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Immunomodulatory Activity Analyses of Cell-Free Supernatant of *Lactobacillus plantarum* LP299v Strain in RAW 264.7 Macrophage Cells

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RAW 264.7;
Immunomodulatory activity

Abstract

The human immune system is essential for defending the body against harmful internal and external elements; immunity includes innate and acquired immunity. Macrophages, the innate immune system's key components, are crucial for the clearance of dead cells and tumor cells, as well as foreign substances by triggering phagocytosis. They also play a role in the adaptive response with the cytokines and mediator molecules they secrete. Lactic acid bacteria (LAB), an important probiotics class, have a strong potential to improve host health and can be used as a functional food. There have been reports of certain LAB strains having immunostimulating effects. However, the effects of cell-free supernatants (CFS) gathered from some LAB strains on macrophage activation have become an important research area in recent years. This study's main objective was to characterize the immunostimulatory activities of *Lactobacillus plantarum* LP299v in the RAW 264.7 macrophage cell line. For this purpose, the immunomodulatory activity of CFS of the related strain was evaluated by MTT, neutral red assay, and Griess reaction respectively, in terms of proliferation, phagocytosis ability, and nitric oxide (NO) production parameters using the macrophage cell line. Studies have shown that this strain significantly increased proliferation, phagocytosis, and NO levels in RAW264.7 macrophage cells. When considered, these results suggest that the cell-free supernatant, obtained from *Lactobacillus plantarum* LP299v selected in this study, may be helpful for candidate compounds with immunostimulatory activity.

Lactobacillus Plantarum LP299v Suşu Hücre İçermeyen Süpernatanının RAW 264.7 Makrofaj Hücrelerinde İmmünmodülatör Aktivitelerinin Analizleri

Anahtar kelimeler
Lactobacillus;
Hücresiz süpernatan;
RAW 264.7;
İmmunomodülatör aktivite.

Öz

İnsan bağışıklık sistemi, vücudu, zararlı iç ve dış unsurlara karşı savunmak için gereklidir; bağışıklık, doğuştan gelen ve edinilmiş bağışıklığı içerir. Doğuştan gelen bağışıklık sisteminin temel bileşeni olan makrofajlar, ölü hücrelerin ve tümör hücrelerinin yanı sıra fagositozu tetikleyerek yabancı maddelerin temizlenmesi için çok önemlidir. Ayrıca salgıladıkları sitokinler ve aracı moleküller ile adaptif yanıtta da rol oynarlar. Probiyotiklerin önemli bir sınıfı olan laktik asit bakterileri (LAB), konakçı sağlığını iyileştirmek ve fonksiyonel bir gıda olarak kullanılmak için güçlü bir potansiyele sahiptir. Bazı LAB suşlarının bağışıklık sistemini uyarıcı etkileri olduğu bildirilmiştir. Bununla birlikte, bazı LAB suşlarından elde edilen hücresiz süpernatantların (CFS) makrofaj aktivasyonu üzerindeki etkileri son yıllarda önemli bir araştırma alanı haline gelmiştir. Bu çalışmanın temel amacı, *Lactobacillus plantarum* LP299v'nin RAW 264.7 makrofaj hücre hattındaki immünostimülatör aktivitelerini karakterize etmektir. Bu amaçla, ilgili suşun CFS'sinin immünomodülatör aktivitesi, makrofaj hücre hattı kullanılarak proliferasyon, fagositoz yeteneği ve nitrik oksit (NO) üretimi parametreleri açısından sırasıyla MTT, nötral kırmızı testi ve Griess reaksiyonu ile değerlendirilmiştir. Çalışmalar, bu suşun RAW264.7 makrofaj hücrelerinde proliferasyon, fagositoz ve NO seviyelerini önemli ölçüde artırdığını göstermiştir. Bu sonuçlar birlikte ele alındığında, bu çalışmada seçilen *Lactobacillus plantarum* LP299v'den elde edilen hücresiz süpernatanın immünostimülatör aktiviteye sahip aday bileşikler için yararlı olabileceğini göstermektedir.

1. Introduction

The human immune system is essential for defending the body against viruses, bacteria, and harmful substances; and consists of innate and acquired immune components (Zheng et al., 2020; Mazziotta et al., 2023). Innate immunity stands for

immune cells' non-specific response to exogenous factors. Acquired immunity is involved in the induction of a particular antigen-specific response. A range of immune cells, including macrophages, are involved in innate immunity (dendritic cells, natural killer cells, neutrophils, and eosinophils). Macrophages play a central role in the response to endogenous factors, taking part in removing tumor cells, dead cells, and exogenous factors via phagocytic activity (Hirayama et al., 2017). Additionally, macrophages have been implicated in adaptive immunity through secreting a variety of mediator molecules, including inducible nitric oxide synthase, cyclooxygenase-2, prostaglandin E2, and nitric oxide (NO), as well as pro-inflammatory cytokines like tumor necrosis factor, interferon, and different interleukins (Geum et al., 2020; Kang et al., 2021; Wu et al., 2022). NO is a soluble endogenous gas that functions in pathways such as immune regulation, inflammation, and destruction of tumor cells, functioning as an effector molecule or metabolic regulator (Palmieri et al., 2020; Vidanapathirana et al., 2022).

