

## RESEARCH ARTICLE

***Bifidobacterium lactis* Inhibits iNOS Expression in LPS-stimulated RAW 264.7 Macrophages**Bertoka Fajar Surya Perwira Negara<sup>1,2</sup>, Jae-Suk Choi<sup>1,2,\*</sup><sup>1</sup>Seafood Research Center, IACF, Silla University, 606, Advanced Seafood Processing Complex, Wonyang-ro, Amnam-dong, Seo-gu, Busan, 49277, Republic of Korea<sup>2</sup>Department of Food Biotechnology, College of Medical and Life Sciences, Silla University, 140, Baegyang-daero 700beon-gil, Sasang-gu, Busan, 46958, Republic of Korea

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## Abstract

**BACKGROUND:** *Bifidobacterium* is a genus of lactic acid bacteria that lives in the large intestine of humans and animals. The health benefits of this genus are well established; however, the anti-inflammatory activity of this genus, specifically *Bifidobacterium lactis*, has not been well defined. Therefore, in this study, we evaluated anti-inflammatory activity of *B. lactis* hydrolysates using lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages.

**METHODS:** RAW 264.7 cells were cultured using Dulbecco's Modified Eagle's Medium in 5% CO<sub>2</sub> incubator at 37°C. One µg/mL of LPS was used to stimulate RAW 264.7 cells. Nitric oxide (NO) production, inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2) were measured to evaluate anti-inflammatory activity of *B. lactis* hydrolysates. The cytotoxicity of the inhibitor was also measured in present study through 3-(4,5-dimethylthiazol-

2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay.

**RESULTS:** The results showed that *B. lactis* hydrolysates at 25–200 µg/mL inhibited NO production. In concentration-dependent manner, *B. lactis* hydrolysate showed inhibition of iNOS expression. However, no inhibition on COX-2 expression was observed. The MTS assay of the *B. lactis* hydrolysates showed no side effects on the cell viability at all concentrations.

**CONCLUSION:** The current study revealed that *B. lactis* hydrolysates possess specific anti-inflammatory effects by inhibiting iNOS expression without cytotoxicity and therefore could potentially be developed as a new iNOS inhibitor.

**KEYWORDS:** *Bifidobacterium lactis*, macrophages, hydrolysates, iNOS, COX-2

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## Introduction

Inflammation is an immune system mechanism and defensive reaction to external infection and tissue damage, promoting the repair of tissue structure and removing invading pathogens. Inflammation can be categorized into chronic and acute inflammation and presents double-sidedness. Several chronic diseases such as gastroenteritis, allergy, atherosclerosis, septic shock, arthritis, and cancer

are caused by the inflammatory mediator-induced chronic inflammation.(1) Increase in blood flow and cellular metabolism, cell cellular influx, leakage of fluid, and release of soluble mediators are all the common indicator of inflammation in human body.(2) There are two types of inflammation. Acute inflammation begins when a particular damage occurs, resulting in the production of soluble mediators and short duration, while chronic inflammation is caused by a failure to eradicate an irritant or low-intensity irritant that has been present for a long time.(3)

Several pro-inflammatory mediators, such as nitric oxide (NO), inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2), stimulate inflammation.(4) The pro-inflammatory mediators are produced in big value and are developed by iNOS and COX-2 while inflammation happen, and it also causes pain in inflammation.(5) The increasing production of iNOS, a type of reactive oxygen species (ROS), is related to various diseases, including cancer and chronic inflammation.

NO, produced by a family of nitric oxide synthases (NOS), is a signal of inflammation and an inflammatory mediator.(6) NO is a pro-inflammatory mediator that causes inflammation when overproduced. A common family of NOS enzymes is iNOS, which is generally expressed to the response of lipopolysaccharide (LPS) in macrophages, pro-inflammatory cytokines, tumour necrosis factor (TNF)- $\alpha$ , smooth muscle cells, astrocytes, and hepatocytes. (7) Together with reactive oxygen species (ROS), NO can damage DNA and mutagenesis. Moreover, LPS is a lipid and a polysaccharide commonly found in Gram-negative cell walls. In various cell types, LPS stimulates acute inflammation by releasing inflammatory cytokines. The LPS also acts as an activator of macrophages.(8) In several studies, LPS-stimulated macrophages have been used to activate macrophages and to evaluate the anti-inflammatory activities of active compounds.(9)

COX-2 is also known to mediate inflammation. Under normal conditions, this enzyme is barely detectable. In many different types of tumours and transformed cells, lipopolysaccharide and mutagenic substances, as pro-inflammatory mediators, increase COX-2 and boost cytokines.(10,11)

Arachidonic acid is also converted to prostaglandin (PG) by COX. Moreover, the production of PG, a pro-inflammatory agent, in various models of inflammation, was stimulated by COX-2.(12) COX-2 expression appears to have a role in carcinogenesis. Therefore, it is crucial to identify new inhibitors of both enzymes to inhibit inflammation.

