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The anti-oxidative capacity of fermented lemon peel and its inhibitory effects on Lipopolysaccharide (LPS)-induced RAW 264.7 cell inflammatory response and cell apoptosis

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Abstract

Chronic inflammation plays a key role in the development and progression of several chronic diseases. Inhibiting the inflammatory cascade, thereby minimising the damage caused by the inflammatory mediators, can be one of the strategies in chronic disease management. In addition, inflammation is closely related to apoptosis, and inflammation can cause apoptosis. Lemon peel has been reported to have antioxidant and anti-inflammatory biological activities. This study aimed to investigate the antioxidant activity of fermented lemon peel (FLP) by Lactobacillus plantarum PNU and the effect of its extract (FLPE) on LPS-induced inflammatory response in RAW 264.7 cells. The results show that FLP has better antioxidant activity than unfermented lemon peel (UFLP). Compared with UFLP extract, FLPE more effectively inhibited the release of NO and pro-inflammatory cytokines (IL-1β, IL-6, TNF-α and IFN-γ) and down-regulated pro-inflammatory genes (IL-1β, IL-6, NF-κB p65, COX-2, IFN-γ, iNOS, IL-5), and pro-apoptotic genes (caspase-3, caspase-9, p53, p21 and Bax), meanwhile, promoted the release of anti-inflammatory cytokine (IL-10) and up-regulated anti-inflammatory genes(IL-10 and IL-4), and anti-apoptotic gene (Bcl2) in LPS-induced RAW 264.7 cells. Therefore, this study elucidates the anti-inflammatory activity mechanism of fermented lemon peel by studying the balance of inflammatory response and the inhibition of apoptosis. It provides an important reference for the future research and treatment of chronic inflammation and related diseases, as well as the development of fermented foods with anti-inflammatory effects.

Keywords: lemon peel; fermentation; RAW 264.7 cell; inflammation; apoptosis.

Practical Application: Inhibiting the inflammatory cascade, thereby minimising the damage caused by the inflammatory mediators, can be one of the strategies in chronic disease management. In addition, inflammation is closely related to apoptosis, and inflammation can cause apoptosis. Therefore, this study elucidates the anti-inflammatory activity mechanism of fermented lemon peel by studying the balance of inflammatory response and the inhibition of apoptosis. It provides an important reference for the future research and treatment of chronic inflammation and related diseases, as well as the development of fermented foods with anti-inflammatory effects.

1 Introduction

Inflammation, triggered by a variety of harmful stimuli, a biological defensive response of the immune system. Activated macrophages respond to these stimuli by releasing inflammatory mediators (Xing et al., 2022) A persistent stimulation resulting in the release of inflammatory factors leads to chronic inflammation (Ahn et al., 2015; Hwang et al., 2014). Inflammation, especially chronic inflammation, however, plays an inextricable role in tumor occurrence and development. There is evidence that chronic inflammatory lesions are often secondary to tumorigenesis, and inflammatory cells are present in tumor tissue biopsy samples. Simply put, inflammation induces apoptosis (Ritter & Greten, 2019). Therefore, the treatment of chronic inflammation is urgent. Traditional steroid and non-steroid anti-inflammatory drugs are widely used, but they have serious side effects on the digestive tract, kidneys, and central nervous system (Islam et al., 2013). For the management of chronic inflammation, there has been an ongoing search for novel, safe, effective anti-inflammatory agents or functional foods.

Lemon is a fruit rich in vitamins, minerals, and flavonoids. The lemon peel has many biological attributes, including antioxidant and anti-inflammatory properties, have also been linked to contribute to weight loss. By delaying aging, preventing diseases, and improving immunity, it plays an important role (Abdel Rahman et al., 2019; Asadi et al., 2019; Shimizu et al.,

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2019; Xi et al., 2017). However, lemon peels are usually discarded during food production and then burned (Boswell, 2021). In order to reduce environmental damage (greenhouse effect) and improve waste utilization (Billant, 2021), we use lactic acid bacteria to ferment lemon peels to develop functional foods that people can eat (Cullen, 2021; Russell et al., 2021). Fruits or fruit peels are thought to have improved biological activity after fermentation, according to studies (Cheng et al., 2020; Hu et al., 2022; Ruiz Rodríguez et al., 2021). Moreover, studies have shown that antiproliferative and apoptotic effects of probiotic whey dairy beverages in human prostate cell lines (Rosa et al., 2020). Nevertheless, there are few research reports on the benefits of fermented lemon peels. Accordingly, a cellular inflammation model was used in this study to analyze the effects of fermented lemon peel on inflammatory responses.

In the systemic inflammatory response syndrome, lipopolysaccharide (LPS) is an important pathogenic factor, a molecule found on the outer membrane of Gram-negative bacteria. It is therefore also used to induce inflammation in experimental models (Rebollo-Hernanz et al., 2019; Wu et al., 2018). It is important to note that macrophages play a key role in initiating and maintaining inflammation. When stimulated by LPS, macrophages induce the secretion of various inflammatory mediators, including interleukins (ILs) and tumor necrosis factor-alpha (TNF-α), and an inflammatory cascade, then ensues (Ahn et al., 2015; Hwang et al., 2014). The severity of inflammation can be determined by detecting these inflammatory mediators quantitatively. In this study, an inflammation model induced by LPS in RAW264.7 macrophages was used to investigate the effects of fermented lemon peel on inflammatory mediator secretion. The role of genes involved in inflammation and apoptosis and their mechanisms were also studied. The study aimed to establish a theoretical foundation for developing fermented lemon peel to prevent or treat chronic inflammation.

2 Materials and methods

2.1 Activation of the strain for fermentation

The lemon peel was fermented with *Lactobacillus plantarum* PNU (KCCM 11352P) isolated from Jeonju Kimchi (Lee et al., 2016), and deposited at the Korea Culture Center of Microorganisms (KCCM, Seoul, Korea). The strains were inoculated into MRS liquid medium with 2% inoculum and cultured at 37 °C for overnight and were used after secondary activation.

2.2 Preparation of fermented and unfermented lemon peels

Fresh, mold-free lemon peels were converted to lemon peel powder for fermentation by freeze-drying. Lemon peel powder was mixed with water in a ratio of 1:20 and inoculated with 4% (10^8 CFU/mL) bacterial inoculum to prepare fermented lemon peel (FLP). Following the addition of sugar (40%) to the mixture, it was fermented for 24 hours. The procedure to prepare unfermented lemon peel (UFLP) was the same as the procedure to prepare FLP. However, the bacterial inoculum was not added (Pan et al., 2022).

2.3 Assessment of 2,2-diphenyl-1-picrylhydrazyl (DPPH) inhibition rate of fermented and unfermented lemon peel

FLP and UFLP were centrifuged to collect supernatants, which were then kept at 4°C until needed. The 96-well plate was filled with 100 μL of FLP and UFLP, methanol, and 150 μM DPPH solution respectively, and a dark reaction carried out for 30 min. In order to calculate the DPPH inhibition rate, we measured the absorbance at 517 nm and applied the formula below (Equation 1):

$$DPPH\ inhibition\ rate\ (\%) = [1-\frac{(A0-A1)}{(A2-A3)}]\times 100 \quad (1)$$

A0: sample + DPPH; A1: sample + methanol; A2: methanol + DPPH; A3: methanol + methanol

2.4 Assessment of total phenol (TP) content

Phosphomolybdic acid and phosphotungstic acid are easily reduced by phenolic compounds and turn blue under alkaline conditions. The Folin-Ciocalteu Reagent, a mixture of phosphomolybdic and phosphotungstic acid, was used to detect the TP content. The standard curve was drawn using gallic acid as the reference (standard concentration was 0.03125-1 mg/mL), and the standard curve was used to calculate the TP content of FLP and UFLP.

