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Lactococcus lactis-fermented spinach juice suppresses LPS-induced expression of adhesion molecules and inflammatory cytokines through the NF-κB pathway in HUVECs

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Abstract. Spinach (*Spinacia oleracea* L.), a green leafy vegetable, is widely regarded as a functional food due to its biological activities; however, to the best of our knowledge, there are no previous studies that have investigated the protective effects of fermented spinach against endothelial dysfunction and its underlying mechanisms. Therefore, this study investigated the effects and possible mechanisms of action of fresh spinach juice (S.juice) and fermented S.juice on lipopolysaccharide (LPS)-induced inflammatory responses in human umbilical vein endothelial cells (HUVECs). The HUVECs were treated with S.juice and fermented S.juice for 18 h before LPS exposure, and the levels of cytokines and chemokines, such as monocyte chemoattractant protein-1 (MCP-1) and interleukin-6 (IL-6), were detected using enzyme-linked immunosorbent assays (ELISA). Furthermore, to examine the changes in inflammatory responses to the two treatments, immunofluorescence analysis was used to visualize the nuclear translocation of nuclear factor-κB (NF-κB). Western blot analysis was also performed to detect the differences in the expression of endothelial cell adhesion molecules, specifically vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1). Both S.juice and fermented S.juice inhibited the LPS-induced expression of MCP-1 and IL-6, and suppressed VCAM-1 and ICAM-1. Additionally, fermented S.juice inhibited the LPS-induced activation of NF-κB and degradation of the inhibitor of NF-κB (IκBα) in an LPS dose-dependent manner. These results suggest that the anti-inflammatory effect of vitamin K_2-enriched fermented S.juice is mediated by the suppression of the NF-κB pathway, suggesting its potential as a novel therapeutic candidate for inflammatory cardiovascular disease.

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Abbreviations: CFU, colony-forming unit; DAPI, 4',6-diamidino-2-phenylindole; ELISA, enzyme-linked immunosorbent assay; HPLC, high-performance liquid chromatography; HUVEC, human umbilical vein endothelial cell; ICAM-1, intercellular adhesion molecule 1; IκBα, inhibitor of nuclear factor-κB; IL, interleukin; LAB, lactic acid bacteria; LPS, lipopolysaccharide; MRS, De Man, Rogosa, and Sharpe; NF-κB, nuclear factor-κB; S.juice, spinach juice; VCAM-1, vascular cell adhesion molecule

Key words: fermented spinach juice, human umbilical vein endothelial cells, *Lactococcus lactis*, lipopolysaccharide, *Spinacia oleracea*, vascular inflammation

Introduction

Atherosclerosis is a chronic vascular inflammatory disease resulting from the buildup of cholesterol-rich fatty deposits (plaques) in the artery walls and is a major contributor to cardiovascular mortality (1). Lipopolysaccharide (LPS), the principal surface membrane component in the majority of Gram-negative bacteria, is known to cause vascular inflammation (2). The endothelial inflammatory response promotes leukocyte adhesion and increases vascular permeability by increasing the expression levels of several cell adhesion molecules, including vascular cell adhesion molecule 1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and E-selectin, and via the release of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin (IL)-6, IL-8, and monocyte chemoattractant protein-1 (MCP-1) (3-5). The activation of nuclear factor-κB (NF-κB) is a critical step in the inflammatory response, as it regulates the expression of various inflammatory mediators including cytokines and chemokines, which promotes cell adhesion and increases endothelial permeability (4,6,7). Thus, medications or nutraceuticals that downregulate the expression of inflammatory cytokines/chemokines and adhesion molecules, and inhibit the NF-κB pathway, are promising candidates for the treatment and prevention of atherosclerotic diseases. Anti-inflammatory drugs are not equally effective in all patients and are associated with adverse effects that limit their use. Phytochemicals such as flavonoids, phenylpropanoids, isothiocyanates, sulforaphanes, indole alkaloids, and sterol glucosides can inhibit the production of pro-inflammatory cytokines and subsequently their signaling pathways (8). Therefore, dietary agents with potent anti-inflammatory activity and with few or no adverse effects are promising for treating inflammatory diseases (9).

Spinach (*Spinacia oleracea* L.) is a green leafy vegetable that a is rich source of vitamins, minerals, phenolic compounds, and carotenoids. Its nutrient and phytochemical contents are associated with a wide range of bioactivities, including antioxidant, anti-inflammatory, hepatoprotective, anti-cancer, anti-obesity, and hypolipidemic activity (10,11). Vitamin K is naturally found in green leafy vegetables as phylloquinone (vitamin K_1), while menaquinones (vitamin K_2; MK-*s*, where *s* represents the number of isoprenyl side-chain units) are produced by micro-organisms including intestinal bacteria (12). Furthermore, higher concentrations of vitamin K_1 have been reported in spinach compared with other foods and beverages (13). Vitamin K is a class of fat-soluble vitamins and plays important roles in the coagulation cascade, anti-inflammatory pathways, and in the regulation of serum calcium levels and bone metabolism, all of which have an effect on cardiovascular health (14). Compared with vitamin K_1, vitamin K_2 has more physiological benefits as it increases the regulation of transcription factors that participate in steroid hormone synthesis, and is associated with increased bone metabolism, inhibition of vascular calcification, reduction of cholesterol, and suppression of peripheral inflammation (15-17).

Lactic acid bacteria (LAB) as starter cultures, probiotics, and producers of vitamins are vital components of various food fermentation processes (18). Among LAB, *Lactococcus lactis* are natural producers of vitamin K_2 (19). Vitamin K_2 production has been observed in various bacteria that are involved in established food fermentation processes, and the exact mechanism of vitamin K_2 production has been elucidated (20). However, earlier studies mainly focused on the vitamin K_2 levels in fermented dairy products, with only a few studies focusing on vitamin K_2 production by LAB itself (21-23). Moreover, to the best of our knowledge, there are no available reports on the fermentation of green vegetables such as spinach by *L. lactis*.

In this study, it was hypothesized that biotransformation of vitamin K_1 to K_2 in spinach juice (S.juice) during *L. lactis*-mediated fermentation can improve its health benefits. Therefore, the objective the present work was to determine the anti-inflammatory effects of fermented S.juice including rich vitamin K_2 against the LPS-stimulated human umbilical vein endothelial cells (HUVECs) and to elucidate the potential anti-vascular inflammatory mechanism.

