BIOSCIENCES BIOTECHNOLOGY RESEARCH ASIA, September 2016. Vol. **13**(3), 1291-1302

Immunomodulatory and Macrophage Activating Activity of *Lactobacillus fermentum* DLBSA204 in Response to Respiratory Infection in a Cellular Model

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http://dx.doi.org/10.13005/bbra/2269

(Received: 17 June 2016; accepted: 20 July 2016)

***Lactobacillus fermentum* DLBSA204 is an isolated lactic acid bacterium (LAB) from human breast milk. This study focused on the effects of *L. fermentum* DLBSA204 on macrophage cell proliferation with MTS assay using murine RAW 264.7 macrophage-like cell line as well as related molecular changes associated with macrophage activation. It was demonstrated that *L. fermentum* DLBSA204 can activate macrophage cell by increasing Nitric Oxide and cytokine expression level. Moreover, this LAB was able to increase phagocytic activity of macrophage. *L. fermentum* DLBSA204 was also able to inhibit the adhesion of *Streptococcus pneumoniae* on lung cells. In conclusion, *L. fermentum* DLBSA204 works as an immunomodulator and macrophage activator.**

Keywords: *Lactobacillus fermentum*, immunomodulator, macrophage, *Streptococcus pneumoniae.*

Human breast milk has been known to protect infants from infections and diseases caused by various types of pathogenic bacteria. This protective property is due to the immunomodulatory compounds which are also a major factor in the initiation and development of neonatal gut microbiota[1, 2, 3]. The human breast milk also contains potential probiotic that possess antimicrobial[4] and immunomodulatory property which may boost the infant's immunity[5].

Probiotics are live microorganisms, which when consumed in an adequate dose, confer health benefits to its host. Members of the genera *Lactobacillus* is known to be one of the main constituents of normal microflora in the human body. They especially colonize human oral cavity, rectum[6], and breast milk[4, 7, 8] where they contribute profoundly to the health of its host and in the case of breast milk, to the infant. Some of these health benefits include prevention and treatment of common diseases in children related with respiratory tract infections[9, 10].

Respiratory tract is divided into 2 parts; the upper and lower respiratory tract. Infection of both upper and lower respiratory tract are caused by various viruses and bacteria, with the most common being *Streptococcus pneumoniae*. Treatments for these infections are drugs for symptomatic treatment, herbal remedies, zinc supplementation and antibiotic [11, 12]. A clinical study with 326 eligible children has demonstrated probiotic treatment that consisted of *Lactobacillus acidophillus* NFM (N110) or *L. acidophillus* NCFM in combination with *Bifidobacterium animalis* subsp *lactis* Bi-07 (N112) for 6 months was able to help treat respiratory tract infection by reducing

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incidence and duration of fever, rhinorrhea and cough[9]. Other probiotic, *Lactobacillus rhamnosus* GG has also been known to help treat respiratory tract infection by modulating host immune response[13]. From these examples, it is evident that the ability to modulate the host's immune system is critical to treatment of respiratory tract infection. The stimulation of immune system by probiotic bacteria is species and strain-dependent. Bifidobacterium and Lactobacilli, which are both probiotic bacteria, possess different mitogenic activity on splenocytes[14]. Different strains of Lactobacilli also induce different cytokine expression in *in vitro* and *in vivo* study, such as TNF-á and IL-6[15, 16]. Therefore, efficacy of each specific strain has to be investigated.

In most studies, *in vitro* immunomodulatory activity was observed using bacterial cultures exposed to RAW 264.7 macrophage cells[17, 18]. In this study, the biological activity of *L. fermentum* DLBSA204, a lactic acid bacteria (LAB) strain isolated from human breast milk, was evaluated in RAW 264.7 and A549 cells. This LAB was evaluated for its potential in promoting macrophage activation, competition for adhesion with *S. pneumonia*, and antioxidant activity *in vitro*.