Lactic acid bacteria (LAB) are live microorganisms that are categorized as Gram-positive, non-respiring, or non-sporulation bacteria. LAB have been associated with food fermentation, owing to the production of lactic acid. Moreover, owing to their versatile metabolism, LAB have been extensively used as probiotics and in the production of interesting compounds.(13) In particular, *Lactobacilli* and *Bifidobacteria* are two LAB candidates for the intervention of inflammation.(14) *Bifidobacteria* can reduce conditioned pathogens and host microbial homeostasis, maintain the

gut mucosal barrier's integrity, and prevent intestinal inflammation.(15,16)

Several studies reported that *Bifidobacteria* affect the mucus by increasing the production, enhancing barrier integrity, and exerting a trophic effect.(17) Moreover, *Bifidobacteria* also increase host defense to resist infection and lower hypersensitivity reactions to bacteria and food. Notably, *Bifidobacterium lactis* possess anti-inflammatory properties differently in monocytes and cells.(18) In the previous study, hydrolysed *B. lactis* with 2 mg/mL of lysozyme possessed anti-edema and anti-erythema activities in phorbol 12-myristate 13-acetate-treated mice.(19) The finding of this study reveal that *B. lactis* is a potential source of NO inhibitors. However, *B. lactis* hydrolysates have not been evaluated *in vitro*. Therefore, current study evaluated potential of *B. lactis* hydrolysates as an anti-inflammatory on their suppressive effect on the production of NO and expression of mRNA (iNOS and COX-2). Cytotoxicity assay of the *B. lactis* hydrolysates was also performed.

## Methods

### Chemicals, Strain and Media

High-performance liquid chromatography (HPLC)-grade and culture media were used in this study. HPLC-grade water (Millipore, Billerica, MA, USA) was used to prepare all solutions and media. *B. lactis* (KCTC 5854) was purchased from KCTC (Korean Collection for Type, Daejeon, Korea) and cultured in MRS broth (Difco<sup>TM</sup>, BD Biosciences, San Diego, CA, USA). The RAW 264.7 macrophage cell line was obtained from KCLB (Korean Cell Line Bank, Seoul, Korea).

### Preparation of *B. lactis* Hydrolysates

The hydrolysates of *B. lactis* were prepared following previous study.(20) In brief, *B. lactis* was incubated and maintained in MRS broth (Difco<sup>TM</sup>) at 37°C in a Bactron Anaerobic Chamber System (Shel Lab, Sheldon Manufacturing Inc., Cornelius, OR, USA). *B. lactis* was harvested until it resulted in 3.0 % absorbance at 625 nm. Thus, *B. lactis* was freeze-dried (VC2200; Gyrozen Co., Ltd. Daejeon, Korea).

Two mL of distilled water was used to dissolve the lyophilizate, which was then centrifuged for 10 min at 5,400 RCF (1.6R; Thermo Fisher Scientific Inc., Waltham, MA, USA). For the enzymatic hydrolysis, 2 mL of tris-ethylenediaminetetraacetic acid buffer and 2.0 mg/mL of lysozyme were mixed with the cell pellets. The mixture

was then incubated for 30 min at 37°C. The cell lysate was obtained by centrifuging the mixture for 10 min at 5,400 RCF. Ethanol was used to dilute the *B. lactis* lysate for the *in vitro* anti-inflammatory activity assay.

### Macrophage Cell Culture

Dulbecco's Modified Eagle's Medium (DMEM) was used to culture macrophage cell line supplemented with 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 10% heat-inactivated fetal bovine serum (FBS), 1 mM sodium pyruvate, 100 U/mL penicillin, 2 mM L-glutamine, 100 µg/mL streptomycin, and 0.2 % NaHCO<sub>3</sub>. RAW 264.7 were cultured in 5% CO<sub>2</sub> or 95 % air at 37°C using Forma 3111 Humidified Chamber (Thermo Fisher Scientific Inc., Waltham, MA, USA). The RAW 264.7 were washed twice. Then, RAW 264.7 was stimulated by 1 µg/mL of LPS.