Materials and methods

Chemicals and reagents. Antibodies against VCAM-1 (cat. no. 13662), ICAM-1 (cat. no. 4915), and phospho-NF-κB p65 (cat. no. 3033), NF-κB p65 (cat. no. 8242), phospho-IκB-α (cat. no. 2859), IκB-α (cat. no. 9242), β-actin (cat. no. 4967) (all dilution, 1:1,000), and goat-anti-rabbit IgG-horseradish peroxidase-conjugated (HRP) secondary antibodies (dilution, 1:5,000; cat. no. 7074) were purchased from Cell Signaling Technology, Inc. Enzyme-linked immunosorbent assay (ELISA) kits for MCP-1 (Human MCP-1 ELISA Set; cat. no. 555179) and IL-6 (Human IL-6 ELISA Set; cat. no. 555220) were purchased from BD Bioscience. LPS was purchased from Sigma Chemical Co. The NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (cat. no. 78833; Thermo Fisher Scientific, Inc.) were used. Triton X-100 and 10% formalin solution were obtained from Sigma-Aldrich; Merck KGaA. Additionally, 4',6-diamidino-2-phenylindole (DAPI) and Alexa Fluor dyes (488 and 555) were obtained from Thermo Fisher Scientific, Inc.

Isolation and identification of L. lactis. A strain of *L. lactis* (KCCM12759P) was isolated from sediment collected from the fish-farm tank at the National Institute of Fisheries Science (Pohang, Republic of Korea). The sediment sample was serially diluted, plated on De Man, Rogosa and Sharpe (MRS; Sigma-Aldrich; Merck KGaA) Agar with bromocresol purple, and incubated anaerobically at 30˚C for 24 h. For 16s rDNA gene sequencing analysis, the isolated yellow colonies were transferred to MRS broth, cultured, and DNA was subsequently extracted using the Wizard Genomic DNA Purification kit (Promega Corporation) following to the manufacturer's method. The 16s rDNA gene was amplified using 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGCTACCTTGTTACGACTT-3') primers. PCR was performed using the AccPower PCR Premix kit (Bioneer Corporation) on a Takara PCR Thermal Cycler Dice Gradient system (Takara Bio, Inc). The PCR conditions were as follows: Initial denaturation at 95˚C for 5 min, 30 cycles of denaturation at 95˚C for 30 sec, annealing at 55˚C for 30 sec and extension at 72˚C for 90 sec and final extension at 72˚C for 5 min. The PCR product was analyzed using 1% agarose gel electrophoresis, purified with a NucleoSpin Gel and PCR clean-up kit (cat. no. 740609.50; Macherey Nagel, Inc.), and sequenced using an ABI 3730 sequencer (Applied Biosystems). Homology search was performed using the Basic Local Alignment Search Tool (BLAST) program from the NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The 16s rDNA of a selected isolate showed 99% similarity with *L. lactis* (acc. no. MG754653.1). The *L. lactis* used in this study was deposited at the Korean Culture Center of Microorganisms (KCCM12759P) and stored in vials of MRS broth with 50% (v/v) glycerol at -70˚C until use.

Preparation of fermented spinach juice samples. Spinach (*Spinacia oleracea* L.) was obtained in April 2020 from an herbal medicine cooperative situated in Gyeongsang Province, Republic of Korea. The isolated *L. lactis* strain ($1x10^8$ CFU/ml) was inoculated into 200 ml sterile S.juice and into MRS medium as control, and incubated anaerobically at 26˚C with agitation for 48 h. S.juice was obtained by pressing fresh spinach, lyophilized, and 5% (w/v) of S.juice was calculated as the weight of the dried product, corresponding to the volume of the juice for fermentation. After fermentation, the culture solution including the pellet and the residues of spinach were evaporated three times at 50˚C for 1 h using a rotary evaporator (N-1000; Eyela); the cell pellet was subsequently washed with 10 mM PBS (pH 7.4). Vitamin K_2 (menaquinone), a fat-soluble vitamin, is produced during fermentation by bacteria such as lactic acid bacteria (20). Vitamin K_2 is commonly recovered from microbial cells by liquid-liquid extraction or supercritical fluid extraction and stored at -70˚C until in experiments for vitamin K analysis (22,23).

Quantitative analysis of vitamin K in L. lactis. The vitamin K analysis was performed according to Liu *et al* (21) and Berenjian *et al* (24) with minor modifications. Briefly, the cell pellet was added to 6 ml of resuspension solution (10 mM PBS containing 1% lysozyme) and incubated at 37˚C for 1 h. Subsequently, 24 ml of extraction buffer (*n*-hexane-isopropanol=2:1, v/v; Sigma-Aldrich; Merck KGaA) was added, vortexed twice at 25˚C for 30 sec, and centrifugation at 3,000 x g for 10 min. Next, the lower phase was collected, an equal volume of *n*-hexane was added, then evaporated at 25˚C for 30 min. Subsequently, the dried pellet was dissolved by adding 2 ml isopropanol; the sample was then filtered with a 0.45 μm syringe filter (MilliporeSigma) for high-performance liquid chromatography (HPLC) analysis using an Agilent C18 column (4.6x250 mm; Agilent Technologies, Inc.), maintained at a temperature of 40˚C during the analysis. The flow rate, injection volume, and detection wavelength were 0.4 ml/min, 50 μl, and 254 nm, respectively. Reagent-grade vitamin K_1 and K_2 (Sigma Chemical Co.), dissolved in methanol (0.5% w/v), were used as reference standards. The mobile phase was a mixture of methanol, isopropanol, and *n*-hexane (25:37.5:37.5; v:v:v). The calibration curve was obtained by plotting the peak area vs. concentration. The slope, intercept, and correlation coefficients for the calibration curve were then determined. The analysis was performed in triplicate (25).

Cell culture and treatment. HUVECs were purchased from Lonza Group, Ltd. and cultured in EBM-2 basal medium (cat. no. CC-3156; Lonza Group, Ltd.) supplemented with EGM-2 SingleQuots supplement pack (cat. no. CC-4176; Lonza Group, Ltd.). HUVECs were incubated at 37˚C with 5% CO_2, and the medium was replaced every 48 h.

Cytotoxicity assays. The cytotoxicity of S.juice and fermented S.juice was measured using the Cell Counting Kit-8 assay (CCK-8; Dojindo Molecular Technologies, Inc.). HUVECs were cultured in 48-well plates ($1x10^5$ cells/well) and pretreated with S.juice or fermented S.juice at 2 concentrations (200 and 400 μg/ml) for 1 h at 37˚C, followed by stimulation with LPS (10 μg/ml) for an incubation of 18 h at 37˚C. Subsequently, 400 μl of CCK-8 working solution was added to each well and incubated at 37˚C for 1.5 h. Cell viability was subsequently measured using CCK-8 solution and a microplate reader set at a detection wavelength of 450 nm (Tecan Group, Ltd.).

Enzyme-linked immunosorbent assay (ELISA). HUVECs were seeded in 24-well plates ($1x10^5$ cells/well) and treated with LPS in the presence or absence of S.juice and fermented S.juice at 200 or 400 μg/ml for 18 h. The cell-free supernatant fractions were collected, and the levels of MCP-1 and IL-6 released into the culture supernatant were measured using the Quantikine ELISA kits according to the manufacturer's instructions.