Probiotics are microbial food additives (live microorganisms) that cause positive effects on host health when consumed in appropriate amounts and under the right conditions (Mazziotta et al., 2023). The microorganism group in which the effects of probiotics on consumer health have been investigated in the most detailed way is lactic acid bacteria (LAB). LAB and some metabolites of this group of microorganisms have been shown to improve immunity, promote intestinal health, lower serum cholesterol levels, and function in anticancer and antioxidant mechanisms (Das et al., 2022; Mazziotta et al., 2023).

Recently, interest in the use of probiotics to support immunity has increased and in this context, research on the immunomodulatory effects of cell-free supernatants of probiotics has emerged as a new research area. Studies have reported that some LAB strains, such as Lactobacilli, Lactococcus, and Bifidobacterium, have immunostimulatory effects on macrophage cells of CFS (Lee et al., 2022; Lee et al., 2023). In this study, we evaluated the immunostimulatory activity of *Lactobacillus plantarum* LP299v on RAW 264.7 macrophage cells and related mechanisms, proliferation (MTT assay), phagocytic capacity (neutral red), and NO production (Griess reaction) parameters.

2. Materials and Methods

2.1 Bacterial Strain and Cultivation Condition

In this study, we used *Lactobacillus plantarum* LP299v, kindly provided by Probest Digestive (Abdi İbrahim, Turkey). The strain was grown in De Man-Rogosa-Sharpe (MRS) broth (Merck, Darmstadt, Germany) and on MRS agar. LP299v strain was cultured under aerobic conditions (5% CO2 and 21% oxygen) at 37°C (Tranberg et al., 2021).

2.2 Cell-Free Supernatant (CFS) Preparation

To examine the immunomodulatory effects of the selected strain of this study, CFS was prepared following the method used in a previously published study (Lee et al., 2022). Briefly, *Lactobacillus plantarum* strain LP299v was cultured in MRS medium at 37 °C for 18 hours. Next, centrifugation was done at 4000 x g for 20 min. to extract CFS from bacterial cultures, which were then filtered via a 0.2 µm filter, followed by freeze-drying. The CFS was then redissolved in the DMEM medium in which the cells were grown and used for cell culture-based immunomodulatory effect analyses

2.3 Cell Culture

Macrophages are versatile immune cells associated with a variety of diseases; their phenotypes and functions vary depending on the surrounding environments. RAW 264.7 cells are widely used in immunostimulatory studies as a macrophage cell line that can reflect normal macrophage functions (Taciak et al., 2018). In our study, we investigated the effects of cell-free supernatant, obtained from *Lactobacillus plantarum* strain LP299v, on proliferation and phagocytosis ability, in addition to nitric oxide production in RAW 264.7 cells.

RAW 264.7 cells were kindly provided by Dokuz Eylül University Oncology Institute (Passage 10). Cells were seeded in DMEM medium containing high glucose concentration (4.5 g/L), accompanied by 10% (v/v) heat-inactivated fetal bovine serum (FBS), and 1% (v/v) penicillin/streptomycin. Then, the cells were harvested using a scraper (GeneDirex, Inc) after trypsin treatment (0.25%)/EDTA (0.05%) (Biological Industries, Cromwell, USA) for 5 min at 37°C when their confluency reached 70% and passaged at a 1:4 dilution every 2-3 days (Xu et al., 2019). Cell viability was checked with trypan blue and experiments were continued if the viability level was above 90%; all experiments were carried out between passages 10-14.

2.4. Macrophage Proliferation Analysis

RAW 264.7 cells were seeded in 96 well plates with 1.10^4 cells per well concentration. Following the overnight incubation (cells were in a 70% confluent state), the cell culture medium was replaced with fresh medium, containing the determined concentration range of CFS (1, 2, and 5 mg/mL) for the administration groups. We also changed and refreshed the media of control wells. At the end of the 24-hour incubation process, MTT (Serva, Germany) solution, with the final concentration of 0.5 mg/ml (10 µL), was added to wells. Three hours after the incubation, the formation of formazan crystals was controlled and the crystals were dissolved using 100 µL of DMSO. The color obtained was measured spectrophotometrically with a ChroMate® ELISA microplate reader, adjusted at a wavelength of 492 nm. Each experiment was performed in triplicate. The wells containing only the medium, in which CFS was not applied, acted as the control, and the cell viability percentage was determined using the following formulation by comparing it with the control group.

$$\% \text{ Cell Viability} = [(A\text{sample}) / (A\text{control})] \; x100 \quad (1)$$

$A_{control}$ stands for control well's absorbance and A_{sample} stands for the CFS application well absorbance.