2.5 Assessment of total flavonoid (TF) content

The principle of color change in the reaction of sodium hydroxide and flavonoids, was used to detect the total flavonoid content. Quercetin was used as the reference (standard concentration is 0-1280 μg/mL) to draw the standard curve. On the basis of the standard curve, the TF content of FLP and UFLP was calculated.

2.6 Preparation of the methanol extract of FLP and UFLP

FLP and UFLP were freeze dried. Three extractions at room temperature were performed on the dried samples using 100% methanol (1:3). By using a rotary vacuum evaporator at 50 °C, the extracts were concentrated under reduced pressure to yield fermented lemon peel extract (FLPE) and unfermented FLPE (UFLPE) powders that were dissolved in dimethyl sulfoxide solution to perform experiments.

2.7 RAW 264.7 cell activation

Cells were obtained from the Korea Cell Line Bank in Seoul, Korea, as RAW 264.7 cells. Incubation of the cells took place at 37 °C in a 5% CO_2 incubator with Dulbecco's Modified Eagle's Media (DMEM, Gibco, Thermo Fisher Scientific, Waltham, MA, USA) containing 1% penicillin-streptomycin solution (PS, Gibco), and 10% inactivated fetal bovine serum (FBS, Gibco). Further, subcultures were performed 2 to 3 times a week on cultured cells.

2.8 Toxicity testing

Cultured RAW 264.7 cells were seeded in 96-well plates for 24 h at 2×10^5 cells/mL. After removing the medium, incubation

of the 96 well plate was conducted for 48 hours with medium supplemented with various concentrations of FLPE and UFLPE, and 1 μg/mL LPS (Sigma-Aldrich Corporation, St. Louis, MO, USA). Toxicity testing was carried out based on previous research experimental methods (Pan et al., 2020).

2.9 NO production

Cultured RAW 264.7 cells were seeded into 6-well plates for 24 hours at 2×10^5 cells/mL. Subsequently, DMEM containing different concentrations (0.4 and 0.8 mg/mL) of FLPE and UFLPE were added along with and LPS (1 μg/mL) to each well and incubated for 48 hours. In other words, the groups and processing are as follows, Control: no treatment, LPS: lipopolysaccharide (1 μg/mL), FH: LPS + FLPE (0.8 mg/mL), FL: LPS + FLPE (0.4 mg/mL), UH: LPS + UFLPE (0.8 mg/mL), UL: LPS + UFLPE (0.4 mg/mL). The cell culture medium was collected for NO production assays, treated with equal amounts of Griess reagent (Enzo Life Sciences, Inc., Farmingdale, NY, USA), measurement of absorbance at 550 nm was performed with a Wallac Victor3 1420 Multilabel Counter.

2.10 Assessment of cytokine concentrations

2×10^5 cells/mL of RAW 264.7 cells were seeded into 6-well plates for 24 h. Incubation was carried out for 48 hours with different concentrations (0.4 and 0.8 mg/mL) of samples in a medium containing LPS (1 μg/mL). A wide range of enzyme-linked immunosorbent assays kits (BioLegend, San Diego, CA, USA) were used to measure enzyme levels in cell culture media, including IL-10, IL-1, IL-6, TNF-α and interferon (IFN)-γ.

2.11 Quantitative real-time polymerase chain reaction (qRT-PCR) for assessing mRNA levels

RAW 264.7 cells were seeded into 6-well plates for 24 hours at 2×10^5 cells/mL. After removing the medium, the samples were supplemented with different concentrations (0.4 and 0.8 mg/mL) and LPS (1 μg/mL), incubated for 48 hours. RNA extraction and amplification were performed according to the protocol of the previous study, and relative transcript levels of mRNA were calculated using the $2^{-\Delta\Delta Cr}$ method (Pan et al., 2020). Table 1 lists the primers used in this study.

2.12 Identification of proteins using western blot assay

RAW 264.7 cells were seeded into 6-well plates for 24 hours at 2×10^5 cells/mL. Media supplemented with various concentrations (0.4 and 0.8 mg/mL) of FLPE and UFLPE, and LPS (1 μg/mL) were added to each well after the medium was removed, then incubated for 48 hours. The protein was extracted, quantified, denatured, and electrophoresed according to previous research and experimental methods (Pan et al., 2020). From Santa Cruz Biotechnology (Santa Cruz, CA, USA), the first antibodies for IL-6, NF-KappaB p65, cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), caspase 3, caspase 9, p21, p53, Bax, Bcl2, and E-actin were purchased. As a final step, the bands of the proteins were visualized by an Amersham imager 680 (GE Healthcare, Chicago, IL, USA).

Table 1. Primer sequences of RT-qPCR assay in this study.

Gene Name	Primer sequence
IL-6	F: 5'-ATGAAGTTCCTCTCTGCAA-3'
	R: 5'-AGTGGTATCCTCTGTGAAG-3'
IL-1β	F: 5'-AAGGGCTGCTTCCAAAC-3'
	R: 5'-CTCCACAGCCACAATGA-3'
IFN-γ	F: 5'-GCTTTGCAGCTCTTCCTCAT-3'
	R: 5'-GTCACCATCCTTTTGCCAGT-3'
NF-κB p65	F: 5'-ATGGCAGACGATGATCCCTAC-3'
	R: 5'-CGGAATCGAAATCCCCTCTGTT-3'
iNOS	F: 5'-ATGGCTTGCCCCTGGAA-3'
	R: 5'-TATTGTTGGGCTGAGAA-3'
COX-2	F: 5'-GGTGCCTGGTCTGATGATG-3'
	R: 5'-TGCTGGTTTGGAATAGTTGCT-3'
IL-5	F: 5'-GCACAGTTTTGTGGGGTTTT-3'
	R: 5'-AAAGAGAAGTGTGGCGAGGA-3'
IL-10	F: 5'-CCAAGCCTTATCGGAAATGA-3'
	R: 5'-TTTTCACAGGGGAGAAATCG-3'
IL-4	F: 5'-TCAACCCCCAGCTAGTTGTC-3'
	R: 5'-TGTTCTTCGTTGCTGTGAGG-3'
Caspase-3	F: 5'-TTTTTCAGAGGGGATCGTTG-3'
	R: 5'-CGGCCTCCACTGGTATTTTA-3'
Caspase-9	F: 5'-CTAGTTTGCCCACACCCAGT-3'
	R: 5'-CTGCTCAAAGATGTCGTCCA-3'
p53	F: 5'-ATGGAGGAGCCGCAGTCAGA-3'
	R: 5'-TGCAGGGGCCGCCGGTGTAG-3'
p21	F: 5'-ATGTCAGAACCGGCTGGGG-3'
	R: 5'-GCCGGGGCCCCGTGGGA-3'
Bax	F: 5'-TGCTTCAGGGTTTCATCCAG -3'
	R: 5'-GGCGGCAATCATCCTCTG-3'
Bcl2	F: 5'-AAGATTGATGGGATCGTTGC-3'
	R: 5'-GCGGAACACTTGATTCTGGT-3'
GAPDH	F: 5'-AGGTCGGTGTGAACGGATTTG-3'
	R: 5'-GGGGTCGTTGATGGCAACA-3'

2.13 Data analysis

Averaging the results of three or more parallel experiments was conducted. Plots and analyses were performed using GraphPad Prism (GraphPad Prism 9.3.1) and SPSS 22 softwear (SPSS Inc., IL, USA). Means and standard deviations (SD) are used to express experimental results. Differences in means between groups were assessed by unpaired T test or two-way ANOVA or one-way ANOVA using Duncan's multiple range test. And there is a significant difference if p is less than 0.05 or less than 0.1.