Western blotting. The whole cell proteins were isolated using radioimmunoprecipitation assay (RIPA) lysis (cat. no. MB-030-0050; Rockland Immunochemicals Inc.) containing a 1x protease/phosphatase inhibitor cocktail (cat. no. 5872; Cell Signaling Technology, Inc.). The protein quantification was performed using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Inc.). Then, 30 μg of proteins were separated by 12% SDS-PAGE and transferred to a PVDF membrane. The membrane was blocked with EveryBlot Blocking Buffer (cat. no. 12010020; Bio-Rad Laboratories, Inc.) for 1 h at room temperature, then incubated with 1:1,000 diluted primary antibodies: VCAM-1, ICAM-1, phospho-NF-κB, NF-κB, phospho-IκB-α, IκB-α, and β-actin at 4˚C overnight. Following washing with TBST, the membrane was then incubated with 1:5,000 diluted goat-anti-rabbit IgG-HRP-conjugated secondary antibody for 1 h at room temperature. The bands were detected using an enhanced chemiluminescence detection kit; Clarity Max western ECL substrate (cat.no. 1705062; Bio-Rad Laboratories, Inc.) and were analyzed using the ImageJ software (version 1.52; National Institutes of Health).

Nuclear and cytosolic fractionation. An NE-PER Nuclear and Cytoplasmic Extraction Reagent kit (Thermo Fisher Scientific, Inc.) was used to extract nuclear and cytoplasmic proteins. After treatment with S.juice and fermented S.juice, the HUVEC were homogenized in Cytoplasmic Extraction Reagent I buffer supplemented with protease inhibitor cocktail (Thermo Fisher Scientific, Inc.). After homogenization, the cells were isolated by centrifugation at 16,000 x g for 5 min at 4˚C in the presence of Cytoplasmic Extraction Reagent II (Thermo Fisher Scientific, Inc.). The supernatant of cytoplasmic extract and left pellet were homogenized in Nuclear Extraction Reagent buffer supplemented with protease inhibitor cocktail (Thermo Fisher Scientific, Inc.). The cells were subsequently isolated by centrifugation at 16,000 x g for 10 min at 4˚C; the protein content of the supernatant (representing the nuclear extract) was quantified using Pierce™ Coomassie Plus (Bradford) Assay kit (cat. no. 23236; Thermo Fisher Scientific, Inc.).

Immunofluorescence. After treatment with S.juice and fermented S.juice, HUVECs were fixed with 10% formalin for 10 min at room temperature and immersed in 0.1% Triton X-100 (cat. no. T8787; Sigma-Aldrich; Merck KGaA) in PBS for 15 min at room temperature. Subsequently, the cells were blocked with 3% bovine serum albumin (BSA) (cat. no. 7906; Sigma-Aldrich; Merck KGaA) in PBS for 1 h at room temperature and incubated overnight at 4˚C with primary antibody (NF-κB p65; dilution, 1:200; cat. no. 8242; Cell Signaling Technology, Inc.). For fluorescence detection, the cells were incubated for 1 h at 4˚C in the dark with secondary goat anti-rabbit IgG Alexa Fluor 555 and 488 antibody (dilution 1:500; cat. no. 4413; Cell Signaling Technology, Inc.) and goat anti-rabbit IgG Alexa Fluor 488 antibody (dilution 1:200; cat. no. 4412; Cell Signaling Technology, Inc.). Next, the cells were stained with DAPI solution for the imaging of the cell nuclei through fluorescence microscopy (IX71; Olympus Corporation) at the same gain and exposure time.

Statistical analysis. All the experiments were performed at least three times and all graph bar data are presented as

Table I. Vitamin K_1 and K_2 contents of S.juice and *Lactococcus lactis*-fermented S.juice.

Sample	LAB counts (CFU/ml) Initial	LAB counts (CFU/ml) Final	Vitamin K_1 content (μg/ml)	Vitamin K_2 content (μg/ml)
S.juice	-	-	1.75	0.04
Fermented S.juice	$1x10^8$	$3.58x10^{10}$	1.77	1.89

S.juice, spinach juice; LAB, lactic acid bacteria; CFU, colony-forming units.

mean ± standard error of the mean (SEM). The mean values of different groups were compared using one-way analysis of variance (ANOVA) followed by Tukey's tests (GraphPad Prism 8; GraphPad Software, Inc.). For all data, statistical significance was defined as P<0.05.

Results

Effect of L. lactis fermentation on vitamin K contents of S.juice. Table I shows the respective vitamin K_1 and vitamin K_2 contents of S.juice and fermented S.juice. Single-strain *L. lactis* starters were used to ferment S.juice for 48 h at 26˚C. The initial LAB cell count was ~$1x10^8$ CFU/ml. After 48 h of fermentation with the *L. lactis* strains, the LAB cell count in fermented S.juice was $3.58x10^{10}$ CFU/ml. The vitamin K_1 and K_2 levels of fermented S.juice as determined using HPLC were 1.77 and 1.89 μg/ml, respectively (Table I). The vitamin K_2 content of fermented S.juice was approximately 47-fold higher than in S.juice. There were no significant differences between the vitamin K_1 contents of S.juice and fermented S.juice.

Cytotoxic effects of S.juice and fermented S.juice in HUVECs. The potential cytotoxic effect of S.juice and fermented S.juice against HUVECs was evaluated using CCK-8 assays (Fig. 1). The cell viability was not affected by S.juice and fermented S.juice at either of the test concentrations (200 and 400 μg/ml), indicating no toxicity against HUVECs at these concentrations.

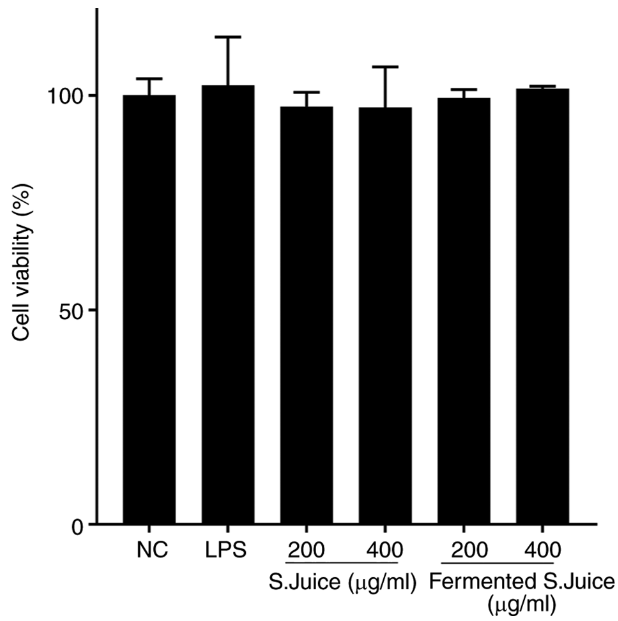

Figure 1. Cytotoxic effects of S.juice and *Lactococcus lactis*-fermented S.juice in human umbilical vein endothelial cells. The cells were treated with different concentrations of S.juice and fermented S.juice (200 and 400 μg/ml) for 1 h, followed by stimulation with LPS (10 μg/ml) for 18 h. The cell viability was determined using the Cell Counting Kit-8 assay. The results are presented as the mean ± SEM (n=3). S.juice, spinach juice; NC, negative control; LPS, lipopolysaccharide.