2.5. Neutral Red Uptake Assay for Macrophage Phagocytosis

RAW 264.7 cells were seeded in 96 well plates at a concentration of 1.10^4 cells per well. Following the overnight incubation, the cells were incubated using the determined concentrations of CFS for 24 hours. We performed analyzes according to the kit protocol of the EZcountTM Neutral Red Cell Assay (HiMedia Laboratories, Maharashtra, India). Using a microplate reader, we measured the absorbance at 490 nm (Xu et al., 2019). We evaluated these absorbances according to the untreated control wells and determined the phagocytic activity.

2.6. Production of Nitric Oxide (NO)

After the overnight incubation, we treated the RAW 264.7 cells, seeded in 96 well plates with a concentration of 1.10^4 cells per well, with CFS at the determined concentrations for 24 hours. Then the we collected the conditioned medium and analyzed it with the Promega Griess Reagent System (Wisconsin, USA) following the manufacturer's protocol (Xu et al., 2019). The results were evaluated according to the untreated control wells.

2.7. Data Analysis

All experiments were carried out in triplicates and values were reported as means ±standard error of the mean (SEM). Data analyses of all groups were carried out using Prism v 8.0 (GraphPad Software, San Diego, CA, USA). Statistical difference was assessed with one-way analysis of variance (ANOVA) followed by Tukey post-hoc test.

3. Results

3.1. Macrophage Proliferation Analysis

Three different concentrations of CFS were added to RAW 264.7 cells for a 24-hour incubation to assess whether the LAB strain CFS could increase proliferation in cells.. Cell viability was found to be 100 ± 0.063 for the control group, 100.84 ± 0.085, 107.073 ± 0.083, and 111.632 ± 0.079 for 1, 2, and

5 mg/mL administrations, respectively. Therefore, it was determined that CFS obtained from the LAB strain did not show any cytotoxic effect on RAW 264.7 cells. However, it increased cell proliferation depending on the application dose (Figure 1). The results are similar to the studies carried out in the literature on this subject and it is seen that the application of 5 mg/mL of the relevant CFS significantly supports macrophage proliferation ($p<0.05$).

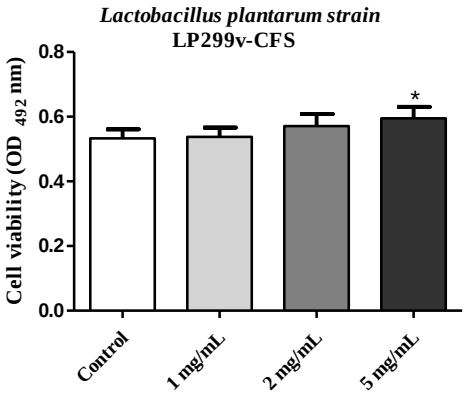

Figure 1. The effect of CFS applications on the viability of RAW 264.7 cells, at different concentrations (Bar represents the mean of cell viability (OD 492)
± SEM; n = 3 statistical difference is represented as ;*p < 0.05.)

3.2. *Neutral Red Uptake Assay for Macrophage Phagocytosis*

The phagocytic activity of LAB strain CFS was assessed via a neutral red uptake assay. The activity was significantly increased for 2 and 5 mg/mL in CFS-treated RAW 264.7 cells in comparison to untreated control cells (Figure 2). The result was parallel with phagocytic activity results obtained in studies with LAB strain CFS (Lee et al., 2022).

3.3. Production of Nitric Oxide (NO)

To show whether the corresponding LAB strain CFS increases the production of NO in cells, we added the obtained CFS fraction to RAW 264.7 cells. Then, the cells were incubated for 24 hours. Since NO is associated with macrophage functions like the elimination of certain tumor cells, pathogens, and cell debris, it is clear that increased NO production is involved in the activation of the immune response in macrophages (Huang et al., 2014). It was determined that especially 2 and 5 mg/mL CFS applications caused a significant increase in NO production, compared to the blank and control groups. Similar to our study, applications with CFSs obtained from LAB strains dramatically boosted the production of NO by 2.84–25.73 µM (Lee et al., 2022).

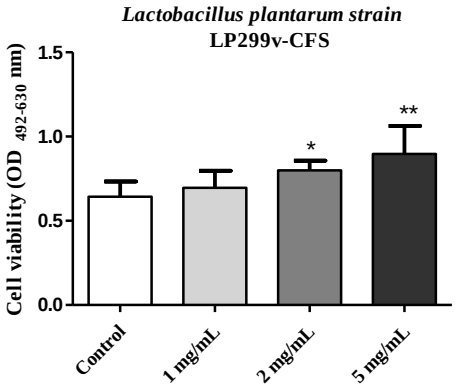

Figure 2. Effect of CFS applications on phagocytic activity in RAW 264.7 cells, at different concentrations (Bar represents mean cell viability (OD 492-630) ± SEM; n = 3 statistical difference is represented as *, ** respectively ; $p < 0.05$, $p < 0.01$).

