Effect of Probiotic Lactobacillus Strains on Phagocytic Activity of Murine Macrophage Cells Infected by Aggregatibacter Actinomycetemcomitans

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Abstract

Periodontal diseases are prevalent inflammatory diseases affecting the periodontium stimulated by an oral pathogen, Aggregatibacter actinomycetemcomitans. Recently, there is an increasing interest in utilizing probiotic as a bacteriotherapy treatment for oral diseases. Macrophages are innate immune cells that perform phagocytosis and synthesis various cytokines when they encounter foreign particles. The aim study is to determine the immunomodulation capacity of heat-killed probiotic Lactobacillus casei subsp. rhamnosus NBRC 3831 and L. casei NBRC 15883 on the activation of RAW 264.7 murine macrophages in phagocytic activity and cytokines secretion to counteract A. actinomycetemcomitans strain infection. In this study, we analyzed the effects of heat-killed L. casei subsp. rhamnosus NBRC 3831, and L. casei NBRC 15883 on the innate immune response. The activity was evaluated based on the viability of macrophages, cytokines secretion, and macrophages’ phagocytic action. The introduction of probiotic lactobacilli significantly induced the phagocytic activity of macrophages as compared to A. actinomycetemcomitans strains Y4 and ATCC 29524. Whereby L. casei NBRC 15883 showed the highest (p < 0.01) phagocytic activity compared to other groups. Besides, there was lower macrophage death when co-incubated with two tested lactobacilli strains, which indicated that lactic acid bacteria had the potential to trigger macrophages proliferation. Interestingly, the number of viable macrophages that pre-activated by probiotic before the addition of A. actinomycetemcomitans was higher than the untreated group. Moreover, Lactobacillus sp. was found to promote and modulate the pro-inflammatory cytokines (IL-1β, IL-6, and IL-8) by murine macrophages against A. actinomycetemcomitans infection after 24 and 48 h incubation in a strain and dose-specific pattern. In conclusion, probiotic Lactobacillus induce the phagocytosis activity, macrophage cell proliferation, and the release of IL-1β, IL-6, and IL-8 cytokines to modulate the immune response. These findings might provide crucial information on the efficacy of probiotic Lactobacillus in developing a promising candidate against A. actinomycetemcomitans infections.

Keywords: Aggregatibacter actinomycetemcomitans, probiotic, phagocytosis, cytokine

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Introduction

Aggregatibacter actinomycetemcomitans is a pathogenic periodontal bacterium with the characteristics of facultative anaerobic Gram-negative coccobacillus and non-motile state. The consequences of A. actinomycetemcomitans infection in the oral cavity include juvenile localized periodontitis and followed by alveolar bone destruction. A. actinomycetemcomitans bacteria have immunosuppressive factors that can inhibit neutrophils apoptosis and also cause macrophage cell lysis. Moreover, A. actinomycetemcomitans can also lead to non-periodontal infections. These include endocarditis, pneumonia, septicemia, infectious arthritis, osteomyelitis, and other systemic diseases. This pathogen expressed several virulence factors such as leukotoxin and lipopolysaccharide (LPS) to facilitate its colonization to biotic and abiotic surfaces and evade periodontium, evade the host immune defense system, and subsequently result in tissue destruction. Also, A. actinomycetemcomitans withstand from antibiotics used in periodontal therapy by constituting persistent biofilms on oral surfaces.

The primary cause of the oral disease is mainly due to dysbiosis of the oral microbiota or defective in host immunoregulatory response that results in a dramatic shift from a symbiotic microbiota, and subsequently leads to inflammation of periodontium. Macrophages and monocytes are phagocytes that perform a significant part in the innate immunity (first immunologic line of defense), which engulf and digest cellular debris or foreign microorganisms. The response of macrophages against any type of infection is pivotal in predicting the consequence of the infection for the host. Activated macrophages will stimulate inflammation by producing pro-inflammatory cytokines such as IL-1β, IL-6, IL-8, IL-17, and tumor necrosis factor-alpha (TNF-α) counteract the invading pathogens directly. Besides, these cytokines can induce adaptive immunity and recruit more immune cells to migrate into the inflammation site. Although these cytokines can aid in enhancing cellular immunity activation but they can also damaging host tissues if there is impaired host immune response.