S.juice and fermented S.juice suppress the levels of pro-inflammatory cytokines and chemokines in LPS-stimulated HUVECs. Inflammatory cytokines and chemokines, such as MCP-1 and IL-6, play critical roles in inflammation (26). Therefore, to investigate the potential anti-inflammatory effects of LPS-stimulated HUVECs, the levels of MCP-1 and IL-6 were determined. Stimulation with LPS significantly increased the production of inflammatory cytokines such as MCP-1 and IL-6 in HUVECs (Fig. 2). However, the treatment with fermented S.juice of 400 μg/ml markedly inhibited the levels of MCP-1 and IL-6 compared with the treatment with S.juice, in a dose-dependent manner.

S.juice and fermented S.juice downregulate the expression of adhesion molecules in LPS-stimulated HUVECs. Adhesion molecules such as VCAM-1 and ICAM-1 play major roles in the initial adhesion and subsequent trans-endothelial migration of leukocytes into inflamed vessels (26). In this study, the effects of S.juice and fermented S.juice on the expression VCAM-1 and ICAM-1 following LPS treatment were determined by western blotting. The expression of both VCAM-1 and ICAM-1 in LPS treatment was significantly increased compared with the control (Fig. 3A and 3B). However, the increase in the VCAM-1 and ICAM-1 levels following LPS treatment was attenuated both by S.juice and fermented S.juice in a dose-dependent manner, with the fermented S.juice exerting a greater effect.

S.juice and fermented S.juice suppress the LPS-induced activation of NF-κB. NF-κB plays an important role in regulating the production of inflammatory mediators (27). Cytokines promote inflammation by promoting NF-κB phosphorylation and IκB-α degradation (28). In this study, the effects of the S.juice and fermented S.juice on NF-κB activation following LPS treatment were investigated. The levels of NF-κB activation and IκB-α degradation were greater following LPS treatment, compared with the control (Fig. 4). However, the pre-treatment with S.juice and fermented S.juice significantly inhibited the

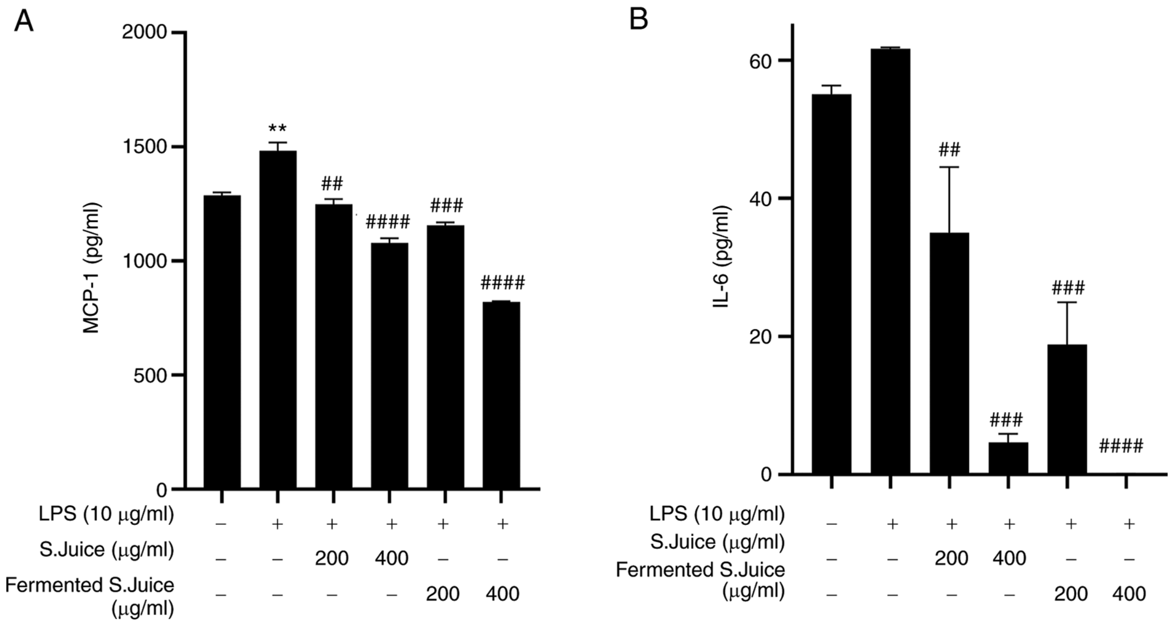

Figure 2. Effects of S.juice and fermented S.juice on the LPS-induced expression of (A) MCP-1 and (B) IL-6 in HUVECs. HUVECs were pretreated with S.juice and fermented S.juice (200 and 400 µg/ml) for 1 h, and subsequently treated with LPS (10 µg/ml) for 18 h. The expression levels of MCP-1 and IL-6 were determined using ELISA. The results are presented as the mean ± SEM (n=3). **P<0.01 vs. the control group. ##P<0.01, ###P<0.001 and ####P<0.001 vs. the LPS group. S.juice, spinach juice; LPS, lipopolysaccharide; MCP-1, monocyte chemoattractant protein-1; IL-6, interleukin 6; HUVECs, human umbilical vein endothelial cells.

