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Bioactive Compounds in Health and Disease

Anti-inflammatory activity of *Lactobacillus* spp. and *Rhodopseudomonas palustris* probiotics

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ABSTRACT

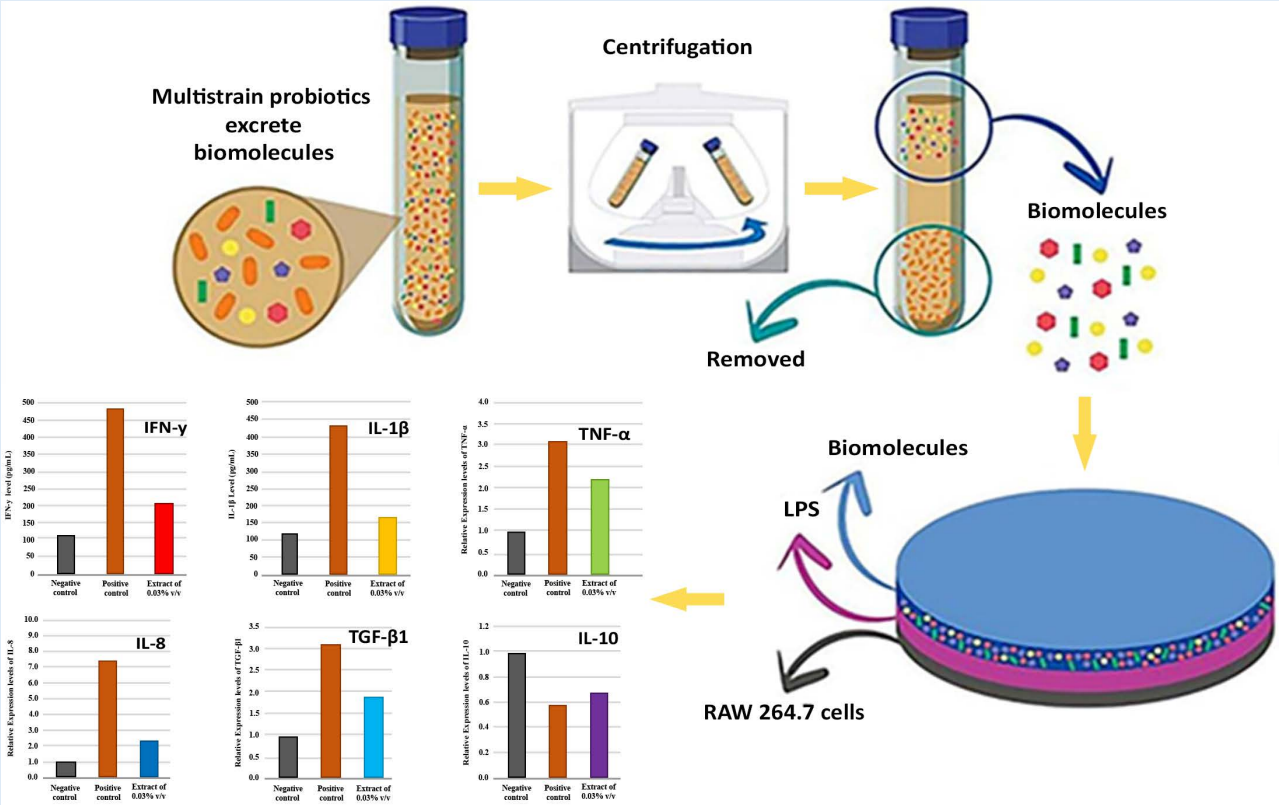
Background: Probiotics have been used for many years to promote human health by mitigating inflammation. However, their mechanisms have not been fully elucidated. During inflammation, excessive and/or prolonged production of pro-inflammatory cytokines is related with various inflammatory diseases and cancer. Several probiotics have been reported as playing a role in suppressing the level of pro-inflammatory cytokines, as the human body attempts to recover itself. The aim of this study was to evaluate the anti-inflammatory activity of probiotics consisting of *Lactobacillus* spp. and *Rhodopseudomonas palustris* on macrophage RAW 264.7 cells.

Methods: The probiotics mixture was centrifuged to separate supernatant, i.e., the probiotics extract, from the cells. The probiotics extract was then evaluated for its effects on cell viability and for anti-inflammatory activity of LPS inflammation-induced RAW 264.7 cells.