3 Results

3.1 Antioxidant capacities of FLP and UFLP

The results of the evaluation of the antioxidant capacities of FLP and UFLP are shown in Figure 1. The DPPH inhibition rate, TP, and TF contents indicated that FLP showed a better antioxidant capacity than UFLP.

3.2 Toxic effects of FLPE and UFLPE on RAW 264.7 cells

When the sample concentration was 0.8 mg/mL, the cell viability after FLPE and UFLPE treatment reached 81.10 ± 0.24%

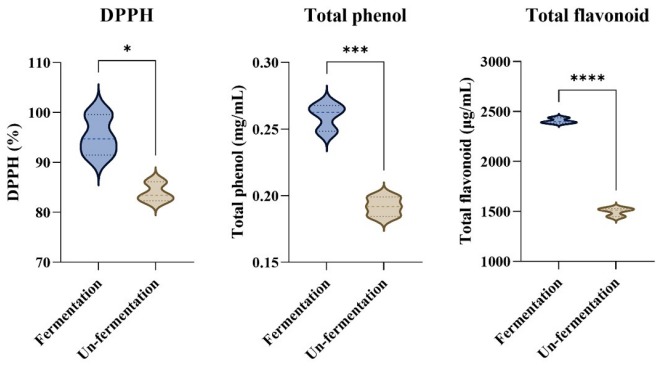

Figure 1. Antioxidant capacity of fermented lemon peel (FLP) and unfermented lemon peel (UFLP). The *, ***, **** symbol means significantly different ($p < 0.1$), ($p < 0.001$), ($p < 0.0001$), respectively, by unpaired T test.

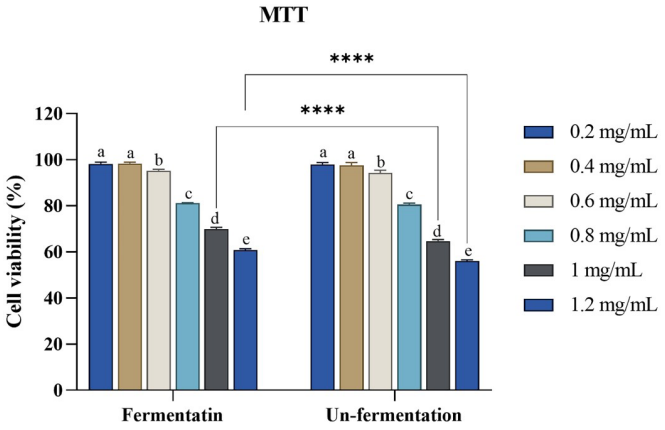

Figure 2. Effects of FLPE and UFLPE on the survival of RAW 264.7 cells. LPS: lipopolysaccharide (1 μg/mL), FH: LPS (1 μg/mL) + FLPE (0.8 mg/mL), FL: LPS (1 μg/mL) + FLPE (0.4 mg/mL), UH: LPS (1 μg/mL) + UFLPE (0.8 mg/mL), UL: LPS (1 μg/mL) + UFLPE (0.4 mg/mL). [a-f] Means with different letters above the bars are significantly different ($p < 0.05$) by Duncan's multiple range test. The **** symbol means significantly different ($p < 0.0001$) by 2 way ANOVA.

and 80.51 ± 0.68% (Figure 2), respectively. Therefore, the high concentration of the sample was set at 0.8 mg/mL. When the sample concentration was in the range of 0.2-0.4 mg/mL, the cell viability was higher than 97%, and different concentrations did not show any significant differences. Therefore, the highest concentration in this range, 0.4 mg/mL, was selected as the low concentration of the sample for subsequent experiments.

3.3 NO production by RAW 264.7 cells after FLPE and UFLPE treatment

The NO production of the cells increased significantly ($p < 0.05$) after LPS treatment in comparison with the control group (Table 2), whereas after FLPE and UFLPE treatment, the NO production was decreased significantly ($p < 0.05$), with FLPE treatment significantly lowering NO production when compared to UFLPE treatment.

Table 2. Effects of fermented lemon peel extract (FLPE) and unfermented lemon peel extract (UFLPE) on NO production in RAW 264.7 cells.

	NO production (μM)
Control	19.29 ± 0.23^{e}
LPS	28.39 ± 0.44^{a}
FH	19.72 ± 0.23^{de}
FL	20.14 ± 0.06^{d}
UH	21.21 ± 0.71^{c}
UL	25.16 ± 0.15^{b}

Control: no treatment, LPS: lipopolysaccharide (1 μg/mL), FH: LPS + FLPE (0.8 mg/mL), FL: LPS + FLPE (0.4 mg/mL), UH: LPS + UFLPE (0.8 mg/mL), UL: LPS + UFLPE (0.4 mg/mL). [a-e] Means with different letters within a column are significantly different ($p<0.05$) by Duncan's multiple range test.

3.4 FLPE and UFLPE effects on the levels of inflammatory cytokines in RAW 264.7 cell culture medium

A high concentration of inflammation cytokines (IL-6, IL-1β, TNF-α and IFN-γ) was observed in the culture medium of RAW 264.7 cells after LPS stimulation (Figure 3), as well as the lowest level of anti-inflammatory cytokine IL-10. A significant decrease or increase in these cytokines was observed in cell culture media after treatment with FLPE and UFLPE, with FH having the best effect, near normal levels of RAW 264.7 cells.

3.5 Effects of FLPE and UFLPE on the mRNA and the protein expressions of inflammation-related genes in RAW 264.7 cells

According to Figure 4, under the stimulation of LPS, the mRNA expressions of IL-6, IL-1β, NF-κB p65, COX-2, IFN-γ, iNOS and IL-5, as well as the protein expression of IL-6, NF-κB p65, COX -2, iNOS and TNF-α were high, and the mRNA expressions of IL-10 and IL-4 were low in the RAW 264.7 cells. Meanwhile, we found that FLPE and UFLPE treatment significantly ($p < 0.05$) suppressed LPS-induced inflammation in the RAW 264.7 cells, and under the administration of FH, the mRNA and protein expression levels of the above-mentioned inflammation-related genes were closest to the normal expression levels in RAW 264.7 cells.

3.6 Effects of FLPE and UFLPE on the mRNA and protein expressions of apoptosis-related genes in RAW 264.7 cells

In the LPS group, caspase 3, caspase 9, p21, p53, and Bax were the most expressed mRNAs and proteins, while Bcl2 was the least expressed mRNAs and proteins (Figure 5). We found that FLPE and UFLPE treatments had significant effects on cell cycle and apoptosis-related representative genes, and the anti-apoptotic effect of FH was the best, which was closest to the expression of each apoptosis-related gene in RAW 264.7 cells without LPS stimulation.