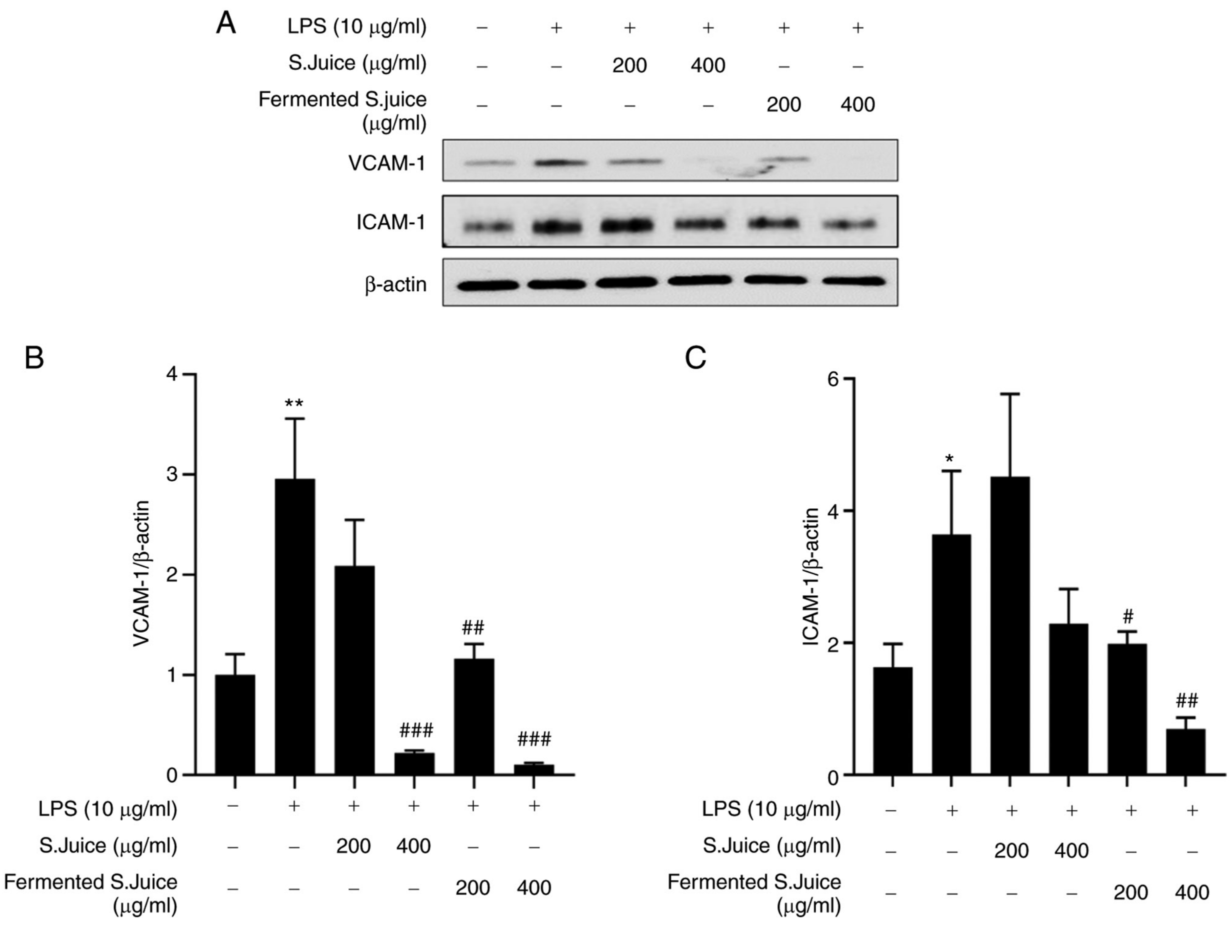

Figure 3. Effect of S.juice and fermented S.juice on LPS-induced expression of (A) the main adhesion molecules such as (B) VCAM-1 and (C) ICAM-1 in HUVECs. HUVECs were treated with S.juice or fermented S.juice (200 and 400 µg/ml) for 1 h, followed by stimulation with LPS (10 µg/ml) for 18 h. HUVECs not treated with either of the juices were used as the control. The results for VCAM-1 and ICAM-1 were analyzed using the ImageJ software. The results are presented as the mean ± SEM (n=3). *P<0.05 and **P<0.01 vs. the control group. #P<0.05, ##P<0.01 and ###P<0.001 vs. the LPS group. S.juice, spinach juice; LPS, lipopolysaccharide; VCAM-1, vascular cell adhesion molecule 1; ICAM-1, intercellular adhesion molecule-1; HUVECs, human umbilical vein endothelial cells.

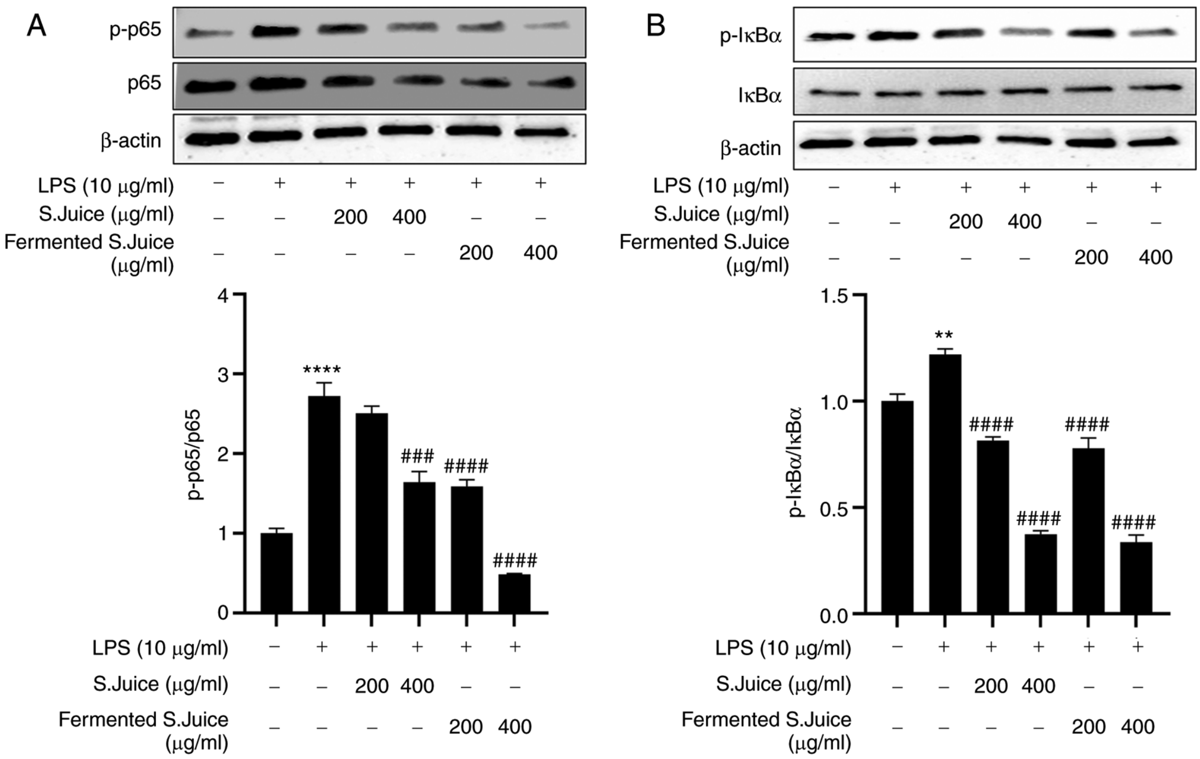

Figure 4. Detection of the protein levels of NF-κB p65, NF-κB p-p65, IκBα and p-IκBα using western blotting. S.juice and fermented S.juice inhibit (A) LPS-induced activation of NF-κB and (B) degradation of IκB-α. Human umbilical vein endothelial cells were pretreated with different concentrations of S.juice and fermented S.juice (200 and 400 μg/ml) for 1 h and stimulated with LPS (10 μg/ml) for 18 h. The results are presented as the mean ± SEM (n=3). **P<0.01 and ****P<0.0001 vs. the control group. $^{###}$P<0.001, and $^{####}$P<0.0001 vs. the LPS group. S.juice, spinach juice; LPS, lipopolysaccharide; nuclear factor κB, NF-κB; inhibitor of NF-κB, IκB-α; p-, phosphorylated.

activation of NF-κB (Fig. 4A) as well as the degradation of IκB-α (Fig. 4B) induced by LPS in a concentration-dependent manner.