### Cytotoxicity Assay

Cytotoxicity was determined following the previously published study.<sup>(21)</sup> The MTS test was used to determine the cellular toxicity of different doses of *B. lactis* hydrolysate depending on the reduction of MTS to formazan through mitochondrial dehydrogenases. The amount of formazan generated was measured using an enzyme-linked immunosorbent assay (ELISA) reader (Spectra Max 250, Sunnyvale, CA, USA) at 492 nm. The cytotoxicity was expressed in a percentage indicating the viability of cell.

### Measurement of NO Production

The production of NO was evaluated by knowing the increasing of NO<sup>2-</sup> in the culture supernatants. Briefly, 24-well culture plates were used to seed RAW264.7 cells (1×10<sup>6</sup> cells/well) and incubated for 2 h. After incubation, the medium was used to wash RAW264.7. Culture supernatants were collected, and *B. lactis* hydrolysates (10, 25, 50, 100, or 200 µg/µL) were added. One-hundred microliters of Griess reagent was added to the culture supernatants and

incubated at room temperature for 10 min. ELISA reader was used to measure optical densities (OD) at 540 nm, and serial dilutions of NaNO<sub>2</sub>, as a standard curve, was used to determine the nitrite concentration.

### RNA Preparation and Real Time-Polymerase Chain Reaction (RT-PCR)

The RAW 264.7, cultured using the above methods, were seeded (2×10<sup>5</sup>/well) in six-well culture plates and incubated in a 5% CO<sub>2</sub> incubator at 37°C for 24 h. Thus, the culture was washed three times with the medium, and the *B. lactis* hydrolysate was added. After the cells had been cultivated for the specified lengths of time, the culture supernatants were collected.

Total RNA was extracted using a Machery-Nagel GmbH & Co. KG Total RNA Isolation Kit (Düren, Germany), and ImProm-II<sup>tm</sup> reverse transcription system was employed to synthesize cDNA in a 20 µL mixture of RNA (1 µg), KCl (50 mM), deoxyribonucleotide triphosphates (1 mM), MgCl<sub>2</sub> (5 mM), RNase inhibitor (20 µL), oligo-(dT) primers, Tris/HCl (10 mM), and MuLV reverse transcriptase (50 µL). The primers used in this study were shown in Table 1. The mixture was incubated for 5 min at 94°C, and then heated for iNOS in a thermocycler (PTC-1148, Bio-Rad Laboratories, Inc., Hercules, CA, USA). For β-actin and COX-2, the mixture was incubated for 2 min at 94°C, and then heated in a thermocycler for 40 cycles. Electrophoresis (Mupid®-2plus; ADVANCE Co., Ltd., Tokyo, Japan) was performed on 2 % agarose gels (Agarose LE, Georgia Chem, Atlanta, GA, USA) in running buffer solution (1 mM EDTA, 40 mM Tris/HCl, and 40 mM acetic acid) for 50 min at 100 V. UV gel documentation system (I-MAX-MC2000, Core Bio, Ann Arbor, MI, USA) was used to visualize DNA retention. β-actin was used to normalize and the quantification of mRNA expression (iNOS and COX-2). ImageJ software (National Institute of Health, Bethesda, MD, USA) was used to calculate the ratio (%) of mRNA.

**Table 1. Nucleotide sequences of the primers used for PCR amplification in this study.**

Parameters		Primer Sequences
iNOS	Sense	5'-CCCTTCCGAAGTTTCTGGCACCACC-3'
	Antisense	5'-GGCTGTCAGAGCCTCGTGGCTTTGG-3'
COX-2	Sense	5'-CTGACCCACTTCAAGGGAGTCTGG-3'
	Antisense	5'-CCATCCTGGAAAAGGCGCAGTT-3'
β-actin	Sense	5'-TGACCGAGCGTGGCTACAGC-3'
	Antisense	5'-ACCGCRCATTGCCGATAGTG-3'

## Statistical Analysis

All experiments were done with three replications independently. One-way ANOVA (analysis of variance) was employed to obtain the significance of the results at a 95% level of probability ( $p < 0.05$ ) (SPSS, Chicago, IL, USA).