A new concept recently tried in oral disease prevention and treatment is by using probiotics, which are live microbial agents. According to FAO and WHO (Food and Agriculture Organization of the United Nations and World Health Organization), a probiotic is defined as a “live microorganism which when administered in adequate amounts, confers a health benefit on the host”. Compared to conventional oral therapies, probiotic bacteria are more favorable to counteract and degrade the mature biofilm formed by A. actinomycetemcomitans and reduce antibiotic usage that can result in antibiotic-resistant issues. Lactic acid bacteria (LAB), belong to the genus Lactobacillus, can produce antimicrobial molecules such as lactic acid, hydrogen peroxide, and bacteriocins to inhibit oral pathogen growth. Previous study showed that some lactobacilli strains can trigger the secretion of pro-inflammatory cytokines like TNF-α, IL-8, and IL-12 and anti-inflammatory cytokines like IL-10. There is still an inadequate understanding of probiotic Lactobacillus casei subsp. rhamnosus NBRC 3831, and L. casei NBRC 15883 in the modulation of macrophage-mediated immune responses. Thus, this study will provide additional scientific evidence on the ability of these two probiotic species as an alternative approach to counteract the periodontal pathogen and to replace antibiotic usage in periodontal therapy.

Materials and methods

1.1 Bacterial strains

Periodontal pathogen Aggregatibacter actinomycetemcomitans strain Y4 was gained from Kyushu Dental University, Japan. In contrast, ATCC 29524 strain was obtained from the American Type Culture Collection (ATCC, USA). While probiotic bacteria Lactobacillus casei subsp. rhamnosus NBRC 3831, and Lactobacillus casei NBRC 15883 were received from Kyushu Institute of Technology, Japan. Each strain of A. actinomycetemcomitans pathogen was cultured in Brain Heart Infusion (BHI) agar (Oxoid Ltd. UK) and incubated at 37°C for 16 h in the anaerobic state, while probiotic Lactobacillus species were cultured in De Man, Rogosa, and Sharpe (MRS) agar (Fluka, Sigma-Aldrich, St. Louis, MO) at 37°C for 20 h under anaerobic conditions.

1.2 Preparation of dead bacteria cells

A 24 h culture of A. actinomycetemcomitans strains and lactobacilli broth were centrifuged at 5000 rpm (Hettich, North America) for 15 min at 24°C. Then, sterile distilled water was used to wash the cell pellet twice. The multiplicity of infection (MOI) 1:5 and MOI 1:50 (macrophage: bacteria) was prepared using the bacteria concentrations that equivalent to 2 x 10^6 cell/mL and 2 x 10^7 cell/mL, respectively. A. actinomycetemcomitans cells pellet was resuspended with sterile distilled water and adjusted to an optical density (OD) of 0.018 for MOI 1:5 and OD 0.18 for MOI 1:50 at wavelength 560 nm by using UV-spectrophotometer (Thermo Scientific™ GENESYS™ 20). On the other hand, pellet suspension of lactobacilli was set to OD 0.004 for MOI 1:5 and OD 0.04 for MOI 1:50 at 600 nm. Finally, all bacteria dilutions was autoclaved at 120°C for 15 min using a high-pressure steam sterilizer.

1.3 Macrophage cell line

The murine macrophage cell line RAW264.7 (ATCC® TIB-71™) was obtained from American Type Culture Collection (ATCC, USA). The macrophages were cultured in Dulbecco's Modified Eagle Medium (DMEM) high glucose medium (Nacalai Tesque, Inc., Japan) complemented by 10% fetal bovine serum (Tico Europe, Netherlands), 1% of antibiotics (10,000 Units/mL penicillin and 10,000 µg/mL streptomycin) (Gibco, USA) and incubated at 37°C in a humidified incubator with 5% CO2.
1.4 Macrophage cell viability assay