S.juice and fermented S.juice suppress the NF-κB p65 signaling pathway in HUVECs. Once activated, the NF-κB p65 subunit is translocated from the cytoplasm to the nucleus and regulates the expression of target genes (28). The effect of S.juice and fermented S.juice on LPS-induced nuclear translocation of NF-κB p65 was investigated. Immunofluorescence assays demonstrated that the LPS-induced nuclear translocation of NF-κB p65 was inhibited by both S.juice and fermented S.juice (Fig. 5). Overall, this indicated that S.juice and fermented S.juice inhibited the production of inflammatory factors by suppressing the NF-κB pathway. Furthermore, fermented S.juice attenuated the LPS-induced expression of NF-κB p65 to a greater extent than S.juice.

Discussion

The present study is, to the best of our knowledge, the first characterization of the mechanism involved in the anti-inflammatory effects of fermented S.juice in HUVECs. Although spinach has been reported to have strong protective effects against vascular inflammation, the anti-inflammatory activity of fermented spinach products remains unclear (10). Fermentation of spinach by *L. lactis* may synthesize into bioactive forms such as vitamin K_2, MK4, MK7, MK9 and to more forms (20). First, it has been demonstrated that vitamin K_2, a major bioactive component of the fermented S.juice, inhibits the endothelial inflammatory response induced by LPS (16,27). The anti-inflammatory effects include the suppression of the secretion of pro-inflammatory cytokines and chemokines and the downregulation of molecules that facilitate adhesion to endothelial cells (10). Our mechanistic analysis revealed that the anti-inflammatory effect of fermented S.juice was possibly mediated via the suppression of NF-κB activation. Thus, to the best of our knowledge, this is the first study to show that fermented S.juice attenuates HUVECs-associated inflammatory responses possibly via the inhibition of the NF-κB signaling pathway.

Spinach is a rich source of vitamins, minerals, phenolic compounds, and carotenoids that are responsible for its various bioactivities (10). The main vitamin in spinach is vitamin K_1, which is used for the microbial biosynthesis of vitamin K_2, including that in intestinal bacteria (12,13). An *in silico* study of LAB genome revealed that the genes encoding enzymes of the vitamin K_2 biosynthesis pathway are different and vary depending on the strain (22,29). LAB play vital roles in food fermentation processes including vitamin K_2 production (30); among the LAB, *L. lactis* is associated with particularly high levels of vitamin K_2 production (19,31). Our results indicate that the vitamin K_2 levels in S.juice increase during fermentation by *L. lactis*. The fat-soluble vitamin K class, including vitamin K_1 and K_2, regulates vascular calcification, bone metabolism, and inflammation, all of which may affect cardiovascular health (32). A previous study reported that the intake of vitamin K_2, but not vitamin K_1, reduced the risk of coronary heart disease mortality, all-cause mortality, as well as severe aortic calcifications (33). The typical recommended

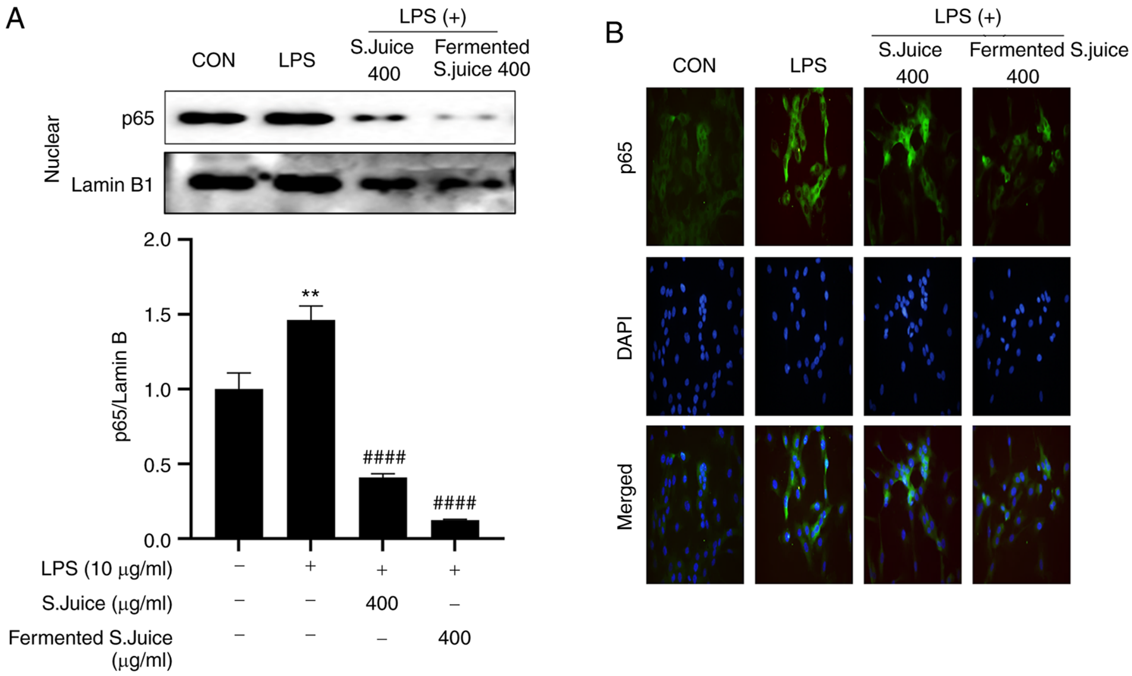

Figure 5. Effect of S.juice and fermented S.juice on nuclear levels of NF-κB p65. (A) LPS stimulation was associated with increased nuclear translocation of NF-κB p65, which was attenuated in HUVECs following treatment with S.juice and fermented S.juice treatment. (B) Immunofluorescence staining shows the translocation of NF-κB (magnification, x200) to the nucleus in HUVECs. The results are presented as the mean ± SEM (n=3). **P<0.01 vs. the control group. ####P<0.001 vs. the LPS group. S.juice, spinach juice; nuclear factor κB, NF-κB; inhibitor of NF-κB; HUVECs, human umbilical vein endothelial cells; CON, control' LPS, lipopolysaccharide.

dietary intake of vitamin K in North America varies from 50 to >600 μg/day for vitamin K_1, and from 5 to 600 μg/day for vitamin K_2 (34). Kawashima *et al* (35) reported anti-atherosclerotic effects in hypercholesterolemic rabbits treated with vitamin K_2 (MK-7; 1 to 10 mg/kg body weight/day) including reduced intimal thickening and ester-cholesterol deposition in the aorta, as well as a slower progression of atherosclerotic plaques. Vitamin K_2 (MK-4) decreased the levels of inflammatory markers such as IL-6, MCP-1, and TNF-α in the liver (36); this might be attributed to the anti-inflammatory effects of vitamin K_2. Vitamin K_2 (MK-4; 0.1-10 μM) reduced the levels of IL-6, IL-1β, and TNFα in LPS-induced MG6 mouse microglia-derived cells (37). Excess vitamin K attenuated the general plasma inflammatory index (38). The overall expression of pro-inflammatory cytokines and chemokines decreased in this study; therefore, vitamin K_2 might directly suppress the vascular NF-κB pathway; this is in line with a previous report (37). In the present study, the treatment levels of fermented S.juice were 200 and 400 μg/ml; 400 μg/ml fermented S.juice contained approximately 211 μg of vitamin K_2 (MK-4).