## Results

### Cell Growth Inhibition by Inhibitor

The short-term cytotoxicity (24 h) of *B. lactis* hydrolysate was measured using MTS assay. At any concentration (10, 25, 50, 100, and 200  $\mu\text{g/mL}$ ), the inhibitory effects were not observed on cell viability of the *B. lactis* hydrolysate (Figure 1).

### Effect of Inhibitor on NO Production

Using an activated RAW 264.7, *B. lactis* hydrolysate was tested for anti-inflammatory activity by measuring NO production inhibition. *B. lactis* hydrolysate at 25–200  $\mu\text{g/mL}$ , in a dose-dependent manner, exhibited potent inhibition of NO production (Figure 2). However, at 10  $\mu\text{g/mL}$ , the *B. lactis* hydrolysate did not inhibit NO production.

### Effect of Inhibitor on iNOS and COX-2 Expression

The anti-inflammatory effect of *B. lactis* hydrolysate was examined *in vitro* through mRNA expression (iNOS and COX-2) suppressive effect. The macrophage was treated with either blank, negative control (only treated with LPS), or 10, 25, 50, 100, or 200  $\mu\text{g}/\mu\text{L}$  of *B. lactis* hydrolysate

(with LPS), and the expression ratios of the iNOS mRNA were 100%, 93.2%, 71.6%, 65.4%, 42.4% and 12.9%, respectively (Figure 3). When the cell lines were treated with blank, negative control (only treated LPS), or 10, 25, 50, 100, or 200  $\mu\text{g}/\mu\text{L}$  of *B. lactis* hydrolysate (with LPS), the expression ratios of the COX-2 mRNA were 100%, 99.6%, 87.9%, 102.9%, 105.3%, and 103.3%, respectively (Figure 4). In a concentration-dependent, *B. lactis* hydrolysate inhibited iNOS mRNA expression; however, in LPS-stimulated RAW 264.7 cells, the COX-2 mRNA expression was not inhibited by *B. lactis* hydrolysate.

## Discussion

In inflammation, several protectives of immune system are involved including pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). These conditions are controlled by pathogen-recognition receptors (PRRs). Moreover, these receptors can activate the pro-inflammatory such as NO and ROS. The activation of pro-inflammatory leads inflammation into chronic inflammation.(22,23)

ROS are produced during the inflammation. As the inflammation goes by, the overproduction of ROS leads the inflammation to chronic inflammation as effects of protein, nucleic acids, and lipids damage. This condition also activates inflammatory mediators. Chronic inflammation causes several diseases including asthma, bronchitis, liver fibrosis, pancreatic, and neurodegenerative disorders.

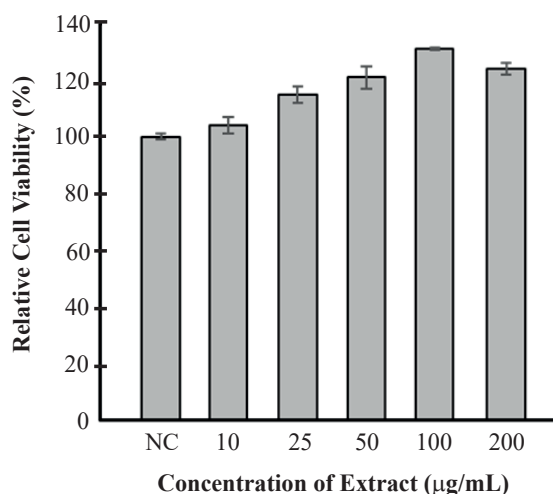


Figure 1. The effects of *B. lactis* hydrolysates at different concentrations on the viability of RAW 264.7 cells after 24 h of treatment based on the MTS assay. The values shown represent the mean  $\pm$  standard deviation of the mean.