Results: The results showed that the probiotics extract was able to decrease the level of IFN-γ, IL-1β, TNF-α, IL-8, TGF-β1 pro-inflammatory cytokines or mRNAs, and increase the level of IL-10 anti-inflammatory mRNA.

Conclusion: The probiotics extract was identified to have anti-inflammatory activity. Thus, the probiotics are promising to be used for treatment of chronic inflammatory conditions.

Keywords: probiotics, ***Lactobacillus* spp.**, ***Rhodopseudomonas palustris*, RAW 264.7 cells, anti-inflammatory activity**



INTRODUCTION

Inflammation is an immunological body response to harmful stimuli, such as damaged self-cells, irritants, infections, and toxins. Among many cells involved in the inflammation process, macrophage plays a major role [1]. During inflammation, macrophage is concentrated at the site of inflammation and releases various pro-inflammatory cytokines such as interferon gamma (IFN-γ), interleukin 1 beta (IL-1β), tumor necrosis factor alpha (TNF-α), interleukin 8 (IL-8), and transforming growth factor beta 1 (TGF-β1). Besides various pro-inflammatory cytokines, macrophages also produce anti-inflammatory cytokines [2]. The cytokines could be used as biomarkers for diagnosis, prognosis, and therapeutics [1]. The excessive and/or prolonged pro-inflammatory cytokines production is related to various inflammatory diseases such as lung inflammation, rheumatoid arthritis, and diabetes mellitus. It is also related to various cancer types such as lung cancer, colon cancer, and skin cancer [3]. A recent example of excessive and prolonged production of pro-inflammatory cytokines can be found during cytokine storm related to Covid-19 [4].

Synthetic drugs are extensively used to treat inflammation. However, prolonged treatment with such drugs is usually accompanied with their prominent adverse reactions. Hence, one of the advantageous approaches to treat prolonged inflammation is using probiotics to suppress the pro-inflammatory cytokines production [5]. For this reason, the use of probiotics is gaining great interest. Certain microorganisms produce specific metabolites which may beneficially suppress excessive and/or prolonged production of pro-inflammatory cytokines, thus giving an anti-inflammatory effect [6]. Probiotics are also known to have limited side effects that are contributed by their pleiotropic immune modulatory behavior [7].

This study aimed to evaluate the anti-inflammatory activity of probiotics extract on macrophage RAW 264.7 cells. The probiotics in the present study consist of the symbiotic culture of *L. bulgaricus*, *L. casei, L. rhamnosus*, *L. fermentum*, *L. plantarum,* and *R. palustris* with each contributing for 2×10^6 colony-forming unit (CFU)/mL in a medium containing 1.1% v/v of *Aloe vera* juice, 1.1% v/v of honey, 3.1% v/v of molasses, 0.2% b/v of citric acid, and water, with a pH of 3.2.

To this end, the macrophage RAW 264.7 cells were first inflammation-induced with lipopolysaccharide (LPS). The probiotics extract was then added into the cells. The level of inflammatory cytokines or the relative expression level of mRNAs was measured with enzyme-linked immunosorbent (ELISA) or with real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) assays.

METHODS

Preparation of probiotics extract: The probiotics mixture was centrifuged for 15 min at 4000 rpm to separate supernatant, i.e., the probiotics extract, from the cells. The probiotics extract was then evaluated for its effects on cell viability and for anti-inflammatory activity of LPS inflammation-induced RAW 264.7 cells.

Preparation of RAW 264.7 cells: Dulbecco's Modified Eagle Medium (DMEM, Biowest L0060) supplemented with 10% fetal bovine serum (Gibco 10082147) and 1% penicillin-streptomycin (Biowest L0022-100) was used to grow RAW 264.7 cells (ATCC TIB-71). Incubation was conducted at 5% of CO_2, 37°C until the cells reached confluence. The cells were then washed with phosphate-buffered saline (PBS) and harvested using trypsin-EDTA (Biowest L0931-500) [8].

Cell viability assay: Cells with a density of 5×10^5 cells/well (180 µL) were seeded in a 96 well plate and incubated for 24 h at 5% of CO_2, 37 °C. After the cells were confluent, the culture media was removed, and 180 µL of medium containing LPS at a final concentration of 1 µg/mL was added into each well and the cultures were incubated for 18 h. Then, 20 µL of probiotics extract at several final concentrations (0.03-2.00% v/v) was added into each well. For negative control, after the culture media was removed, 200 µL of medium (without LPS) was added into each well, and for positive control, after the culture media was removed, 200 µL of medium containing LPS at a final concentration of 1 µg/mL was added into each well. The cultures were then incubated for 24 h. Then, 20 µL of [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazol-ium)] (MTS) Cell Proliferation Assay Kit (Abcam ab197010) was added into each well. After 3 h of incubation, the absorbance at 490 nm was measured using a microplate reader [8].