Trypan blue dye (Sigma-Aldrich, USA) exclusion technique was applied to evaluate the cytotoxic effect of bacterial cells against the RAW264.7 murine macrophage. In short, the autoclave-killed bacteria broth was centrifuged at 3,000 × g for 10 min and suspended in DMEM media supplemented with 10% FBS. Then, macrophages were co-incubated with bacteria at the MOI 50:1 and 5:1 (bacteria: macrophage), respectively, in 6-well plates (Nest Biotechnology Co., Ltd., China) for 1 to 2 h in a 5% CO₂ incubator. The cell mixtures washed thrice with DMEM containing 1% penicillin/streptomycin and incubated in 3 mL of DMEM without antibiotic for an additional 18 h at 37°C in a 5% CO₂ incubator. Subsequently, these cells were resuspended in 0.4% trypan blue (Sigma-Aldrich, USA) solution and then transferred 10μL of cell suspension to a hemocytometer counting chamber (Neubauer). Cell concentration was calculated by using formula 16:

\[
\text{Viable cells per mL} = \frac{\text{Number of cells counted}}{\text{Number of squares counted}} \times \text{dilution factor} \times 10^4
\]

1.5 Phagocytosis assay

Each well of the 6 well-plate was seeded with 4 × 10⁵ cell/mL of RAW264.7 (ATCC® TIB-71™) cells in DMEM media supplemented with 10% FBS and then incubated for overnight at 37°C in a 5% CO₂ incubator to allow cells attached to the surface of the well. Then, the RAW264.7 cells suspension was mixed with each pellet suspension (2 mL) of dead autoclave cells of periodontal pathogens (A. actinomycetemcomitans strain Y4 and strain ATCC 29524) or lactobacilli (L. casei subsp. rhamnosus NBRC 3831, and L. casei NBRC 15883), respectively, to make a mixture at an MOI of 1000:1 (bacterial: macrophage) in DMEM. The RAW264.7 macrophages cell suspension, without the addition of any bacterial suspension, was served as a negative control. After that, all the samples were incubated at 37°C in a 5% CO₂ incubator for 20 h. An assay reagent, latex beads IgG–fluorescein isothiocyanate (FITC; Cayman Chemical Co.), was used to assess the phagocytic activity of macrophage. At first, pre-warmed DMEM was used to dilute the latex beads–rabbit IgG–FITC complex for 100 times. The supernatant of the culture assay was substituted with diluted latex beads reagent (after 24 h cultured) and incubated in the 5% CO₂ incubator at 37°C for 2h. The binding of IgG molecules with crystallizable fragment receptors expressed on the cellular membrane of the activated macrophages will then stimulate the ingestion of FITC beads. Therefore, the uptake of beads by macrophages was determined as the phagocytic activity of macrophages by identifying the FITC fluorescence at 498 nm of the excitation wavelength and 522 nm of the fluorescence wavelength. An inverted fluorescence microscope (Olympus Vökeetwil, Switzerland) was used to count the activated macrophage cells that contained the ingested beads. The percentage of phagocytic activity was determined by using the formula 10:

\[
\text{Phagocytic activity (％)} = \frac{\text{total number of macrophages containing ingested fluorescence beads}}{\text{total number of macrophages}} \times 100
\]

1.6 Macrophage pre-activation and invasion assays

For the pre-activation assay, RAW264.7 macrophages were co-cultured with probiotic Lactobacillus in 6 well-plate at MOI 5:1 and MOI 50:1 (macrophage: bacteria), respectively, at 37°C in 5% CO₂ incubator for 24 h. After that, the supernatant of the culture mixture was removed and substituted with dead A. actinomycetemcomitans strains in fresh DMEM supplemented with 10% FBS to achieve MOI 50:1. Next, the cell mixture seeded in a 6-well plate was incubated at 37°C in a 5% CO₂ incubator for 1h to promote the phagocytic activity by macrophage cells. Then, DMEM media containing 1% penicillin/streptomycin was used to wash each of the wells twice to kill and eliminate the bacterial cells from the medium and then was substituted with DMEM added with 10% FBS and incubated for an additional 24 and 48 h respectively in 5% CO₂ incubator. Subsequently, the cell suspensions were centrifuged at 2000 rpm for 20 min. Then the supernatant was collected and preserved at −80°C for further analysis used in ELISA. Simultaneously, the cell pellet was resuspended in 0.4% trypan blue (Sigma-Aldrich, USA) solution to count for the viable macrophages.10