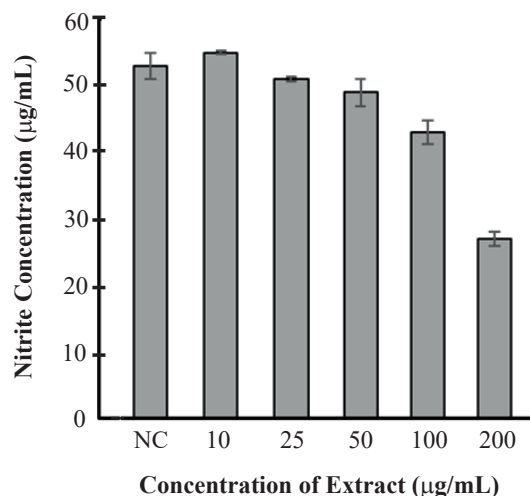
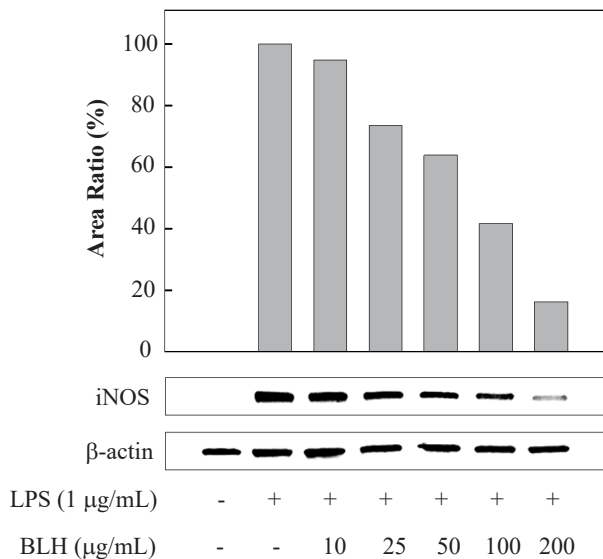


Figure 2. The effects of *B. lactis* hydrolysates at different concentrations on NO production in LPS-stimulated RAW264.7 cells. The values shown represent the mean  $\pm$  standard deviation of the mean.

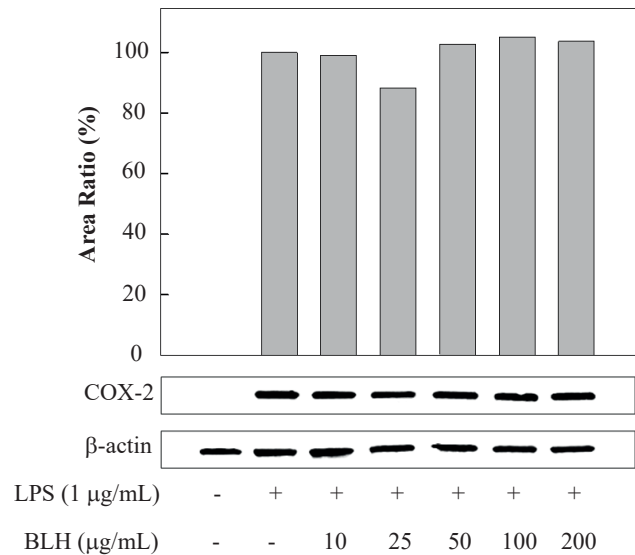


**Figure 3.** The effects of of *B. lactis* hydrolysates (BLH) on iNOS expressions in LPS-induced RAW 264.7 cells. Cells were treated with different concentrations of *B. lactis* hydrolysates for 1 h and then LPS was added and cells were incubated for 24 h.

In addition, on macrophages, chronic inflammation stimulates Toll-like receptors expression and inflammatory mediators are produced excessively.(24) In normal inflammatory reactions, the synthesis of pro-inflammatory mediators diminishes with time, whereas the number of anti-inflammatory mediators increases, limiting the inflammatory response itself.(25) Macrophages are the cell types involved in inflammation. They produce various inflammatory mediators, including NO, through iNOS and COX-2 expression.(26)

Naturally, the immune system has some mechanisms to reduce inflammation such as regulating chemical mediators (vasoactive peptides and amines), various immune cells, acute-phase proteins, pro-inflammatory cytokines, and eicosanoids.(27) These mechanisms avoid tissue damage and repair the tissue functions. The process of recovery from inflammation involves complex pathways. Anti-inflammation is needed to accelerate the recovery.

Anti-inflammatory is a compound that not only reduces inflammation but also inhibits the pro-inflammatory mediators. As a compound that has an effect on diminishing inflammation, anti-inflammatory, in several cases, has side effects. The use of anti-inflammatory decreases the function of the gastrointestinal tract.(28) Moreover, anti-inflammatory has side effects on the small and large intestine in humans including bleeding, perforation, and large intestinal ulcers.(29) These side effects indicate that the use of anti-inflammatory in the long term will be harmful. It is potential to find new anti-inflammation with less side effects. In the



**Figure 4.** The effects of of *B. lactis* hydrolysates (BLH) on COX-2 expressions in LPS-induced RAW 264.7 cells. Cells were treated with different concentrations of *B. lactis* hydrolysates for 1 h and then LPS was added and cells were incubated for 24 h.

current study, we examined the anti-inflammatory potential of *B. lactis* hydrolysates in LPS-stimulated macrophages by the inhibition of iNOS and COX-2.