Treatment with probiotics extract: Cells with a density of 5×10^5 cells/well (180 µL) were seeded in a 96 well plate and incubated for 24 h at 5% of CO_2, 37 °C. After the cells reached confluence, the culture media was removed, and 180 µL of medium containing LPS at a final concentration of 1 µg/mL were added into each well and the cultures were incubated for 18 h. Then, 20 µL of probiotics extract at the selected concentration, i.e., the concentration giving the greatest % cell viability based on *cell viability assay*, was added into each well. The cultures were incubated for 24 h, then collected, and centrifuged for 15 min at 4000 rpm. The supernatant was taken for ELISA assays of the level of IFN-γ and IL-1β cytokines , and the pellet was taken for qRT-PCR assays of the level of TNF-α, IL-8, TGF-β1, and IL-10 mRNAs.

The level of IFN-γ and IL-1β cytokines upon inflammation induction (i.e., of the positive controls) and upon treatment with the probiotics extract was measured

using ELISA Kits (E-EL-M0048 and E-EL-M0037, Elabsci). Briefly, 100 µL of probiotics extract was added into each well and the plates were incubated at 37 °C for 90 min. The unbound components were discarded, and the plates were washed with PBS buffer, then 100 µL/well of biotinylated detection antibody was added into each well, and the plates were incubated at 37 °C for 60 min. After the unbound components were discarded and the plates were washed with PBS buffer, 100 µL/well of avidin-horseradish peroxidase conjugate was added into each well, and the plates were incubated at 37 °C for 30 min. After the unbound components were discarded and the plates were washed, 90 µL/well of tetramethylbenzidine substrate was added into each well and the plates were incubated at 37 °C for 30 min. After color change, 50 µL/well of H_2SO_4 solution was added into each well to stop the reaction and the absorbance at 450 nm was measured using a microplate reader. The absorbance was proportional to the level of IFN-γ and IL-1β cytokines.

Meanwhile, the level of TNF-α, IL-8, TGF-β1, and IL-10 mRNAs upon inflammation induction (i.e., of the positive controls) and upon treatment with probiotics extract was measured using qRT-PCR [9]. Briefly, total mRNA was extracted from the harvested RAW 264.7 pellet using RNA Isolation Kit (Zymo, R2073). cDNA was then generated from 200 ng of the total isolated mRNA using iScript Reverse Transcription Supermix for RT-PCR (Bio-Rad 170-8841). The copy number of TNF-α, IL-8, TGF-β1, and IL-10 genes was measured with qRT-PCR using primers from Origene (Table 1). The quantity of the inflammatory cytokine genes was expressed as their relative expression levels to that of β-actin reference gene.

Table 1. Primers used for qRT-PCR assays.

Gene	Forward (5'-3')	Reverse (5'-3')	Product Length (Base pairs)	Reference
TNF-α	CCTCACACTCAGATCATCTTCTC	GTTGTCTTTGAGATCCATGCC	145	NM_013693.3
IL-8	GAAGTGATAGCAGTCCCA	AGCTAAAATCAGCAAAGTGTC	258	NM_011339.2
TGF-β1	AACCAAGGAGACGGAATACAG	TGGAGCTGAAGCAATAGTTGG	241	NM_011577.2
IL-10	GAAGACAATAACTGCACCCA	AACCCAAGTAACCCTTAAAGTC	163	NM_010548.2
β-actin	AAGATCAAGATCATTGCTCCTCC	TAACAGTCCGCCTAGAAGCA	164	NM_007393.5

RESULTS

In the present study, the anti-inflammatory activity of the probiotics extract was evaluated on the inflammation-induced macrophage RAW 264.7 cells, one of the commonly used cell models for anti-inflammatory studies [10].