4 Discussion

Inflammation is a cascade of physiological or pathological defensive responses produced by the body against various inflammatory stimuli including infection and tissue damage. It is possible for excessive inflammation to damage many organs

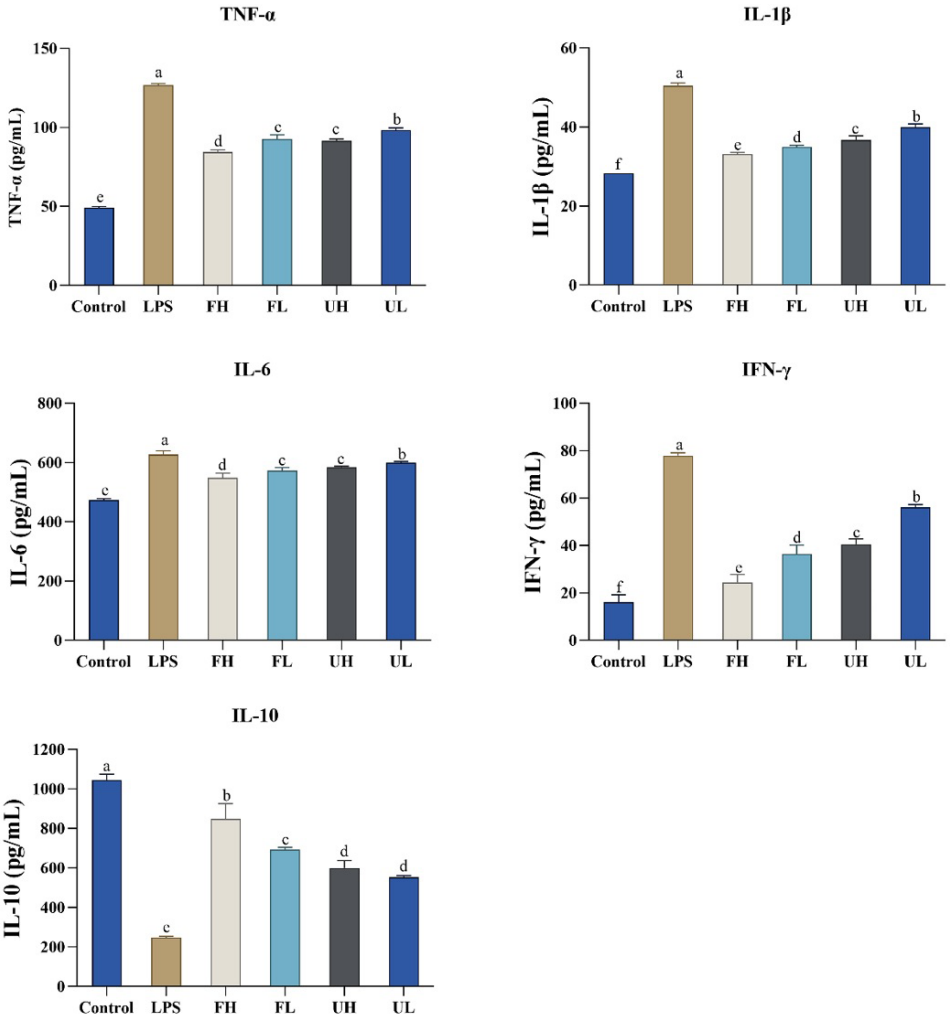

Figure 3. Effects of FLPE and UFLPE on the levels of cytokines TNF-α, IL-1β, IL-6, IFN-γ and IL-10 in RAW 264.7 cell culture medium. LPS: lipopolysaccharide (1 μg/mL), FH: LPS + FLPE (0.8 mg/mL), FL: LPS + FLPE (0.4 mg/mL), UH: LPS + UFLPE (0.8 mg/mL), UL: LPS + UFLPE (0.4 mg/mL). [a-f] Means with different letters above the bars are significantly different ($p < 0.05$) by Duncan's multiple range test.

of the body and may even be life-threatening in severe cases (Chen et al., 2017), thus, making it important and necessary to combat it. Studies have shown that citrus lemon peel powder reduces intestinal barrier defects and inflammation in mice with colitis (Tinh et al., 2021). Additionally, fermented dry *citrus unshiu* peel extract inhibits the inflammatory response induced by LPS, according to study (Kim et al., 2019). Our study examined the antioxidant properties of fermented lemon peel. Furthermore, we evaluated the anti-inflammatory and anti-apoptotic effects of fermented lemon peel on RAW 264.7 cells infected with LPS. The results clearly show that fermented lemon peel has good antioxidant capacity, and simultaneously exhibited anti-inflammatory and anti-apoptotic effects by inhibiting the release of pro-inflammatory cytokines and regulating the expression levels of genes related to inflammation and apoptosis.

Oxygen-free radicals are effectors of inflammatory responses, and the excessive production of these radicals can aggravate inflammatory responses. Some studies have pointed out that the pro-inflammatory cytokines released during an inflammatory response can activate macrophages and leukocytes to secrete a large number of peroxide-free radicals, thereby worsening inflammation. Therefore, antioxidants that scavenge free radicals could have the potential to reduce inflammation Samarghandian et al. (2016). the DPPH assay, TP, and TF contents are indicators of the antioxidant potential of a substance (Ghasemzadeh & Jaafar, 2014; Eor et al., 2021). It has been determined that the antioxidant properties of fermented lemon peel are superior to that of unfermented lemon peel in this study. Therefore, we postulated that the fermented lemon peel with good antioxidant activity could exert anti-inflammatory effects by quenching oxygen-free radicals.

Various immunopathological changes have been linked to NO, a reactive free radical. As an important pro-inflammatory mediator, NO levels are suggestive of the severity of inflammation. A large amount of NO is released by LPS in RAW 264.7 cells, thereby triggering multiple inflammatory pathological responses (Ji et al., 2021). TNF-α and IL-1β that trigger the cascade of inflammatory mediators. Of these, as an important pro-inflammatory cytokine, TNF-α, regulates immune cells, and induces fever and cell apoptosis by producing IL-1β and IL-6,