Vascular inflammatory responses contribute to the pathogenesis of atherosclerosis (39), and endothelial cells play an important role in vascular inflammation (40). Pro-inflammatory stimuli such as LPS activate the endothelial inflammatory response, resulting in the secretion of pro-inflammatory cytokines that contribute to the pathogenesis of cardiovascular disease (41). Endothelial inflammatory responses are characterized by the overproduction of inflammatory mediators, including pro-inflammatory chemokines (e.g., MCP-1), cytokines (e.g., IL-6), and adhesion molecules (e.g., VCAM-1 and ICAM-1). In the present study, LPS significantly upregulated the expression of MCP-1, IL-6, VCAM-1, and ICAM-1; however, this pro-inflammatory effect of LPS was significantly inhibited by pretreatment of HUVECs with S.juice and in particular fermented S.juice.

The inhibitory effect of fermented S.juice on the expression of inflammatory mediators was similar to that of vitamin K_2, an inhibitor of the NF-κB signaling pathway (37). These findings indicated that the LPS-induced secretion of MCP-1, IL-6, VCAM-1, and ICAM-1 in HUVECs was mediated via the NF-κB signaling pathway. In addition, the inhibitory effect of fermented S.juice on inflammatory mediators was likely mediated via inhibition of the NF-κB pathway.

NF-κB is involved in the LPS-induced inflammation response (42). LPS induces the phosphorylation and degradation of IκBα, as well as the phosphorylation, release, and nuclear translocation of NF-κB to activate target gene expression (43,44). To the best of our knowledge, the present study characterized for the first time the effects of S.juice and fermented S.juice on the LPS-induced activation of NF-κB.

These results are not direct evidence that the NF-κB pathway is suppressed by vitamin K_2 to exert a protective effect against vascular inflammation; however, previous data that demonstrated the role of vitamin K_2 in suppressing LPS-induced microglial inflammation (36), combined with our results, suggest that vitamin K_2-enriched fermented S.juice may inhibit LPS-induced inflammation by suppressing the NF-κB signaling pathway.

In conclusion, this is, to the best of our knowledge, the first report of the inhibitory effect of fermented S.juice against LPS-induced inflammation via suppression of NF-κB signaling and downregulation of ICAM-1, VCAM-1, IL-6, and MCP-1 in HUVECs. Our present findings indicate that fermented S.juice might be a potential novel anti-inflammatory agent against vascular inflammation or cardiovascular disease induced by inflammation.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

SMH and SHL conceived and designed the study. ARH performed the experiments. MJS and BMK performed the data analysis. SHL wrote the original draft. SHL, SMH and MJS confirm the authenticity of all the raw data. All authors discussed the results and reviewed the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Awan Z and Genest J: Inflammation modulation and cardiovascular disease prevention. Eur J Prev Cardiol 22: 719-733, 2015.
2. Doherty TM, Shah PK and Arditi M: Lipopolysaccharide, toll-like receptors, and the immune contribution to atherosclerosis. Arterioscler Thromb Vasc Biol 25: e38, 2005.
3. Liang J, Yuan S, Wang X, Lei Y, Zhang X, Huang M and Ouyang H: Attenuation of pristimerin on TNF-α-induced endothelial inflammation. Int Immunopharmacol 82: 106326, 2020.
4. Huang W, Huang M, Ouyang H, Peng J and Liang J: Oridonin inhibits vascular inflammation by blocking NF-κB and MAPK activation. Eur J Pharmacol 826: 133-139, 2018.
5. Zhang L, Wang F, Zhang Q, Kiang Q, Wang S, Xian M and Wang F: Anti-inflammatory and anti-apoptotic effects of Stybenpropol A on human umbilical vein endothelial cells. Int J Mol Sci 20: 5383, 2019.
6. Gao J, Zhao WX, Zhou LJ, Zeng BX, Yao SL, Liu D and Chen ZQ: Protective effects of propofol on lipopolysaccharide-activated endothelial cell barrier dysfunction. Inflamm Res 55: 385-392, 2006.
7. Rao C, Liu B, Huang D, Chen R, Huang K, Ki F and Dong N: Nucleophosmin contributes to vascular inflammation and endothelial dysfunction in atherosclerosis progression. J Thora Cardiovasc Surg 161: e377-e393, 2021.
8. Manach C, Scalbert A, Morand C, Rémésy C and Jiménez L: Polyphenols: Food sources and bioavailability. Am J Clin Nutr 79: 727-747, 2004.
9. Patil KR, Mahajan UB, Unger BS, Goyal SN, Belemkar S, Surana SJ, Ojha S and Patil CR: Animal models of inflammation for screening of anti-inflammatory drugs: Implications for the discovery and development of phytopharmaceuticals. Int J Mol Sci 20: 4367, 2019.
10. Ishii M, Nakahara T, Araho D, Murakami J and Nishimura M: Glycolipids from spinach suppress LPS-induced vascular inflammation through eNOS and NK-κB signaling. Biomed Pharmacother 91: 111-120, 2017.
11. Roberts JL and Moreau R: Functional properties of spinach (*Spinacia oleracea* L.) phytochemicals and bioactives. Food Funct 7: 3337-3353, 2016.
12. Shearer MJ and Newman P: Metabolism and cell biology of vitamin K. Thromb Haemost 100: 530-547, 2008.
13. Booth SL, Davidson KW and Sadowski JA: Evaluation of an HPLC method for the determination of phylloquinine (vitamin K1) in various food matrixes. J Agric Food Chem 42: 295-300, 1994.
14. McCann JC and Ames BN: Vitamin K, an example of triage theory: Is micronutrient inadequacy linked to diseases of aging? Am J Clin Nutr 90: 889-907, 2009.
15. Ichikawa T, Horie-Inoue K, Ikeda K, Blumberg B and Inoue S: Steroid and xenobiotic receptor SXR mediates vitamin K2-activated transcription of extracellular matrix-related genes and collagen accumulation in osteoblastic cells. J Biol Chem 281: 16927-16934, 2006.
16. Ohsaki Y, Shirakawa H, Miura A, Giriwono PE, Sato S, Ohashi A, Iribe M, Goto T and Komai M: Vitamin K suppresses the lipopolysaccharide-induced expression of inflammatory cytokines in cultured macrophage-like cells via the inhibition of the activation of nuclear factor κB through the repression of IKKα/β phosphorylation. J Nutr Biochem 21: 1120-1126, 2010.
17. Yokoyama T, Miyazawa K, Naito M, Toyotake J, Tauchi T, Itoh M, You A, Hayashi Y, Georgescu MM, Kondo Y, *et al*: Vitamin K2 induces autophagy and apoptosis simultaneously in leukemia cells. Autophagy 4: 629-640, 2008.
18. Bintsis T: Lactic acid bacteria as starter cultures: An update in their metabolism and genetics. AIMS Microbiol 4: 665-684, 2018.
19. Bøe CA and Holo H: Engineering *Lactococcus lactis* for increased vitamin K2 production. Front Bioeng Biotechnol 8: 191, 2020.
20. Zhang Z, Liu L, Liu C, Sun Y and Zhang D: New aspects of micrbial vitamin K2 production by expanding the product spectrum. Microb Cell Fact 20: 84-96, 2021.
21. Liu Y, van Bennekom EO, Zhang Y, Abee T and Smid EJ: Long-chain vitamin K2 production in *Lactococcus lactis* is influenced by temperature, carbon source, aeration and mode of energy metabolism. Micro Cell Fact 18: 129, 2019.
22. Chollet M, Guggisberg D, Portmann R, Risse MC and Walther B: Determination of menaquinone production by Lactococcus spp. and propionibacteria in cheese. Int Dairy J 75: 1-9, 2017.
23. Morishita T, Tamura N, Makino T and Kudo S: Production of menaquinones by lactic acid bacteria. J Dairy Sci 82: 1897-1903, 1999.
24. Berenjian A, Mahanama R, Talbot A, Biffin R, Regtop H, Valtchev P, Kavanagh J and Dehghani F: Efficient media for high menaquinone-7 production: Response surface methodology approach. N Biotechnol 28: 665-672, 2011.
25. Irvan, Atsuta Y, Saeki T, Daimon H and Fujie K: Supercritical carbon dioxide extraction of ubiquinones and menaquinones from activated sludge. J Chromatogr A 1113: 14-19, 2006.
26. Bhaskar S, Sudhakaran PR and Helen A: Quercetin attenuates atherosclerotic inflammation and adhesion molecule expression by modulating TLR-NF-κB signaling pathway. Cell Immunol 310: 131-140, 2016.
27. Li X, Tang Y, Ma B, Wang Z, Jiang J, Hou S, Wang S, Zhang J, Deng M, Duan Z, *et al*: The peptide lycosin-I attenuates TNF-α-induced inflammation in human umbilical vein endothelial cells via IκB/NF-κB signaling pathway. Inflamm Res 67: 455-466, 2018.