MATERIALS AND METHODS

Lactobacillus fermentum DLBSA204 was isolated in our laboratory from human breast milk. De Man, Rogosa and Sharpe broth (MRSB); De Man, Rogosa and Sharpe agar (MRSA), t*rypticase soy broth* (TSB) and t*rypticase soy* agar (TSA) were purchased from © Merck KGaA, Darmstadt, Germany. RAW 264.7 (ATCC® TIB71™), A549 (ATCC® CCL185™) and *Streptococcus pneumonia* (ATCC® 49619™) were derived from ATCC, Manassas, USA. Dulbecco's Modified Eagle's Medium (DMEM), F-12K medium, L-glutamine, Fetal Bovine Serum (FBS) and penicillin/ streptomycin (P/S) were acquired from Gibco® (Thermo-fischer scientific, Waltham, MA, USA). TRizol® was obtained from Invitrogen (Thermo-fischer scientific, California, United States), while PGE_2 ELISA kit was obtained from GE Healthcare (Little Chalfont, United Kingdom). One Step RNA PCR Kit RT, RNasin, dNTP mix, oligo dT, $MgCl_2$, Taq polymerase, GoTaq Green master mix, Track It DNA Ladder 100 bp, and CellTiter 96® Aqueous one solution cell proliferation assay (MTS) were purchased from Promega (Madison, USA). Lipopolysaccharide (LPS) from *Escherichia coli* 055:B5, DPPH (2,2-diphenyl-1-picylhydrazyl), Griess reagent and all other chemicals used in this study were obtained from Sigma-Aldrich (Saint Louis, USA) and were of analytical grade.

Preparation of LAB isolate

L. fermentum DLBSA204 was isolated from human breast milk and has been identified with API system that was based on carbohydrate fermentation profile. The identification was done using the API 50 CHL kit (BioMérieux; USA). The result proved that the strain is *Lactobacillus fermentum*. This strain was further cultured at 37°C in MRSB for 18-20 hours. The resulting pellet was collected by centrifugation at 4000xg for 10 minutes using Hettich Mikro 200 centrifuge (Hettich, Massachusetts, United States), and then washed twice with PBS 1x (137 mM NaCl, 2.7 mM Kcl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4). For the experiment, $1x10^9$ colony forming unit (CFU) of bacteria per mL was added to the cell culture. Untreated cells were used as control, while LPS 250 ng/ml was used as positive control to induce inflammation. *S. pneumonia* was cultured in t*rypticase soy broth* (TSB) for 20 hours and was used in adhesion and phagocytic activity assays.

Cell culture

Mouse macrophage cell line RAW 264.7 was cultured in DMEM supplemented with 10% (v/v) FBS and 1% P/S 100 U/ml at 37°C in a humidified incubator with 5% CO_2 (New Brunswick Scientific Co. Inc, New Jersey, United States) as described previously (19). Human lung cancer cell line A549 was grown in F12K medium supplemented with 10% (v/v) FBS, and 1% P/S (100 U/ml) at 37°C in a 5% CO_2 humidified incubator.

Macrophage cell viability assay

MTS assay was used to evaluate *L.fermentum* DLBSA204 as macrophage activator. RAW 264.7 cells were seeded onto 96-well plate. As much as 10^9 CFU/ml of DLBS *L.fermentum* DLBSA204 was added to the culture and incubated for 24h. MTS solution 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (0.5mg mL^{-1}) was added to the cells

for 2h to induce reaction. Absorbance at 490 nm was measured with model 680 microplate reader (BioRad Laboratories, CA, USA).

Effect of *L. fermentum* DLBSA204 on NO production

RAW 264.7 cells were seeded in a six-well plate and incubated at 37°C with 5% CO_2. As much as 10^9 CFU/ml of *L.fermentum* DLBSA204 was added to the culture and incubated for 24h at 37°C. NO in culture supernatant was measured as concentration of nitrite using Griess reagent. One hundred microliter of cultured supernatant was added into the 96-well plate. Then 100 ìl of Griess reagent was added to each well and allowed to stand for 15 min at 25°C. The absorbance at 540 nm was measured with model 680 microplate reader (680, BioRad Laboratories, CA, USA).