1.7 Cytokine determination

The supernatants of the macrophage cells assay that pre-activated with dead lactobacilli at MOI 50:1 and 5:1 (macrophage: bacteria), and then co-incubated with dead periodontal pathogen A. actinomycetemcomitans strains at MOI 50:1 (macrophage: bacteria) were harvested after 24 h and 48 h incubation. The concentration of cytokines quantified using sandwich ELISA kits (Elabscience® rat IL-6 kit, Elabscience® rat IL-1β kit, and SunLong Biotech Co., LTD rat IL-8 kit) according to the instructions of the manufacturer. A standard curve was plotted, and the absorbance of the samples was measured spectrophotometrically at 450 nm using a microplate reader (Tecan, Switzerland).6

1.8 Statistical analysis

The data was calculated as the mean ± standard deviation (SD) with triplicate determinations. GraphPad Prism version 7 (GraphPad Software Inc, California) used to perform means Student’s t-test. A p-value < 0.05 treated as statistically significant.10

Results

Phagocytosis Activity

Phagocytosis is used as a model of microbe-innate immune interactions. In this study, the phagocytosis activity of macrophage cells after 24 h co-cultured with A. actinomycetemcomitans pathogens and Lactobacillus spp. respectively, at MOI 1:1000 (macrophage: bacteria) were assessed using the IgG-FITC phagocytosis assay kit, and the result is shown in Figure 1.
Phagocytosis activity of murine macrophages were not significant when co-incubated with dead *A. actinomycetemcomitans* Y4 and showed lower phagocytosis activity compared to the probiotic *Lactobacillus* spp. at MOI 1:1000 (macrophage: bacteria). Besides, the phagocytosis activity of macrophages stimulated by *A. actinomycetemcomitans* ATCC 29524 was significantly higher (*p < 0.01*) than strain Y4. On the other hand, phagocytosis activities of RAW264.7 macrophages triggered by *L. casei* NBRC 15883 were significantly higher (*p < 0.01*) with 35% phagocytic activity compared to the untreated group and those with *A. actinomycetemcomitans* strains. Moreover, the phagocytosis activities of murine macrophages induced by *L. casei* subsp. *rhamnosus* NBRC 3831 was significantly higher (*p < 0.05*) than *A. actinomycetemcomitans* Y4 but lower than *L. casei* NBRC 15883.

**Viability of Macrophages**

The viability of RAW 264.7 murine macrophages after co-incubation with bacterial cells at MOI of 5 and 50 were counted using the trypan blue exclusion method, and the result is shown in Figure 2. The number of viable macrophage cells after 21 h (18 - 24 h) co-incubation with the tested probiotic *Lactobacillus* strains was higher compared to both *A. actinomycetemcomitans* strains at the MOI 1:5 (Figure 2A) and MOI 1:50 (Figure 2B). Besides, among the two *Lactobacillus* strains, *L. casei* subsp. *rhamnosus* NBRC 3831 illustrated the lower cytotoxic effect against macrophages at low and high concentrations, which indicated by the higher number of viable macrophages at MOI 5 and MOI 50. On the other hand, the number of viable RAW264.7 murine macrophages that co-cultured with dead *A. actinomycetemcomitans* strains Y4 and ATCC 29524 at MOI 1:5 and MOI 1:50 (macrophage: bacteria) were lesser than the untreated cells. There was significantly lower (*p < 0.05*) as compared to the untreated in the *A. actinomycetemcomitans* strains ATCC 29524. For the treatment group, macrophages were pre-activated with probiotic *L. casei* NBRC 15883 and *L. casei* subsp. *rhamnosus* NBRC 3831 in 6-well plate at MOI 5:1 and MOI 50:1 (macrophage: bacteria), respectively prior invaded with oral pathogens *A. actinomycetemcomitans* at MOI 50:1 (macrophage: bacteria) for 24 h and 48 h in 5% CO2 incubator. The viability of macrophages in different treatment groups was also determined using the trypan blue exclusion technique, and the results are shown in Figure 3.