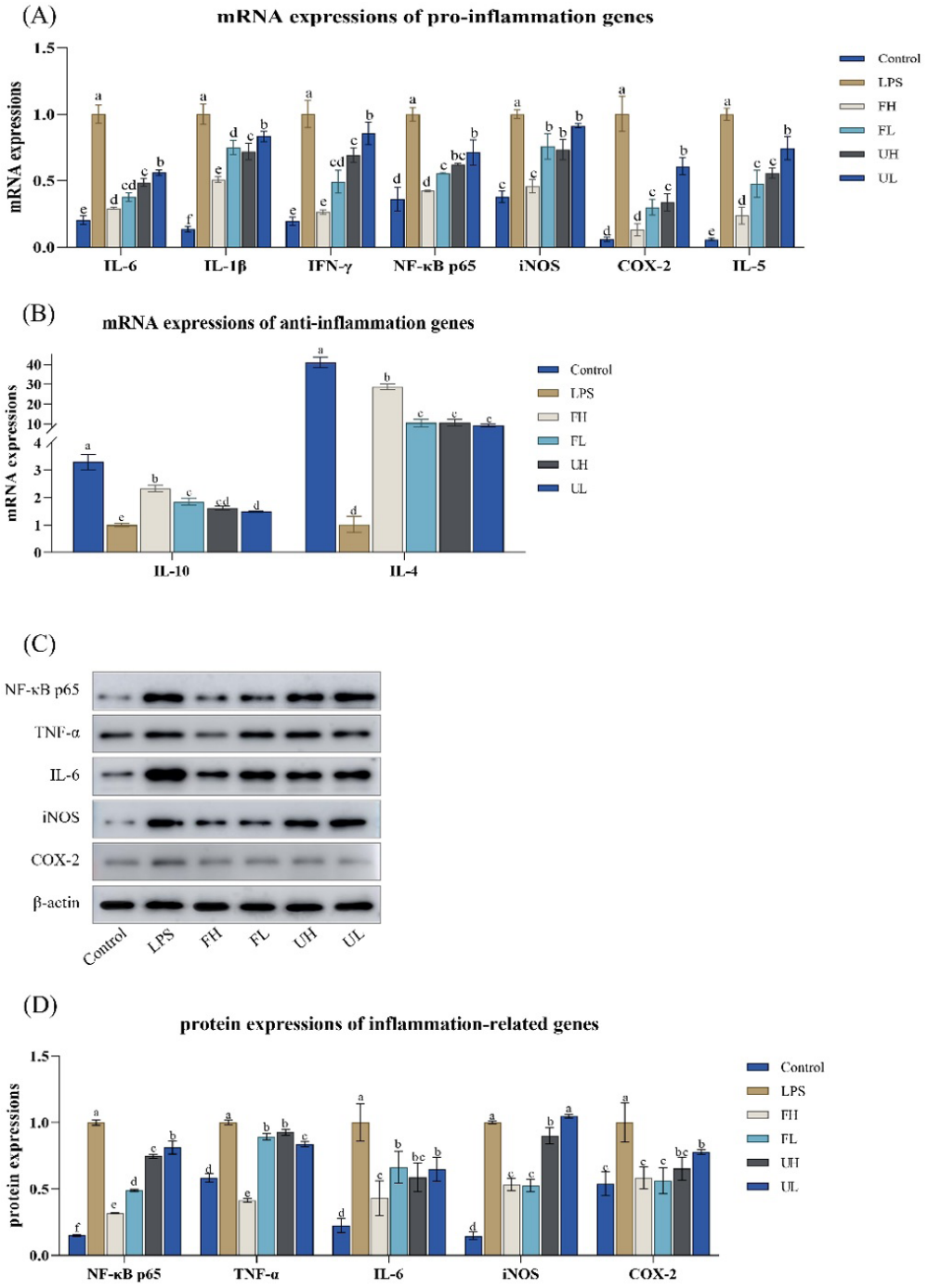

Figure 4. Effects of FLPE and UFLPE on the mRNA expression (A, B) of inflammation-related genes IL-6, IL-1β, NF-κB p65, COX-2, IFN-γ, iNOS, IL-5, IL-10 and IL-4, and protein expression (C, D) of IL-6, NF-κB p65, COX-2, iNOS and TNF-α in RAW 264.7 cells. LPS: lipopolysaccharide (1 μg/mL), FH: LPS + FLPE (0.8 mg/mL), FL: LPS + FLPE (0.4 mg/mL), UH: LPS + UFLPE (0.8 mg/mL), UL: LPS + UFLPE (0.4 mg/mL). [a-f] Means with different letters above the bars are significantly different ($p < 0.05$) by Duncan's multiple range test.

further triggering the inflammation cascade. As a multifunctional cytokine, IL-6 regulates immunological and inflammatory responses, and its expression is positively regulated by IL-1β and LPS (Al-Roub et al., 2021; Dimou et al., 2019). IFN-γ, a glycoprotein secreted by T lymphocytes and NK cells, increases macrophage sensitivity to TNF-α and other cytokine secretions, and its overproduction may lead to local inflammation and tissue destruction (Lee et al., 2013).

NF-κB plays a key role in cell inflammation and immunity by regulating the expression of cytokines and other pro-inflammatory genes. NF-κB p65 is activated and translocated from the cytoplasm to the nucleus, leading to the transcription of pro-inflammatory mediators, such as IL-6, TNF-α, and iNOS (Han et al., 2019; Liu et al., 2016; Wang et al., 2017). iNOS is a cytokine produced by activated macrophages. When iNOS levels are excessive, they promote the release of inflammatory

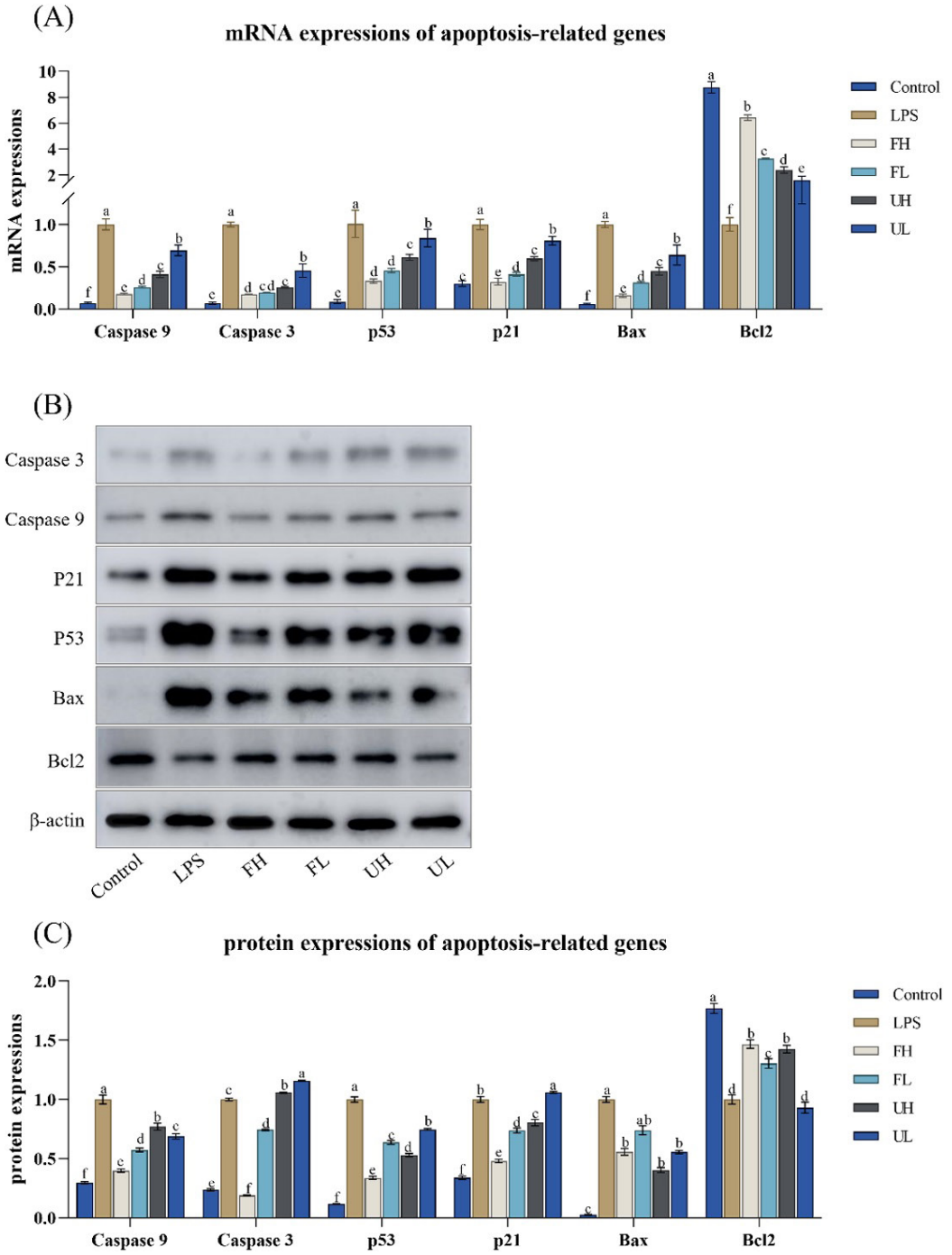

Figure 5. Effects of FLPE and UFLPE on the mRNA (A) and protein (B, C) expressions of apoptosis-related genes caspase 3, caspase 9, p21, p53, Bax and Bcl2 in RAW 264.7 cells. LPS: lipopolysaccharide (1 μg/mL), FH: LPS + FLPE (0.8 mg/mL), FL: LPS + FLPE (0.4 mg/mL), UH: LPS + UFLPE (0.8 mg/mL), UL: LPS + UFLPE (0.4 mg/mL). [a-f] Means with different letters above the bars are significantly different ($p < 0.05$) by Duncan's multiple range test.