Phagocytic activity of RAW 264.7 cells

A ciprofloxacin protection assay was used to quantify the ability of RAW 264.7 cells to ingest *S. pneumonia*. A 24-well plate was inoculated with RAW 264.7 cells suspended in DMEM with density of 25 x 10^6 cell/ml and incubated at 37^0C with 5% CO_2 in a humidified incubator. The cells were treated with 250 ng/ml LPS and *L. fermentum* DLBSA204 10^9 CFU/ml for 24 hours. After incubation, 10^8 CFU/ml of *S. pneumonia* was added to each sample for 15 minutes. Fifteen minutes is the optimal incubation time for macrophage to phagocyte *S. pnuemoniae*. Following incubation, the medium was discarded and the cells were incubated for 2 hours in the presence of 1 ml DMEM supplemented with 25 ìg/ml of ciprofloxacin. Upon completion of incubation, all cells were washed thoroughly with PBS 1x to remove all traces of antibiotic, prior to addition of RIPA buffer (50 mM Tris pH 6.8, 0.5% (wt/vol) sodium dodecyl sulfate, 150 mM sodium chloride, 1% NP-40, 2 mM ethylenediaminatetraacetic acid (EDTA) and 1x protease inhibitor). Recovered *S.pneumoniae* from lysed RAW 264.7 cells were serially diluted and plated on tryptic soy agar (TSA) supplemented with sheep blood. Enumeration of recovered bacteria was done by colony count.

Gene expression analysis

TRIzol® reagent (Thermo Fisher Scientific) was used to homogenize cells and isolate total RNA. cDNA synthesis was performed using a reverse transcription system kit according to the manufacturer's instructions. Polymerase chain reaction (PCR) was performed to amplify a specific gene using oligonucleotide primer designed by Primer 3 software as described previously (20, 21). The gene sequences were obtained from the Genebank database. Genes involved in macrophage activation (primer sequences are shown in table 1) were detected and amplified in a mixture of 12.5ìL Go Taq Green master mix, a pair of target genes with a final concentration of 1 ìM, a pair of internal controls with a final concentration of 0.2 ìM, 3ìL cDNA, and nuclease-free water to a total of 25ìL. PCR process generally consisted of initial denaturation at 94^0C for 3 minutes; 30 cycles of denaturation at 94^0C for 30 seconds, primer annealing at 60-61^0C for 30 seconds, and elongation at 72^0C for 1 minute. An additional elongation was performed at 72^0C for 5 minutes. Reverse transcription polymerase chain reaction (RT-PCR) was performed using PCR Biometra. The targeted gene expression was quantified using Quantity One software and the arbitrary unit was measured by comparing the expression of target gene with internal control expression.

Measurement of Lysozyme Release in Culture media

Lysozyme release was observed from the lysis rate of *Micrococcus lysodeikticus*. RAW 264.7 cells were seeded in a six-well plate and treated with LPS 250 ng/ml and DLBSA204 10^9 CFU/ml. The cells were incubated for 24h at 37^0C in 5% CO_2 in a humidified incubator. Cell supernatant was collected and centrifuged at 4000xg for 10 minutes. The supernatant was collected and mixed with 950 ml of *M. lysodeikticus* suspension of 200 $mg.ml^{-1}$ in 0.05M sodium phosphate buffer (pH 6.2). The mixture of cell supernatant and *M. lysodeikticus* was incubated at 25ºC, and its optical density (OD) was measured at the 5^{th} and 6^{th} minute using a spectrophotometer with absorbance at 530 nm. The change in absorbance was compared to the standard solution of crystalline egg white lysozyme. One activity is equivalent to a decrease of 0.001 absorbance unit per minute.