RAW 264.7 cell viability assay: Cell viability was evaluated using the MTS method based on the yellow tetrazolium salt conversion to a purple formazan product. The results showed that the probiotics extract with final concentrations up to 1% v/v give % cell viability greater than that of the positive control (LPS inflammation induction), i.e., greater than 80% (Table 2), indicating that the probiotics extract with the concentrations up to 1% v/v were nontoxic for cells and hence safe to use.

Table 2. RAW 264.7 cell viability assay.

Parameter evaluated		Cell viability (%)
Negative control (medium only)		100.00 ± 1.63
Positive control (LPS inflammation induction)		80.06 ± 1.81
Extract concentration, % v/v	0.03*	131.30 ± 0.80
	0.06	118.43 ± 2.27
	0.13	106.18 ± 10.31
	0.25	91.69 ± 8.30
	0.50	90.70 ± 3.64
	1.00	87.47 ± 3.60
	2.00	73.89 ± 0.78

Data were presented as mean ± SD of 3 replications. *Concentration giving the greatest % cell viability that is taken for further evaluation.

Inflammation induction of RAW 264.7 cells: The level of IFN-γ and IL-1β cytokines was measured using ELISA, and the level of TNF-α, IL-8, TGF-β1, and IL-10 mRNAs was measured using qRT-PCR.

Upon inflammation induction, the level of pro-inflammatory IFN-γ, IL-1β, TNF-α, IL-8, and TGF-β1 cytokines or mRNAs increased (Figures 1A and B, Figures 2A, B, and C), whereas that of IL-10 anti-inflammatory cytokine decreased (Figure 2D).

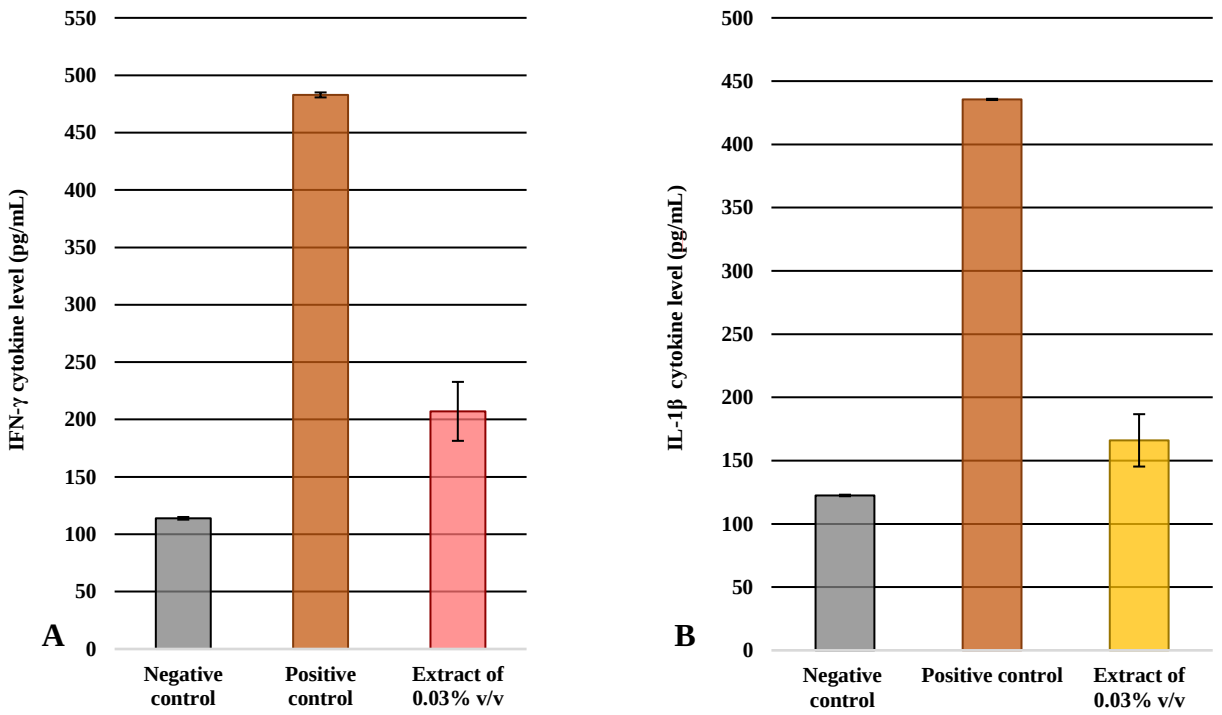


Figure 1. The level of IFN-γ (**A**) and IL-1β (**B**) cytokines upon inflammation induction (the positive control) and upon treatment with probiotics extract of 0.03% v/v of RAW 264.7 cells. The experiments were conducted in triplicate.