cytokines and the production of NO, which leads to various types of inflammatory lesions in the body (Cinelli et al., 2020) An enzyme called COX-2 mediates inflammation and is also expressed in inflammatory cells provoked by LPS, pro-inflammatory cytokines, and tumor promoters (Gandhi et al., 2017). As macrophages and T lymphocytes become activated, IL-5 is secreted. IL-5, as a factor in the differentiation and growth of B lymphocytes and eosinophils, is often associated with autoimmune diseases accompanied by inflammatory responses (Jeon et al., 2014). The cytokines IL-4 and IL-10 play a crucial role in the regulation of the immune system and are anti-inflammatory cytokines. Studies have shown that the high expression of IL-4 and IL-10 in RAW264.7 monocyte-macrophages is beneficial for macrophages to play an immunoregulatory function (Han et al., 2021). Similar conclusions were drawn from our research results, which suggest that fermented lemon peel

effectively controls the inflammatory response of LPS-induced RAW 264.7 cells by regulating the pro- and anti-inflammatory responses, thereby restoring the balance.

Recent studies have found that bacterial infection or LPS induces apoptosis (Ezzat et al., 2021). As protease enzymes, caspases play an important role in apoptosis (McIlwain et al., 2013). Caspase-9 is required to initiate the intrinsic death program, which subsequently activates effector proteases, including caspase-3 (Tsuchiya, 2020). As a result of caspase-3, many key cellular proteins are cleaved, contributing to apoptosis (He et al., 2013). p53 and p21 have been identified as key mediators of cellular responses to DNA damage, apoptosis and cell cycle arrest (Bao et al., 2019). It is possible for p53 to initiate the apoptotic program directly from the cytoplasm, involving the release of Bax and activation of caspases without the presence of the nucleus (Yuan et al., 2016). When p53 is activated, transcription of p21 is induced, which results in reprogramming, senescence, and apoptosis of cells (Solhaug et al., 2012). In the process of cell apoptosis, both the pro-apoptotic protein Bax and the anti-apoptotic protein Bcl2 play important roles. Down-regulating Bax protein expression and up-regulating Bcl2 protein expression have been shown to inhibit macrophage apoptosis, thereby effectively enhancing the immunomodulatory function of macrophages (Zhang et al., 2020). Our results suggest that fermented lemon peel ameliorated LPS-induced macrophage inflammatory response injury by reducing macrophage apoptosis.

5 Conclusion

In the present study, the antioxidant capacity (DPPH clearance rate, TP and TF content) of FLP was stronger than that of UFLP. In addition, both FLPE and UFLPE alleviated the inflammation induced by LPS in RAW 264.7 cells to varying degrees, as well as FH inhibited inflammation and apoptosis of LPS-induced cell more. Meanwhile, after FH treatment, the levels of inflammatory cytokines in the cell culture medium and the mRNA and protein expressions of inflammation and apoptosis-related genes in the cells were the closest to the expression levels of normal RAW 264.7 cells. In conclusion, fermented lemon peel has excellent antioxidant capacity, and prominent inhibits the inflammatory and apoptotic effects of LPS on RAW 264.7 cells.

Conflict of interest

No conflicts of interest in this article.

Availability of data and material

The data that support the findings of this study are available on request from the corresponding author.

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References

Abdel Rahman, A. N., ElHady, M., & Shalaby, S. I. (2019). Efficacy of the dehydrated lemon peels on the immunity, enzymatic antioxidant capacity and growth of Nile tilapia (*Oreochromis niloticus*) and African catfish (*Clarias gariepinus*). *Aquaculture (Amsterdam, Netherlands)*, 505, 92-97. http://dx.doi.org/10.1016/j.aquaculture.2019.02.051.

Ahn, C. B., Cho, Y. S., & Je, J. Y. (2015). Purification and anti-inflammatory action of tripeptide from salmon pectoral fin byproduct protein hydrolysate. *Food Chemistry*, 168, 151-156. http://dx.doi.org/10.1016/j.foodchem.2014.05.112. PMid:25172694.

Al-Roub, A., Al Madhoun, A., Akhter, N., Thomas, R., Miranda, L., Jacob, T., Al-Ozairi, E., Al-Mulla, F., Sindhu, S., & Ahmad, R. (2021). L-1β and TNFα cooperativity in regulating IL-6 expression in adipocytes depends on CREB binding and H3K14 acetylation. *Cells*, 10(11), 3228. http://dx.doi.org/10.3390/cells10113228. PMid:34831450.

Asadi, A., Shidfar, F., Safari, M., Hosseini, A. F., Fallah Huseini, H., Heidari, I., & Rajab, A. (2019). Efficacy of *Melissa officinalis* L. (lemon balm) extract on glycemic control and cardiovascular risk factors in individuals with type 2 diabetes: A randomized, double-blind, clinical trial. *Phytotherapy Research*, 33(3), 651-659. PMid:30548118.

Bao, W. R., Li, Z. P., Zhang, Q. W., Li, L. F., Liu, H. B., Ma, D. L., Leung, C. H., Lu, A. P., Bian, Z. X., & Han, Q. B. (2019). Astragalus polysaccharide RAP selectively attenuates paclitaxel-induced cytotoxicity toward RAW 264.7 cells by reversing cell cycle arrest and apoptosis. *Frontiers in Pharmacology*, 9, 1580. http://dx.doi.org/10.3389/fphar.2018.01580. PMid:30804792.

Billant, J. (2021). Cutting edge technologies to end food waste. *Food Science and Technology (Campinas)*, 35(1), 40-45. http://dx.doi.org/10.1002/fsat.3501_11.x.

Boswell, L. (2021). Redistributing surplus food. *Food Science and Technology (Campinas)*, 35(2), 24-27. http://dx.doi.org/10.1002/fsat.3502_7.x.

Chen, L., Deng, H., Cui, H., Fang, J., Zuo, Z., Deng, J., Li, Y., Wang, X., & Zhao, L. (2017). Inflammatory responses and inflammation-associated diseases in organs. *Oncotarget*, 9(6), 7204-7218. http://dx.doi.org/10.18632/oncotarget.23208. PMid:29467962.

Cheng, Y., Wu, T., Tang, S., Liang, F., Fang, Y., Cao, W., Pan, S., & Xu, X. (2020). Fermented blueberry pomace ameliorates intestinal barrier function through the NF-κB-MLCK signaling pathway in high-fat diet mice. *Food & Function*, 11(4), 3167-3179. http://dx.doi.org/10.1039/C9FO02517K. PMid:32208477.