The number of viable macrophages in treatment groups after 24 h pre-activation with dead *L. casei* NBRC 15883 at ratio of 5 and then invaded with *A. actinomycetemcomitans* strains Y4 (Figure 3A) and strain ATCC 29524 (Figure 3C), respectively at MOI 1:50 (macrophage: bacteria) were significantly higher (*p < 0.05*) than the untreated group. Moreover, the *L. casei* subsp. *rhamnosus* NBRC 3831 pre-activated macrophages at MOI 5 and *A. actinomycetemcomitans* ATCC 29524 invasion, obtained the significant highest number of alive macrophages than other treatment groups (Figure 3G). However, the number of macrophages that pre-treated with *L. casei* subsp. *rhamnosus* NBRC 3831 at ratio of 1:50 (macrophage: bacteria) prior *A. actinomycetemcomitans* strain Y4 (Figure 3F) and strain ATCC 29524 (Figure 3H) infections were lower (*p > 0.05*) compared to other treatment groups.

**Cytokine Secretion in RAW 264.7 Cells**

RAW 264.7 cells were pre-activated with dead *Lactobacillus* strains at different concentrations (MOI 1:5 and 1:50) (macrophage: bacteria) for 24 h and then followed by the stimulation with dead *A. actinomycetemcomitans* strains at MOI 1:50 (macrophage: bacteria). Cytokine production (IL-6, IL-8, and IL-1β) in the culture supernatants were determined using the ELISA method at 24 h and 48 h after initiation of treatment (Figure 4).

All strains of *lactobacilli* in the treatment groups stimulated a significantly increase level of pro-inflammatory cytokine IL-6 at MOI 1:5 (Figure 4A) and MOI of 50 (Figure 4B) after 24 and 48 h incubation, except the treatment group of *L. casei* subsp. *rhamnosus* NBRC 3831 (MOI 1:50) co-cultured with *A. actinomycetemcomitans* ATCC 29524 at both 24 and 48 h incubation (Figure 4B). On the other hand, the synthesis of IL-8 elevated across the treatment groups, and the maximum level of IL-8 was obtained after 48 h incubation at both MOI 1:5 (Figure 4C) and MOI of 1:50 (Figure 4D). While the maximum concentration of cytokine IL-1β was achieved right after 24 h, the production of this cytokine was different across the treatments, as shown in Figure 4E and Figure 4F. Overall, the secretion of IL-6, IL-8, and IL-1β was significantly higher than untreated cells (macrophage only). The highest level of IL-6, IL-8, and IL-1β production was achieved when RAW 264.7 cells were pre-treated with *L. casei* subsp. *rhamnosus* NBRC 3831 prior co-cultured with *A. actinomycetemcomitans* strains.