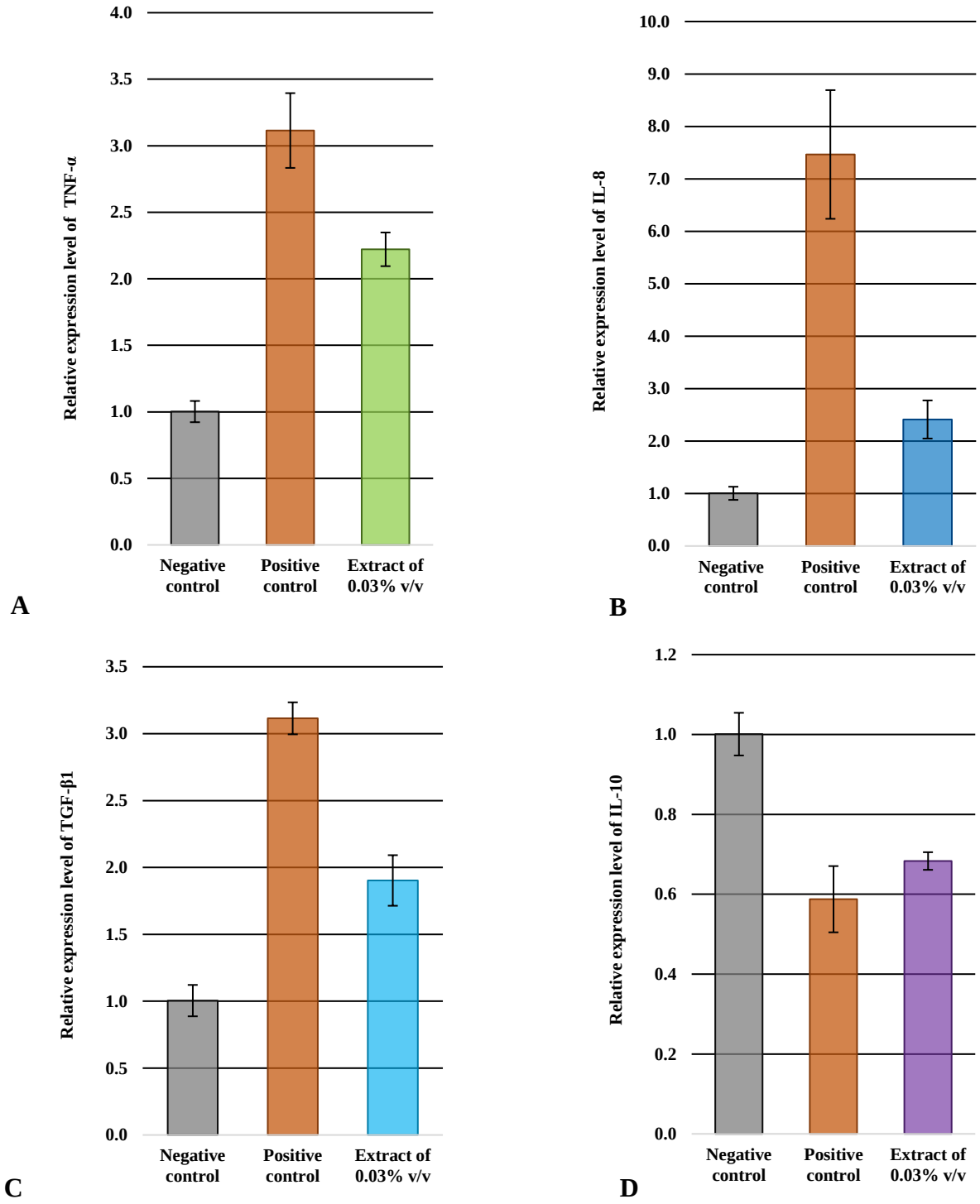


Figure 2. The relative expression level of TNF-α (**A**), IL-8 (**B**), TGF-β1 (**C**), and IL-10 (**D**) mRNAs upon inflammation induction (the positive control) and upon treatment with probiotics extract of 0.03% v/v of RAW 264.7 cells. The experiments were conducted in triplicate.

Treatment with probiotics extract: For the treatment, the probiotics extract at a final concentration of 0.03 v/v that gave the greatest % cell viability, i.e., gave the best condition for cell health/life, was used.