Cinelli, M. A., Do, H. T., Miley, G. P., & Silverman, R. B. (2020). Inducible nitric oxide synthase: Regulation, structure, and inhibition. *Medicinal Research Reviews*, 40(1), 158-189. http://dx.doi.org/10.1002/med.21599. PMid:31192483.

Cullen, L. (2021). Alternative proteins attract investment. *Food Science and Technology (Campinas)*, 35(2), 44-46. http://dx.doi.org/10.1002/fsat.3502_13.x.

Dimou, P., Wright, R. D., Budge, K. L., Midgley, A., Satchell, S. C., Peak, M., & Beresford, M. W. (2019). The human glomerular endothelial cells are potent pro-inflammatory contributors in an in vitro model of lupus nephritis. *Scientific Reports*, 9(1), 8348. http://dx.doi.org/10.1038/s41598-019-44868-y. PMid:31171837.

Eor, J. Y., Son, Y. J., & Kim, S. H. (2021). The anti-inflammatory and anti-oxidative potential of synbiotics in two independent cell lines. *International Journal of Dairy Technology*, 74(3), 518-527. http://dx.doi.org/10.1111/1471-0307.12777.

Ezzat, M. I., Hassan, M., Abdelhalim, M. A., El-Desoky, A. M., Mohamed, S. O., & Ezzat, S. M. (2021). Immunomodulatory effect of Noni fruit and its isolates: insights into cell-mediated immune response

and inhibition of LPS-induced THP-1 macrophage inflammation. *Food & Function*, 12(7), 3170-3179. http://dx.doi.org/10.1039/D0FO03402A. PMid:33734250.

Gandhi, J., Khera, L., Gaur, N., Paul, C., & Kaul, R. (2017). Role of modulator of inflammation cyclooxygenase-2 in gammaherpesvirus mediated tumorigenesis. *Frontiers in Microbiology*, 8, 538. http://dx.doi.org/10.3389/fmicb.2017.00538. PMid:28400769.

Ghasemzadeh, A., & Jaafar, H. Z. (2014). Optimization of reflux conditions for total flavonoid and total phenolic extraction and enhanced antioxidant capacity in Pandan (*Pandanus amaryllifolius* Roxb.) using response surface methodology. *TheScientificWorldJournal*, 2014, 523120. PMid:25147852.

Han, N. R., Kim, H. J., Lee, J. S., Kim, H. Y., Moon, P. D., Kim, H. M., & Jeong, H. J. (2021). The immune-enhancing effect of anthocyanin-fucoidan nanocomplex in RAW264.7 macrophages and cyclophosphamide-induced immunosuppressed mice. *Journal of Food Biochemistry*, 45(4), e13631. http://dx.doi.org/10.1111/jfbc.13631. PMid:33528053.

Han, S., Gao, H., Chen, S., Wang, Q., Li, X., Du, L. J., Li, J., Luo, Y. Y., Li, J. X., Zhao, L. C., Feng, J., & Yang, S. (2019). Procyanidin A1 alleviates inflammatory response induced by LPS through NF-κB, MAPK, and Nrf2/HO-1 pathways in RAW264.7 cells. *Scientific Reports*, 9(1), 15087. http://dx.doi.org/10.1038/s41598-019-51614-x. PMid:31636354.

He, J., Wang, Y., Xu, L. H., Qiao, J., Ouyang, D. Y., & He, X. H. (2013). Cucurbitacin IIa induces caspase-3-dependent apoptosis and enhances autophagy in lipopolysaccharide-stimulated RAW 264.7 macrophages. *International Immunopharmacology*, 16(1), 27-34. http://dx.doi.org/10.1016/j.intimp.2013.03.013. PMid:23541744.

Hu, X., Zeng, J., Shen, F., Xia, X., Tian, X., & Wu, Z. (2022). Citrus pomace fermentation with autochthonous probiotics improves its nutrient composition and antioxidant activities. *Lwt*, 157, 113076. http://dx.doi.org/10.1016/j.lwt.2022.113076.

Hwang, S. J., Kim, Y. W., Park, Y., Lee, H. J., & Kim, K. W. (2014). Anti-inflammatory effects of chlorogenic acid in lipopolysaccharide-stimulated RAW 264.7 cells. *Inflammation Research*, 63(1), 81-90. http://dx.doi.org/10.1007/s00011-013-0674-4. PMid:24127072.

Islam, M. N., Ishita, I. J., Jin, S. E., Choi, R. J., Lee, C. M., Kim, Y. S., Jung, H. A., & Choi, J. S. (2013). Anti-inflammatory activity of edible brown alga Saccharina japonica and its constituents pheophorbide a and pheophytin a in LPS-stimulated RAW 264.7 macrophage cells. *Food and Chemical Toxicology*, 55, 541-548. http://dx.doi.org/10.1016/j.fct.2013.01.054. PMid:23402855.

Jeon, C. M., Shin, I. S., Shin, N. R., Hong, J. M., Kwon, O. K., Kim, H. S., Oh, S. R., Myung, P. K., & Ahn, K. S. (2014). Siegesbeckia glabrescens attenuates allergic airway inflammation in LPS-stimulated RAW 264.7 cells and OVA induced asthma murine model. *International Immunopharmacology*, 22(2), 414-419. http://dx.doi.org/10.1016/j.intimp.2014.07.013. PMid:25066761.

Ji, S. Y., Cha, H. J., Molagoda, I. M. N., Kim, M. Y., Kim, S. Y., Hwangbo, H., Lee, H., Kim, G. Y., Kim, D. H., Hyun, J. W., Kim, H. S., Kim, S., Jin, C. Y., & Choi, Y. H. (2021). Suppression of lipopolysaccharide-induced inflammatory and oxidative response by 5-aminolevulinic acid in RAW 264.7 macrophages and zebrafish larvae. *Biomolecules & Therapeutics*, 29(6), 685-696. http://dx.doi.org/10.4062/biomolther.2021.030. PMid:33820881.

Kim, C., Ji, J., Ho Baek, S., Lee, J. H., Ha, I. J., Lim, S. S., Yoon, H. J., Je Nam, Y., & Ahn, K. S. (2019). Fermented dried Citrus unshiu peel extracts exert anti-inflammatory activities in LPS-induced RAW264.7 macrophages and improve skin moisturizing efficacy in immortalized human HaCaT keratinocytes. *Pharmaceutical Biology*, 57(1), 392-402. http://dx.doi.org/10.1080/13880209.2019.1621353. PMid:31188689.

Lee, D. M., Han, H. S., & Lee, Y. J. (2013). Effect of Hibisci flos on inflammatory cytokines production in lipopolysaccaride-stimulated raw 264.7 macrophages. *The Korea Journal of Herbology*, 28(5), 61-68. http://dx.doi.org/10.6116/kjh.2013.28.5.61.

Lee, K. H., Bong, Y. J., Lee, H. A., Kim, H. Y., & Park, K. Y. (2016). Probiotic effects of Lactobacillus plantarum and Leuconostoc mesenteroides isolated from kimchi. *Journal of the Korean Society of Food Science and Nutrition*, 45(1), 12-19. http://dx.doi.org/10.3746/jkfn.2016.45.1.012.

Liu, D. C., Gong, G. H., Wei, C. X., Jin, X. J., & Quan, Z. S. (2016). Synthesis and anti-inflammatory activity evaluation of a novel series of 6-phenoxy-[1,2,4] triazolo [3,4-a] phthalazine-3-carboxamide derivatives. *Bioorganic & Medicinal Chemistry Letters*, 26(6), 1576-1579. http://dx.doi.org/10.1016/j.bmcl.2016.02.008. PMid:26876930.