**Discussion**

Phagocytosis is a cellular process for ingesting and eliminating particles larger than 0.5 µm in diameter. This study showed the impact of *L. casei* NBRC 15883 and *L. casei* subsp. *rhamnosus* NBRC 3831, and the periodontal pathogen (*A. actinomycetemcomitans* strain Y4 and strain ATCC 29524) on the phagocytic activity of RAW 264.7 cells, which represents the activation of murine macrophages. The phagocytosis activity of murine macrophages was not significant when co-cultured with dead *A. actinomycetemcomitans* Y4. Lower phagocytosis activity is shown as compared to probiotic *Lactobacillus* species at MOI 1:1000 (macrophages: bacteria) (Figure 1), which indicates that *A. actinomycetemcomitans* Y4 might evade the immune response by using the unknown mechanism that prevents the activation of macrophages to engulf them. The *A. actinomycetemcomitans* strains might express their virulence factor such as cytolethal distending toxin (CDT) that functions to inhibit the phagocytic activity of macrophage.17 Although both *A. actinomycetemcomitans* strains Y4 and ATCC 29524 can express CDT, the phagocytosis activity of macrophages stimulated by *A. actinomycetemcomitans* ATCC 29524 was significantly higher (*p < 0.01*) than strain Y4 because strain ATCC_29524 was a commercial strain and artificially induced by ATCC company, and hence less virulence than strain Y4. Therefore, *A. actinomycetemcomitans* strain Y4 had a higher potential
to inhibit phagocytosis by macrophages. This finding was supported by a review study, where strain Y4 is more virulence than other strains of *A. actinomycescomitan* (e.g., ATCC 29524, ATCC 29523, and ATCC 29522) and has high periodontopathic potential in humans and animals due to the presence of the free cellular acid iso-
C150 acid in its content. Besides, the cellular proteins in strain Y4 are most different from other strains of *A. actinomycescomitan*, which can be proved by the higher intensive staining result with ruthenium red that observed on the plasma membrane of strain Y4 cells than the cellular membranes of other Gram-negative bacteria. 

Besides, phagocytosis activity of macrophages stimulated by *L. casei* NBRC 15883 was significantly highest (p < 0.01) compared to untreated and those with *A. actinomycescomitan* Y4 (Figure 1). Thus, probiotic *L. casei* NBRC 15883 can effectively enhance the activation of macrophages to defense against pathogens. In this study, *L. casei* NBRC 15883 is the strongest stimulator to enhance phagocytosis of macrophage compared to *L. casei* subsp. rhamnosus NBRC 3831. The influence of probiotic bacteria on phagocytosis is strain-specific due to the structural variations in the cell wall composition of lactobacilli strains. Thus, their efficacy to trigger the phagocytic activity of macrophages are also different. 

Overall, the lactobacilli exerted a significant increase in phagocytic activity of RAW 264.7 murine macrophages. 

The possibility of bacteria causing death in murine macrophages was tested using non-viable bacterial cells at different concentrations (MOI 5, and 50). The incubation of the RAW 264.7 cells with increasing concentrations of the two lactobacilli strains (*L. casei* NBRC 15883 and *L. casei* subsp. rhamnosus NBRC 3831) for 21 h (18 - 24 h) resulted in increased viability of the macrophages as compared to those that co-incubated with *A. actinomycescomitan* strains using the trypan blue exclusion assay (Figure 2). On the other hand, *A. actinomycescomitan* strains Y4 and ATCC 29524 demonstrated a highly toxic effect against macrophages and caused more macrophage cells to die. This result suggests that the two probiotic strains used in this study had a less cytotoxic effect on macrophages and were able to activate the proliferation of murine macrophages. The capability of the *Lactobacillus* strains to promote macrophage proliferation has been presented by Jaffar et al. (2018), where lyophilzed dead lactobacilli such as *L. plantarum* NBRC 15891 and *F. fermentum* NBRC 15885 had no profound cytotoxic effect on RAW 264.7 cells, whereby the least cytotoxic effect was demonstrated by *L. johnsonii* NBRC 13952. Furthermore, a previous study showed that the three *Lactobacillus* spp. (*L. fermentum* PC1, *L. fermentum* PC2, and *L. acidophilus*) were not toxic to mouse macrophage cells.

Generally, the number of viable macrophage cells in all the treatment groups (Figure 3) was significantly higher than the untreated group, in which macrophages were mixed with only probiotic or oral pathogens. This finding suggests that pre-treatment of macrophages with a higher dose (MOI 50) of *L. casei* subsp. rhamnosus NBRC 3831 resulted in a higher cytotoxic outcome to RAW 264.7 murine macrophages than using a lower dose at MOI 5.

Moreover, all the treatment groups pre-treated with lactobacilli at MOI 5 (Figure 3A, C, E, G) showed a higher number of alive macrophages using higher doses at MOI 50 (Figure 3B, D, F, and H). Thus, suggesting that a lower amount of probiotic *Lactobacillus* strains had a more beneficial effect in fighting *A. actinomycescomitan* infections. Similar to a previous study showed incubation of macrophages with *F. fermentum* NBRC 15885 at MOI of 50 and 500 induced a higher percentage of macrophage cell death than at MOI of 5. 