The results of ELISA assay for determination of the level of IFN-γ and IL-1β cytokines showed that treatment

with the probiotics extract of 0.03% decreases the level of IFN-γ and IL-1β pro-inflammatory cytokines (Figures 1A and B).

Meanwhile, the results of qRT-PCR assay for determination of the level of TNF-α, IL-8, TGF-β1, and IL-10 mRNAs showed that treatment with the probiotics extract of 0.03 v/v decreases the relative expression level of TNF-α, IL-8, and TGF-β1 pro-inflammatory mRNAs (Figure 2A, B, and C), and increases that of IL10 anti-inflammatory mRNA (Figure 2D).

DISCUSSION

The probiotics used in this study consisted of 6 bacteria strains: 5 strains of *Lactobacillus* bacteria, and a *R. palustris*. This symbiotic culture may lead to a complex production of metabolites, in which each microorganism synthesizes its own metabolites [11]. *Lactobacillus* bacteria usually releases bioactive compounds such as lactic acid, amino acids, acetic acid, mannitol, exopolysaccharides, vitamins, bacteriocins, some of which have antioxidative, anti-inflammatory, and anticancer activity [12–15]. Meanwhile, *R. palustris* could produce squalene and lycopene having antioxidant and anti-inflammatory activity [16–18].

In this study, ELISA and qRT-PCR assays were used for evaluation of the effects of the probiotics extract on the alteration of the level of pro-inflammatory and anti-inflammatory cytokines or mRNAs of inflammation-induced macrophage RAW 264.7 cells.

Inflammation induction was done on the RAW 264.7 cells by introduction of LPS, which is a potent stimulating mediator for pro-inflammatory cytokine by altering the expression of genes [19]. It was reported that upon LPS induction, the level of pro-inflammatory cytokines are elevated [20–24].

In this study, upon LPS inflammation induction of RAW 264.7 cells, the level of pro-inflammatory cytokines or mRNAs was elevated from that of the negative control (medium only): by ca. 300% for IFN-γ (from 114 to 483 pg/mL), by ca. 250% for IL-1β (from 122 to 436 pg/mL) (Figure 1A and B), by ca. 200% for TNF-α (from 1.0 to 3.1 relative expression level) and by ca. 650% for IL-8 (from 1.0 to 7.5 relative expression level), and by ca. 200% for TGF-β1 (from 1.0 to 3.1 expression level) (Figures 2A, B, and C). Whereas the level of IL-10 anti-inflammatory mRNA was lowered by ca. 40% (from 1.0 to 0.6 relative expression level) (Figure 2D). These findings were in line with previous studies revealing that LPS induction increases the level of several pro-inflammatory cytokines: IL-1β, TNF-α [21], IL-1β, TNF-α, IL-6 [20, 22], TNF-α, IL-6, IL-8 [23–24].

IFN-γ is an essential cytokine for defense against several pathogenic microorganisms. It is also the main cytokine in the progression of several inflammatory diseases. When macrophages are activated by IFN-γ, they increase the transcription of TNF-α gene [1]. In this study, the level of IFN-γ cytokine was increased by ca. 300% (from 114 to 483 pg/mL) upon LPS inflammation induction. Thus, this result concurs with previous studies reporting the function of IFN-γ as the cytokine for defense against the pathogenic microorganisms attack, which in this case, is in the form of LPS which is the inflammatory toxin in the outer membrane of pathogenic Gram-negative bacteria. Moreover, in accordance with previous studies, the increase in the level of IFN-γ cytokine in this study was accompanied by the increase of relative expression level of TNF-α (Figure 2A) [1]. Next, treatment with the probiotics extract could decrease the level of IFN-γ cytokine by ca. 55% (from 483 to 207 pg/mL) (Figure 1A), indicating the anti-inflammatory activity of the extract.

IL-1β is an important cytokine for the stimulation and initiation of the inflammatory response during inflammation process, contributed by its capability to induce other pro-inflammatory cytokines secretion, such as IL-8 [25]. In agreement with this finding, the level of IL-

1β cytokine in this study was increased by ca. 250% (from 122 to 436 pg/mL) upon LPS inflammation induction and its increase was accompanied by the increase of relative expression level of IL-8 mRNA. Upon treatment with the probiotics extract, the level of IL-1β cytokine was decreased by ca. 60% (from 436 to 166 pg/mL) (Figure 1B), indicating its potential anti-inflammatory activity.