McIlwain, D. R., Berger, T., & Mak, T. W. (2013). Caspase functions in cell death and disease. *Cold Spring Harbor Perspectives in Biology*, 5(4), a008656. http://dx.doi.org/10.1101/cshperspect.a008656. PMid:23545416.

Pan, Y., Tan, J., Long, X., Yi, R., Zhao, X., & Park, K. (2022). Anti-obesity effect of fermented lemon peel on high-fat diet-induced obese mice by modulating the inflammatory response. *Journal of Food Biochemistry*, 46(8), e14200. http://dx.doi.org/10.1111/jfbc.14200. PMid:35484880.

Pan, Y., Zhao, X., Kim, S. H., Kang, S. A., Kim, Y. G., & Park, K. Y. (2020). Anti-inflammatory effects of Beopje curly dock (*Rumex crispus* L.) in LPS-induced RAW 264.7 cells and its active compounds. *Journal of Food Biochemistry*, 44(7), e13291. http://dx.doi.org/10.1111/jfbc.13291. PMid:32458452.

Rebollo-Hernanz, M., Zhang, Q., Aguilera, Y., Martin-Cabrejas, M. A., & Gonzalez de Mejia, E. (2019). Phenolic compounds from coffee by-products modulate adipogenesis-related inflammation, mitochondrial dysfunction, and insulin resistance in adipocytes, via insulin/PI3K/AKT signaling pathways. *Food and Chemical Toxicology*, 132, 110672. http://dx.doi.org/10.1016/j.fct.2019.110672. PMid:31306686.

Ritter, B., & Greten, F. R. (2019). Modulating inflammation for cancer therapy. *The Journal of Experimental Medicine*, 216(6), 1234-1243. http://dx.doi.org/10.1084/jem.20181739. PMid:31023715.

Rosa, L. S., Santos, M. L., Abreu, J. P., Balthazar, C. F., Rocha, R. S., Silva, H. L. A., Esmerino, E. A., Duarte, M. C. K. H., Pimentel, T. C., Freitas, M. Q., Silva, M. C., Cruz, A. G., & Teodoro, A. J. (2020). Antiproliferative and apoptotic effects of probiotic whey dairy beverages in human prostate cell lines. *Food Research International*, 137, 109450. http://dx.doi.org/10.1016/j.foodres.2020.109450. PMid:33233128.

Ruiz Rodríguez, L. G., Zamora Gasga, V. M., Pescuma, M., Van Nieuwenhove, C., Mozzi, F., & Sanchez Burgos, J. A. (2021). Fruits and fruit by-products as sources of bioactive compounds. Benefits and trends of lactic acid fermentation in the development of novel fruit-based functional beverages. *Food Research International*, 140, 109854. http://dx.doi.org/10.1016/j.foodres.2020.109854. PMid:33648172.

Russell, W. R., Neacsu, M., & Duncan, S. H. (2021). Microbiota-directed food formulation. *Food Science and Technology (Campinas)*, 35(1), 26-27. http://dx.doi.org/10.1002/fsat.3501_7.x.

Samarghandian, S., Azimi-Nezhad, M., Borji, A., & Farkhondeh, T. (2016). Effect of crocin on aged rat kidney through inhibition of oxidative stress and proinflammatory state. *Phytotherapy Research*, 30(8), 1345-1353. http://dx.doi.org/10.1002/ptr.5638. PMid:27279282.

Shimizu, C., Wakita, Y., Inoue, T., Hiramitsu, M., Okada, M., Mitani, Y., Segawa, S., Tsuchiya, Y., & Nabeshima, T. (2019). Effects of lifelong intake of lemon polyphenols on aging and intestinal microbiome in the senescence-accelerated mouse prone 1 (SAMP1). *Scientific Reports*, 9(1), 3671. http://dx.doi.org/10.1038/s41598-019-40253-x. PMid:30842523.

Solhaug, A., Vines, L. L., Ivanova, L., Spilsberg, B., Holme, J. A., Pestka, J., Collins, A., & Eriksen, G. S. (2012). Mechanisms involved in alternariol-induced cell cycle arrest. *Mutation Research. Fundamental and Molecular Mechanisms of Mutagenesis*, 738-739, 1-11. http://dx.doi.org/10.1016/j.mrfmmm.2012.09.001. PMid:23031795.

Tinh, N. T. T., Sitolo, G. C., Yamamoto, Y., & Suzuki, T. (2021). *Citrus limon* Peel powder reduces intestinal barrier defects and inflammation in a colitic murine experimental model. *Foods*, 10(2), 240. http://dx.doi.org/10.3390/foods10020240. PMid:33503995.

Tsuchiya, K. (2020). Inflammasome-associated cell death: Pyroptosis, apoptosis, and physiological implications. *Microbiology and Immunology*, 64(4), 252-269. http://dx.doi.org/10.1111/1348-0421.12771. PMid:31912554.

Wang, H., Gu, J., Hou, X., Chen, J., Yang, N., Liu, Y., Wang, G., Du, M., Qiu, H., Luo, Y., Jiang, Z., & Feng, L. (2017). Anti-inflammatory effect of miltirone on inflammatory bowel disease via TLR4/NF-κB/IQGAP2 signaling pathway. *Biomedicine and Pharmacotherapy*, 85, 531-540. http://dx.doi.org/10.1016/j.biopha.2016.11.061. PMid:27903427.

Wu, X., Gao, H., Hou, Y., Yu, J., Sun, W., Wang, Y., Chen, X., Feng, Y., Xu, Q. M., & Chen, X. (2018). Dihydronortanshinone, a natural product, alleviates LPS-induced inflammatory response through NF-kappaB, mitochondrial ROS, and MAPK pathways. *Toxicology and Applied Pharmacology*, 355, 1-8. http://dx.doi.org/10.1016/j.taap.2018.06.007. PMid:29906494.

Xi, W., Lu, J., Qun, J., & Jiao, B. (2017). Characterization of phenolic profile and antioxidant capacity of different fruit part from lemon (*Citrus limon* Burm.) cultivars. *Journal of Food Science and Technology*, 54(5), 1108-1118. http://dx.doi.org/10.1007/s13197-017-2544-5. PMid:28416860.

Xing, L., Fu, L., Toldrá, F., Teng, S., Yin, Y., & Zhang, W. (2022). The stability of dry-cured ham-derived peptides and its anti-inflammatory effect in RAW264.7 macrophage cells. *International Journal of Food Science & Technology*, ijfs.15800. http://dx.doi.org/10.1111/ijfs.15800.

Yuan, Z. H., Liang, Z. E., Wu, J., Yi, J. E., Chen, X. J., & Sun, Z. L. (2016). A potential mechanism for the anti-apoptotic property of koumine involving mitochondrial pathway in LPS-mediated RAW 264.7 macrophages. *Molecules (Basel, Switzerland)*, 21(10), 1317. http://dx.doi.org/10.3390/molecules21101317. PMid:27706063.

Zhang, Z., Zhang, Y., & Zhou, R. (2020). Loss of *Annexin* A5 expression attenuates the lipopolysaccharide-induced inflammatory response of rat alveolar macrophages. *Cell Biology International*, 44(2), 391-401. http://dx.doi.org/10.1002/cbin.11239. PMid:31502716.