Overall, pre-treatment of macrophages with probiotics *L. casei* NBRC 15883 and *L. casei* subsp. rhamnosus NBRC 3831 showed a significant rise in the number of alive macrophages and, at the same time, promote the proliferation of macrophage cells, hence had potential in counteracting *A. actinomycescomitan* strain Y4 and strain ATCC 29524 infections.

Most of the probiotics lead to the macrophages activation to secrete cytokines or several mediators due to lipoteichoic acids and peptidoglycan (PG) in their cell walls. The ability of the tested bacteria to stimulate macrophages was different among strains. *L. casei* subsp. rhamnosus NBRC 3831 was presented to be a more potent stimulator at MOI 5 to increase the secretion of IL-6 (after 48 h) (Figure 4A) and IL-8 (after 48 h) (Figure 4C) compared to strain *L. casei* NBRC 15883. However, a higher concentration (MOI of 50) of *L. casei* subsp. rhamnosus NBRC 3831 need to be used to achieve the maximum level of IL-1β cytokine right after 24 h (Figure 4F). Therefore, this finding suggests that non-viable *Lactobacillus* strains can significantly enhance and modulated cytokines secretion in a strain and dose-dependent pattern, as well as the time course of response. This statement supported by another study where the stimulation of human monocyte-derived macrophages (MDMs) with heat-killed *L. casei* IMAU60214 dose-dependently enhanced TNF-α, IL-1β, IL-6, IL-12p70, IL-10, and TGF-β production with more considerable extracellular accumulation at a ratio (bacteria: MDM) of 250:1 and 500:1 compared to 100:1 and 50:1.

IL-8 is a chemokine that mainly functions in recruiting phagocytes, such as macrophages and neutrophils, during an inflammatory reaction. In this study, the chemokine IL-8 synthesized during the initial phases of the interaction between probiotic, macromycosis, and *A. actinomycescomitan* strains (Figure 4C, D). This reaction sustained for 48 h at much higher concentrations (> 1500 pg/mL) than that of the secretion of other cytokines measured in this study (Figure 4C, D). The macrophages that pre-activated with dead *L. casei* subsp. *rhamnosus* NBRC 3831 at MOI 5 and 50 prior co-cultured with Gram-negative *A. actinomycescomitan* strains Y4, and ATCC 29524 had promoted the highest IL-8 secretion after 48 h compared to other strains (Figure 4C, D). IL-8 had proven helpful in distinguishing patients with severe periodontitis from healthy individuals.

IL-1β is a crucial inflammatory response mediator and involved in cyclooxygenase-2 activation, costimulating the function of T lymphocytes, cell proliferation, differentiation, and apoptosis. As presented in Figure 4, both probiotic *Lactobacillus* strains achieved the highest level of IL-1β right after 24 h stimulation at a ratio of 1:5 (macrophage: bacteria) for macrophages that pre-treated with *L. casei* NBRC 15883 (Figure 4E) and MOI 1:50 (macrophage: bacteria) for *L. casei* subsp. *rhamnosus*
NBRC 3831 (Figure 4F). Furthermore, high secretion of IL-1β may correlate with high phagocytic activity and cell viability induced by L. casei NBRC 15883. This finding was supported by a study that showed the active form of IL-1β was expressed only in the A. actinomycetemcomitans Y4 infected macrophages, whereas no active forms of IL-1β secreted by the macrophages that co-cultured with the lactobacilli. Thus, lactobacilli are capable of activating macrophage cells without having to be cytotoxic to the cells.24 According to Sharma et al. (2014), different cytokines such as TNF-α, IL-1β, granulocyte-macrophage colony-stimulating factor (GM-CSF), and transforming growth factor-beta 1 (TGF-β) can enhance phagocytic activity and facilitate cell proliferation.25

