Sci. Monit.* **11**: BR228-BR234.
- Jin S, Hong J-H, Jung S-H, Cho K-H. 2011. Turmeric and laurel aqueous extracts exhibit in vitro anti-atherosclerotic activity and in vivo hypolipidemic effects in a zebrafish model. *J. Med. Food.* **14**: 247-256.
- Polasa K, Raghuram T, Krishna TP, Krishnaswamy K. 1992. Effect of turmeric on urinary mutagens in smokers. *Mutagenesis* **7**: 107-109.
- Lampe V, Milobedzka J. 1913. Studien über curcumin. *Ber. Dtsch. Chem. Ges.* **46**: 2235-2240.
- Chan MM-Y. 1995. Inhibition of tumor necrosis factor by curcumin, a phytochemical. *Biochem. Pharmacol.* **49**: 1551-1556.
- Chan MM-Y, Huang H-I, Fenton MR, Fong D. 1998. In vivo inhibition of nitric oxide synthase gene expression by curcumin, a cancer preventive natural product with anti-inflammatory properties. *Biochem. Pharmacol.* **55**: 1955-1962.
- Ireson C, Orr S, Jones DJ, Verschoyle R, Lim C-K, Luo J-L, et al. 2001. Characterization of metabolites of the chemopreventive agent curcumin in human and rat hepatocytes and in the rat in vivo, and evaluation of their ability to inhibit phorbol ester-induced prostaglandin E2 production. *Cancer Res.* **61**: 1058-1064.
- Gupta S, Abu-Ghannam N. 2012. Probiotic fermentation of plant based products: possibilities and opportunities. *Crit. Rev. Food Sci. Nutr.* **52**: 183-199.
- Ho JN, Park SJ, Choue R, Lee J. 2013. Standardized ethanol extract of *Curcuma longa* L. fermented by *Aspergillus oryzae* promotes lipolysis via activation of cAMP-dependent PKA in 3T3-L1 adipocytes. *J. Food Biochem.* **37**: 595-603.
- Kim JH, Kim O-K, Yoon H-G, Park J, You Y, Kim K, et al. 2016. Anti-obesity effect of extract from fermented *Curcuma longa* L. through regulation of adipogenesis and lipolysis pathway in high-fat diet-induced obese rats. *Food Nutr. Res.* **60**: 30428.
- Kim SW, Ha KC, Choi EK, Jung SY, Kim MG, Kwon DY, et al. 2013. The effectiveness of fermented turmeric powder in subjects with elevated alanine transaminase levels: a randomised controlled study. *BMC Complement. Altern. Med.* **13**: 58.
- Pianpumepong P, Anal AK, Doungchawee G, Noomhorm A. 2012. Study on enhanced absorption of phenolic compounds of *Lactobacillus*-fermented turmeric (*Curcuma longa* Linn.) beverages in rats. *Int. J. Food Sci. Technol.* **47**: 2380-2387.
- Kim SB, Kang BH, Kwon HS, Kang JH. 2011. Antiinflammatory and antiallergic activity of fermented turmeric by *Lactobacillus johnsonii* IDCC 9203. *Microbiol. Biotechnol. Lett.* **39**: 266-273.
- Tønnesen HH, Karlsten J. 1983. High-performance liquid chromatography of curcumin and related compounds. *J. Chromatogr. A.* **259**: 367-371.
- Liu CF, Tseng KC, Chiang SS, Lee BH, Hsu WH, Pan TM. 2011. Immunomodulatory and antioxidant potential of *Lactobacillus* exopolysaccharides. *J. Sci. Food Agric.* **91**: 2284-2291.
- Ak T, Gülçin İ. 2008. Antioxidant and radical scavenging properties of curcumin. *Chem. Biol. Interact.* **174**: 27-37.
- Merrell JG, McLaughlin SW, Tie L, Laurencin CT, Chen AF, Nair LS. 2009. Curcumin-loaded poly (ϵ -caprolactone) nanofibres: diabetic wound dressing with anti-oxidant and anti-inflammatory properties. *Clin. Exp. Pharmacol. Physiol.* **36**: 1149-1156.
- Reddy BV, Sundari JS, Balamurugan E, Menon VP. 2009. Prevention of nicotine and streptozotocin treatment induced circulatory oxidative stress by bis-1, 7-(2-hydroxyphenyl)-hepta-1, 6-diene-3, 5-dione in diabetic rats. *Mol. Cell. Biochem.* **331**: 127-133.

23. Aggarwal BB, Sundaram C, Malani N, Ichikawa H. 2007. Curcumin: the Indian solid gold, pp. 1-75. In Aggarwal BB, Surh Y-J, Shishodia S (eds.), *The molecular targets and therapeutic uses of curcumin in health and disease*, Springer, Massachusetts, USA.