The anti-inflammatory activity was assessed by testing the effect of *B. lactis* hydrolysate on the production of NO in RAW 264.7. The results revealed that in a dose-dependent manner, *B. lactis* hydrolysate inhibited NO production in the RAW 264.7. The results of this study agree with several studies who reported that NO production in cell line was inhibited by showing anti-inflammatory activity.(30-32)

In many mammalian cells and tissues, L-arginine produces NO and iNOS via constitutive.(6) The expression of neuronal NOS (nNOS) and endothelial NOS (eNOS) is an important factor that regulates homeostasis. NO is synthesized by iNOS through immunological stimuli (interferon (IFN)- $\gamma$ ) or bacterial LPS. Furthermore, iNOS-mediated sustained NO production is linked to inflammation. Regardless of the fact that iNOS activation is not a key event in all diseases, sustained NO production by iNOS results in cytotoxic and proinflammatory effects and contributes to disease pathophysiology. Therefore, finding a new inhibitor of iNOS may be beneficial.

In the current study, the results of NO inhibition support the results of iNOS gene expression. A decrease in NO production reduces iNOS expression. Previous studies found that the reduction in NO decreased the expression of iNOS in cell lines. The decreasing of iNOS by natural compounds can make that compounds as novel candidate for anti-inflammatory treatment.(33) Interestingly, the

number of nitric oxide and prostaglandins can be reduced significantly by selective inhibition of iNOS in the site of inflammation.(34)

However, although iNOS and COX-2 are often found together in inflamed tissues and are generated by many cytokines, our results showed that a decrease in iNOS did not reduce the COX-2 expression. This might be caused by several factors including the lack of activity of the *B. lactis* hydrolysate to inhibit the activation of peroxynitrite, to control the production of cytokine, and to drive chemokines. Peroxynitrite activation of peroxidase mediates the activation of COX-2 by NO.(35) NO increased the COX-2 expression in cell types through peroxynitrite, either directly or indirectly.(36) Moreover, the less activation of transcription factors and chemokines also contributed to the lack activity of the *B. lactis* hydrolysate to suppress COX-2 expression. Several studies proved that the activation of chemokines can stimulate immune systems to produces diminish inflammation through DCs. (37) Furthermore, *B. lactis* hydrolysate might not control the production of cytokines (IL-6, IL-8 and IL-10) and chemokines (IFN- $\gamma$  production). By controlling the production of cytokines and chemokines, the COX-2 expression could be suppressed.(38)

Interestingly, lack of activity of *B. lactis* hydrolysate in reducing COX-2 expression implies that *B. lactis* might not have the side effects of COX-2 inhibitors, such as acute renal failure, modest elevations in blood pressure, and edema.(39) The results of this study indicate that *B. lactis* hydrolysate possesses specific anti-inflammatory by inhibiting NO production and decreasing in iNOS expression. Given that *B. lactis* is categorized as a probiotic and contains varying compositions of intrinsic compounds, its biological activity could be strain-specific.(40)

In addition, we evaluated viability of RAW 264.7 using MTS assay to know the effects of *B. lactis* hydrolysates, as an inhibitor. After 24 h of incubation, *B. lactis* hydrolysates showed no inhibitory effects on cell viability. The results of this study indicate that *B. lactis* hydrolysate possesses anti-inflammatory activities by prohibiting iNOS expression and reducing NO production, without exerting a cytotoxic effect on macrophages.

## Conclusion

In this study, our results showed that the *B. lactis* hydrolysate possesses anti-inflammatory activities without side effects such as cytotoxicity. The mechanisms of anti-inflammatory

activities of *B. lactis* hydrolysate through NO inhibition involved either suppression of iNOS gene expression or scavenging of the produced NO. The findings of this study are useful for the development of new iNOS inhibitors that are applicable to cosmetics and pharmaceuticals.

## Authors Contribution

JSC was involved in planning and supervised the work, performed the calculations and statistical analysis, and aided in interpreting the results and worked on the manuscript. BFSPN performed the measurements, processed the experimental data, performed the analysis, drafted the manuscript and designed the figures. All authors discussed the results and commented on the manuscript.

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