TNF-α is the main cytokine produced by stimulated macrophages. The increased expression of this cytokine contributes to the stimulation of the inflammatory response and is related with the severity of several diseases [1]. Upon LPS inflammation induction in this study, the level of TNF-α mRNA was increased by ca. 200% (from 1.0 to 3.1 relative expression level), indicating that the macrophage was stimulated in response to the inflammation induction by LPS. Treatment with the probiotics extract was found to decrease the level by ca. 30% (from 3.1 to 2.2 relative expression level) (Figure 2A), signifying the anti-inflammatory activity of the extract.

IL-8 is a commonly used cytokine as a diagnostic and prognostic marker in infectious diseases and other inflammatory conditions [26]. It is a major cytokine mainly expressed by macrophages and fibroblasts, participating in rheumatoid arthritis [1]. The increase in the level of IL-8 mRNA by ca. 650% (from 1.0 to 7.5 relative expression level) upon LPS inflammation induction indicated the inflammatory condition of cells. Treatment with the probiotics extract decreased that level by ca. 70% (from 7.5 to 2.4 relative expression level) (Figure 2B), demonstrating the anti-inflammatory activity of the extract.

TGF-β1 plays a crucial role in immune homeostasis and tolerance, suppressing the expansion and activity of many components in the immune system [27]. Stimulation of TGF-β1 is associated with increased inflammation in early stage of the disease, however this cytokine is reported as possibly participating in the inflammation suppression late in the chronic state of multiple sclerosis [1]. In this study, the level of TGF-β1 mRNA was increased by ca. 200% (from 1.0 to 3.1 relative expression level) upon LPS inflammation induction, indicating the induced inflammation in early stage. Treatment with the probiotics extract decreased that level by ca. 40% (from 3.1 to 1.9 relative expression level) (Figure 2C), indicating anti-inflammatory potential of the extract.

IL-10 is commonly known as the most important immuno-suppressive cytokine, and its ability to recover inflammation and stimulate wound healing at peripheral sites is well reported [28]. Its anti-inflammatory activities include the suppression of IFN-γ and TNF-α expression [1]. In this study, the level of IL-10 mRNA was decreased by ca. 40% (from 1.0 to 0.6 relative expression level) upon LPS inflammation induction, indicating the inflammatory condition of cells. Treatment with the probiotics extract could increase the level of the anti-inflammatory mRNA by ca. 15% (from 0.6 to 0.7 relative expression level) (Figure 2D). The increase of IL-10 level in this study was also accompanied by the decrease of the level of IFN-γ cytokine and TNF-α s, signifying further the potential of the extract for recovery of the cells from inflammation condition.

Taken together, these findings revealed that the probiotics extract of *Lactobacillus* spp. and *R. palustris* possesses anti-inflammatory activities, beneficial for the recovery of cells from inflammatory conditions.

This research might impact the way people treat inflammatory conditions, especially chronic inflammatory disorders, i.e., using probiotics, which are safer for long-term use than synthetic anti-inflammatory medications.

CONCLUSION

The probiotics used in the present study showed anti-inflammatory activities, i.e., able to decrease the level of

pro-inflammatory cytokines or mRNAs of the inflammation-induced RAW 264.7 cells and to increase the level of IL-10 anti-inflammatory mRNA. Thus, the probiotics could be promising for the treatment of chronic inflammatory conditions. Hence, the products of probiotics might be used in the future as functional foods that offer health benefits to treat diseases associated with inflammation.

List of abbreviations: LPS: lipopolysaccharide, IFN-γ: interferon gamma, IL-1β: interleukin 1β, TNF-α: tumor necrosis factor α, IL-8: interleukin 8, TGF-β1: transforming growth factor β1, IL-10: interleukin 10.

Author Contribution: TK conceived the project, designed the study, performed the experiments, critically analyzed, interpreted the data, wrote, and revised the manuscript.

Competing Interests: The author declares that there are no competing interests accompanying this study.