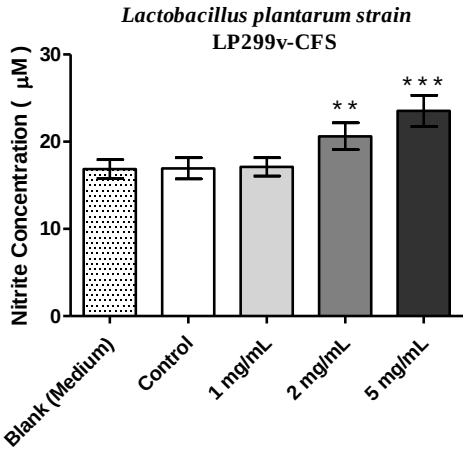

Figure 3. The effect of CFS applications on NO production in RAW 264.7 cells, at various concentrations, (Bar represents the mean of nitrite concentration (µM) ± SEM; n = 3 statistical difference is shown as **, *** respectively; $p < 0.01$, $p < 0.001$).

4. Discussion

The innate immune system serves as the body's initial line of defense and it is crucial in defending it from foreign substances and pathogens. The innate immune response includes cells like dendritic cells, natural killer cells, and macrophages. Macrophages

have been reported to be an important barrier to the immune response in the host defense system via phagocytic activation and play a critical role in this process (Yeşilyurt et al., 2021).

Recently, there has been great interest in CFS involving bioactive soluble factors secreted by probiotics (De Marco et al., 2018; Maghsood et al., 2018). Cell lines have been used as in vitro models for evaluating the physiological benefits of CFS of probiotic strains (De Marco et al., 2018). The focus of this study was the effect of one of the LAB strains, *Lactobacillus plantarum* strain LP299v CFS, on macrophages' immunostimulatory activity. Macrophage phagocytosis is a crucial step in the removal of dead cells and pathogens (Jeong et al., 2020).

In earlier studies, CFS of *Lacticaseibacillus rhamnosus* (*L. rhamnosus*) GG was observed to increase phagocytic activity significantly in the J774 murine cell line (Vincenti, 2010). In another study, Xiu et al. observed that phagocytic activity in RAW 264.7 macrophage cells was induced by exopolysaccharides obtained from *Lacticaseibacillus casei* (*L. casei*) WXD030 (Xiu et al., 2018). The evaluation in our study followed a methodology similar to the one in the study of Lee et al., 2022, which is comprehensive research performed in this field. In this study, the immunostimulatory activity and associated molecular mechanisms of LABs, such as *Limosilactobacillus reuteri* (*L. reuteri*), *Limosilactobacillus fermentum (L. fermentum*), and *Lactococcus lactis* (*Lc. lactis*), isolated from humans, fermented and normal foods, were evaluated. The study showed the cell viability values obtained for 5 mg/mL CFS were between 98.92-103.88%; meanwhile in our study, cell proliferation was found to be quite high at 5 mg/mL CFS application, at 111.632 ± 0.08. In another study, specifically *L. reuteri* MG5462 (21.73 ± 0.95 µM), L. fermentum MG4263 (21.47 ± 1.14 µM), MG4268 (20.87 ± 2.02 µM) and MG4282 (25.73 ± 0.12 µM) isolates showed high NO induction (19.27 ± 2.80 µM) (Lee et al., 2022). Since NO is associated with macrophage function, such as the elimination of cellular debris, pathogens, and tumor cells, increased NO production is known to activate the immune response in macrophages (Huang et al., 2014). Therefore, strains with high NO production, such as *L. reuteri* MG5462, *L. fermentum* MG4263, MG4268, MG4282, and *Lc. lactis* MG4668, MG5278, and MG5474 were selected and further studies were conducted (Lee et al., 2022). In our study, NO production was found to be 17.12 ± 0.98 µM for 1 mg/mL CFS application, and 20.62 ± 1.43 and 23.53 ± 1.58 µM for 2 and 5 mg/mL, respectively. It was determined that 2 and 5 mg/mL CFS application significantly induced NO production in macrophage cells.

Limitations of our study include the fact that it was restricted to macrophage cell lines, and the molecular mechanisms underlying the immunostimulatory activity were not observed. However, with the data provided, our study serves as a platform for further research that focuses on the evaluation of molecular mechanisms involved in the immunomodulatory activity of cell-free supernatant from *Lactobacillus plantarum* LP299v strain in RAW 264.7 macrophage cells.

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