24. Atsumi T, Fujisawa S, Tonosaki K. 2005. Relationship between intracellular ROS production and membrane mobility in curcumin-and tetrahydrocurcumin-treated human gingival fibroblasts and human submandibular gland carcinoma cells. *Oral Dis.* **11**: 236-242.
25. Garden OJ, Wigmore SJ. 2007. Curcumin induces heme oxygenase 1 through generation of reactive oxygen species, p38 activation and phosphatase inhibition. *Int. J. Mol. Med.* **19**: 165-172.
26. Bisht K, Choi WH, Park SY, Chung MK, Koh WS. 2009. Curcumin enhances non-inflammatory phagocytic activity of RAW264.7 cells. *Biochem. Biophys. Res. Commun.* **379**: 632-636.
27. Fang W, Bi D, Zheng R, Cai N, Xu H, Zhou R, et al. 2017. Identification and activation of TLR4-mediated signalling pathways by alginate-derived guluronate oligosaccharide in RAW264.7 macrophages. *Sci. Rep.* **7**: 1663.
28. Kanzler H, Barrat FJ, Hessel EM, Coffman RL. 2007. Therapeutic targeting of innate immunity with Toll-like receptor agonists and antagonists. *Nat. Med.* **13**: 552-559.
29. Vaure C, Liu Y. 2014. A comparative review of toll-like receptor 4 expression and functionality in different animal species. *Front. Immunol.* **5**: 316.
30. Akdis M, Burgler S, Cramer R, Eiwegger T, Fujita H, Gomez E, et al. 2011. Interleukins, from 1 to 37, and interferon- γ : receptors, functions, and roles in diseases. *J. Allergy Clin. Immunol.* **127**: 701-721. e770.
31. Brown KL, Cosseau C, Gardy JL, Hancock RE. 2007. Complexities of targeting innate immunity to treat infection. *Trends Immunol.* **28**: 260-266.
32. Halliwell B. 1996. Antioxidants in human health and disease. *Annu. Rev. Nutr.* **16**: 33-50.
33. Kullisaar T, Zilmer M, Mikelsaar M, Vihalemm T, Annuk H, Kairane C, et al. 2002. Two antioxidative lactobacilli strains as promising probiotics. *Int. J. Food Microbiol.* **72**: 215-224.
34. Lee HY, Park JH, Seok SH, Baek MW, Kim DJ, Lee KE, et al. 2006. Human originated bacteria, *Lactobacillus rhammosus* PL60, produce conjugated linoleic acid and show anti-obesity effects in diet-induced obese mice. *BBA-Mol. Cell Biol. L* **1761**: 736-744.
35. Barclay LRC, Vinqvist MR, Mukai K, Goto H, Hashimoto Y, Tokunaga A, et al. 2000. On the antioxidant mechanism of curcumin: classical methods are needed to determine antioxidant mechanism and activity. *Org. Lett.* **2**: 2841-2843.
36. Hur SJ, Lee SY, Kim YC, Choi I, Kim GB. 2014. Effect of fermentation on the antioxidant activity in plant-based foods. *Food Chem.* **160**: 346-356.
37. Albina JE, Cui S, Mateo RB, Reichner JS. 1993. Nitric oxide-mediated apoptosis in murine peritoneal macrophages. *J. Immunol.* **150**: 5080-5085.
38. Allione A, Bernabei P, Bosticardo M, Ariotti S, Forni G, Novelli F. 1999. Nitric oxide suppresses human T lymphocyte proliferation through IFN- γ -dependent and IFN- γ -independent induction of apoptosis. *J. Immunol.* **163**: 4182-4191.
39. Jun CD, Choi BM, Kim HM, Chung HT. 1995. Involvement of protein kinase C during taxol-induced activation of murine peritoneal macrophages. *J. Immunol.* **154**: 6541-6547.
40. Deng Y, Ren X, Yang L, Lin Y, Wu X. 2003. A JNK-dependent pathway is required for TNF α -induced apoptosis. *Cell* **115**: 61-70.
41. Yang X, Khosravi-Far R, Chang HY, Baltimore D. 1997. Daxx, a novel Fas-binding protein that activates JNK and apoptosis. *Cell* **89**: 1067-1076.
42. Seminara AR, Ruvolo PP, Murad F. 2007. LPS/IFN- γ -induced RAW 264.7 apoptosis is regulated by both nitric oxide-dependent and-independent pathways involving JNK and the Bcl-2 family. *Cell Cycle* **6**: 1772-1778.
43. Wang Y, Xie J, Wang N, Li Y, Sun X, Zhang Y, et al. 2013. *Lactobacillus casei* Zhang modulate cytokine and toll-like receptor expression and beneficially regulate poly I: C-induced immune responses in RAW264.7 macrophages. *Microbiol. Immunol.* **57**: 54-62.