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PUBLICATION TYPE	ISSN	COVERAGE
Journals	25740334, 27692426	2018-2022

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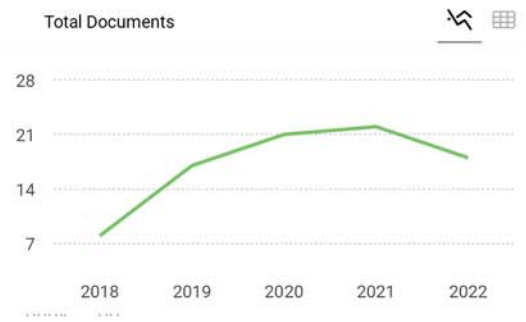

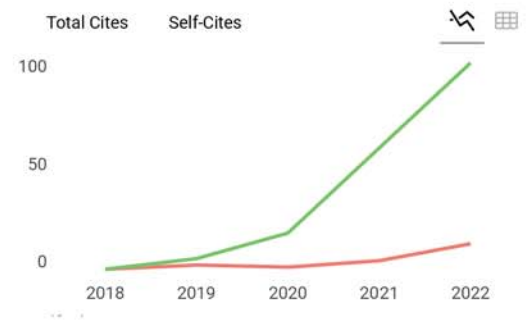

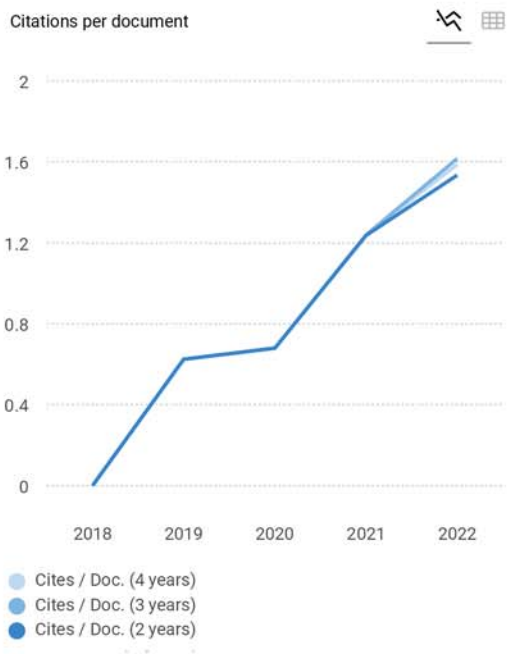

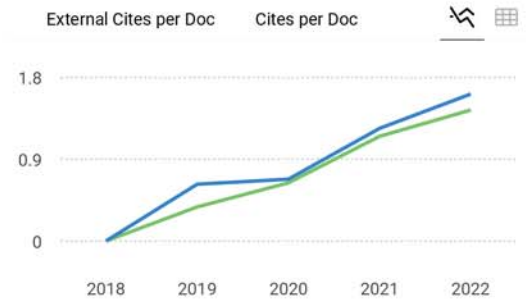

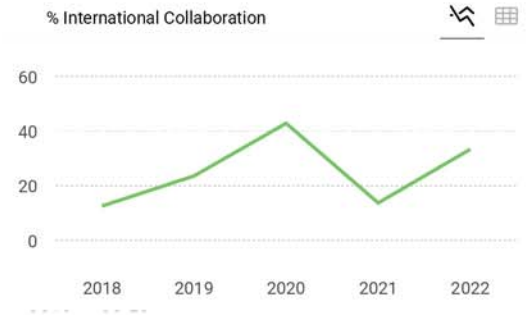

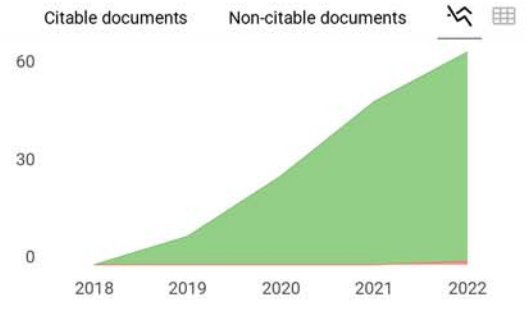

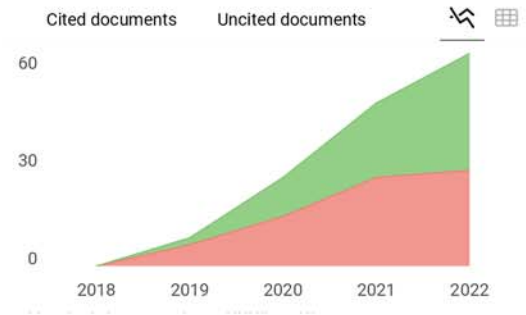

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