Moreover, this finding showed that activated RAW264.7 cells with probiotics in the treatment group had increased IL-1β secretion (Figure 4E, F), although the viability of the macrophages was not affected. The result might be due to the presence of inactive form cytokine that serves as a precursor of IL-1β that requires a pro-inflammatory protease, caspase-1, to be an active form 26, and subsequent pro-inflammatory activities.7

On the other hand, the synthesis of IL-6 was upregulated after 24 h and 48 h activation by both lactobacilli species prior to co-incubated with periodontal pathogens (Figure 4 A, B). Similarly, a previous study by Rocha-Ramírez et al. (2017) had also shown that heat-killed L. rhamnosus GG, L. rhamnosus KLDS, L. helveticus IMAU70129, and L. casei IMAU60214 were able to activate the human macrophages and induce the release of IL-8, TNF-α, IL-6, IL-12, IL-1β, and IL-10 in vitro.

Conclusion

Two dead probiotic Lactobacillus strains promoted phagocytosis activity of RAW264.7 murine macrophages and exerted immunomodulatory effects by activating the macrophages to synthesis several pro-inflammatory cytokines (IL-6 and IL-1β) and chemokines (IL-8) in vitro. These findings suggest that the non-viable probiotics have the potential to enhance the proliferation of macrophages and regulate the innate immune system to counteract A. actinomycetemcomitans strain Y4 and strain ATCC 29524 infections. The limitation of this study is the mechanisms used by the Lactobacillus strains to activate the macrophages are still unclear. Thus, future in vivo studies and more specific analyses are necessary to be done to find out the mode of action of lactobacilli and to validate their ability as an immunomodulator in hosts.

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References

14. Ramos CL, Thorsen L, Schwan RF, Jespersen L.


Tables and figures

**Figure 1:** Phagocytosis activity of macrophage cells after 24 h co-cultured with *A. actinomyctecomitans* pathogens and lactobacilli respectively at MOI 1:1000 (macrophage: bacteria). Untreated group represents macrophages culture without any bacteria exposure. Bars show the mean, error bars indicate standard deviations (n = 3) and significance was analyzed using Student’s t-test when compared to the untreated group (* = p < 0.05, ** = p < 0.01). AAY4 = *A. actinomyctecomitans* Y4; AA29524 = *A. actinomyctecomitans* ATCC 29524; LC83 = *L. casei* NBRC 15883; LCR31 = *L. casei* subsp. *rhamnosus* NBRC 3831.

**Figure 2:** Viability of macrophages after 24 h incubation with bacteria. Macrophages were co-incubated with *A. actinomyctecomitans* strain Y4 (AAY4), strain ATCC 29524 (AA29524), *L. casei* NBRC 15883 (LC83), and *L. casei* subsp. *rhamnosus* NBRC 3831 (LCR31) at MOI 1:5 (A) and 1:50 (B), respectively. Results are presented as mean ± SD (* = p < 0.05).
Figure 3: Viability of macrophages in different treatment groups. RAW 264.7 cells were pre-activated with dead *L. casei* NBRC 15883 (LC83), and *L. casei* subsp. *rhamnosus* NBRC 3831 (LCR31) at MOI 1:5 (A, C, E, G) and 1:50 (B, D, F, H) respectively and then invaded with dead *A. actinomycetemcomitans* Y4 strain (AA4) and ATCC 29524 strain (AA29524) at MOI 50. Values are illustrated as mean ± SD (n = 3) and significance was analyzed using Student’s *t*-test when compared to untreated group (* = *p* < 0.05, ** = *p* < 0.01).
Figure 4: Cytokines IL-6, IL-8, and IL-1β secretion in RAW 264.7 murine macrophages. The macrophages were induced with dead lactobacilli at MOI 1:5 (A), (C), (E), and MOI 1:50 (B), (D), (F) and then followed by dead A. actinomycetemcomitans strains stimulation at MOI 1:50 and were measured using ELISA after 24 h (■) and 48 h (□) stimulation. Results are presented as mean ± SD and significance was analyzed using Student’s t-test when compared to untreated group (* = p < 0.05, ** = p < 0.01, *** = p < 0.001).