28. Han BH, Song CH, Yoon JJ, Kim HY, Seo CS, Kang DG, Lee YJ and Lee HS: Anti-vascular inflammatory effect of ethanol extract from Securinega suffruticosa in human umbilical vein endothelial cells. Nutrients 12: 3448, 2020.
29. Watthanasakphuban N, Virginia LJ, Haltrich D and Peterbauer C: Analysis and reconstitution of the menaquinone biosynthesis pathway in lactiplantibacillus plantarum and lentilactibacillus buchneri. Microorganisms 9: 1476, 2021.
30. Liu Y, Charamis N, Boeren S, Blok J, Lewis AG, Smid EJ and Abee T: Physiological roles of short-chain and long-chain menaquinones (vitamin K2) in Lactococcus cremoris. Front Microbiol 13: 823623, 2022.
31. Garrigues C and Pederson MB: *Lactococcus lactis* strain with high vitamin K2 production. US Patent 8765118B2. Filed August 11, 2011, issued March 15, 2012.
32. Fusaro M, Gallieni M, Rizzo MA, Stucchi A, Delanaye P, Cavalier E, Moysés RMA, Jorgetti V, Iervasi G, Giannini S, *et al*: Vitamin K plasma levels determination in human health. Clin Chem Lab Med 55: 789-799, 2017.
33. Geleijnse JM, Vermeer C, Grobbee DE, Schurgers LJ, Knapen MH, van der Meer IM, Hofman A and Witteman JC: Dietary intake of menaquinone is associated with a reduced risk of coronary heart disease: The Rotterdam study. J Nutr 134: 3100-3105, 2004.
34. Marles RJ, Roe AL and Oketch-Rabah HA: US pharmacopeial convention safety evaluation of menaquinone-7, a form of vitamin K. Nutr Rev 75: 553-578, 2017.
35. Kawashima H, Nakajima Y, Matubara Y, Nakanowatari J, Fukuta T, Mizuno S, Takahashi S, Tajima T and Nakamura T: Effects of vitamin K_2 (menatetrenone) on atherosclerosis and blood coagulation in hypercholesterolemic rabbits. Jpn J Pharmacol 75: 135-143, 1997.
36. Weisell J, Ruotsalainen AK, Näpänkangas J, Jauhiainen M and Rysä J: Menaquinone 4 increases plasma lipid levels in hypercholesterolemic mice. Sci Rep 11: 3014, 2021.
37. Saputra WD, Aoyama N, Komai M and Shirakawa H: Menaquinone-4 suppresses lipopolysaccharide-induced inflammation in MG6 mouse microglia-derived cells by inhibiting the NF-κB signaling pathway. Int J Mol Sci 20: 2317, 2019.
38. Shea MK, Booth SL, Massaro JM, Jacques PF, D'Agostino RB Sr, Dawson-Hughes B, Ordovas JM, O'Donnell CJ, Kathiresan S, Keaney JF Jr, *et al*. Vitamin K and vitamin D status: Associations with inflammatory markers in the Framingham offspring study. Am J Epidemiol 167: 313-320, 2008.
39. Chistiakov DA, Sobenin IA and Orekhov AN: Regulatory T cells in atherosclerosis and strategies to induce the endogenous atheroprotective immune response. Immunol Lett 151: 10-22, 2012.
40. Makó V, Czúcz J, Weiszhár Z, Herczenik E, Matkó J, Prohászka Z and Cervenak L: Proinflammatory activation pattern of human umbilical vein endothelial cells induced by IL-1β, TNF-α, and LPS. Cytometry A 77: 962-970, 2010.
41. Xiao L, Liu Y and Wang N: New paradigms in inflammatory signaling in vascular endothelial cells. Am J Physiol Heart Circ Physiol 306: H317-H325, 2014.
42. Fu Y, Wei Z, Zhou E, Zhang N and Yang Z: Cyanidin-3-O-β-glucoside inhibits lipopolysaccharide-induced inflammatory response in mouse mastitis model. J Lipid Res 55: 1111-1119, 2014.
43. Baker RG, Hayden MS and Ghosh S: NF- κB, inflammation, and metabolic disease. Cell Metab 13: 11-22, 2011.
44. Khakpour S, Wilhelmsen K and Hellman J: Vascular endothelial cell Toll-like receptor pathway in sepsis. Innate Immun 21: 827-846, 2015.