Adhesion competition of *L. fermentum* DLBSA204 and *S.pneumoniae* with Scanning Electron Micrograph (SEM) and Total Plate Count

Observation of adhesion competition was analyzed microscopically using SEM and was counted by plating the attached bacteria in the

appropriate medium. Prior to the observation with SEM, the A549 cells were grown on a cover slide. After 24 hours, the cells were treated with the *L. fermentum* DLBSA204 and *S.pneumoniae*, and incubated for another 24 hours. Then, the cell was washed with PBS buffer for several times and air-dried. The cells were post-fixed in 2% formalin for an hour, instead of glutaraldehyde. The cover slide was soaked in PBS buffer for 15 minutes and dehydrated through graded ethanol solutions. The cell was air-dried, coated with gold and examined with JEOL JSM 6510 scanning electron microscope (Peabody, MA, USA).

In total plate count method, the A549 human lung cells were seeded in a 12-well plate and incubated at 37°C with 5% CO_2. *L. fermentum* DLBSA204 was added to the culture and incubated at 2h and 24h. Then the culture was washed 3 times with sterile PBS and split using trypsin EDTA. The number of viable attached-bacteria was quantified and expressed as CFU. The CFUs were determined by plating the diluted bacterial suspension on MRSB or TSB plates depending on the bacterial strains.

Scavenging activity of DPPH Radical

In this study, DPPH (2,2-diphenyl-1-picylhydrazyl) was used as a stable radical. Bacteria was inoculated to MRSB and incubated at 37°C for 18-20 hours. The pellet of bacteria was collected and resuspended in PBS. One hundred microliter of the sample was added to 2 ml of DPPH in methanol, mixed vigorously and allowed to stand in the dark at room temperature for 30 minutes. The absorbance was measured at 517 nm using a spectrophotometer (Helios, Thermo Fisher Scientific, Waltham, MA USA). The blank standard was prepared by replacing the extract with methanol. Radical scavenging activity of the samples was expressed using the following equation:

$$\text{DPPH radical scavenging activity (\%)} = [1 - (OD_{517,\ sample} / OD_{517,\ blank})] \times 100 \quad (22)$$

Statistical Analysis

The statistical difference between the test and control samples was determined by Student's *t-test* analysis using the Stat-View software package (Abacus Concepts, Piscataway, NJ, USA). Values are expressed as means ± standard deviation for at least two independent experiments.

RESULTS

Effect of DLBSA204 on Macrophage Proliferation

Effect of *L. fermentum* DLBSA204 on the viability of RAW 264.7 macrophage cells was investigated. The proliferation of RAW 264.7 cells in the presence of *L. fermentum* DLBSA204 after 24h increased 21.42% compared to control group treated with medium only (fig.1), while proliferation of RAW 264.7 cell which was treated with LPS increased 34.6%. LPS is used as positive control because it can induce inflammation, which activates macrophage as immune system alert.

Induction of NO Secretion by DLBSA204

Production of NO in the normal RAW 264.7 cell was 14.59 pg/ml, whereas the cell culture that was co-cultured with *L. fermentum* DLBSA204 was measured at 36.35 pg/ml. The production of NO in cell co-stimulated with LPS (250 ng/ml) was increased to 48.58 pg/ml (fig. 2). Higher level of NO after stimulation of *L. fermentum* DLBSA204 compared to control indicated that macrophage has been activated.

Induction of *S. pneumonia* Phagocytosis in RAW 264.7 cells

In order to investigate the phagocytic activity of macrophage with *L. fermentum* DLBSA204 treatment, phagocytic activity assay was conducted. In this experiment, ciprofloxacin was used as an antibiotic that inhibit growth of external *S. pneumonia*. Fifteen minutes incubation of ciprofloxacin is the optimal time to ensure the survival of macrophage. In this study, DLBSA204 treated-cells have a higher phagocytic activity on *S. pneumoniae* with 2.5-fold higher activity compared to the normal cells (fig.3).

Lysozyme measurement

Activated macrophage induces high level of lysozyme[23]. This study measured the release of lysozyme from macrophage as an indicator of macrophage activation. Lysozyme level in the supernatant of *L. fermentum* DLBSA204 treated-macrophage cells was higher than the untreated cells (fig.4). Decreased turbidity was observed in the substrate buffer (as a measure of lysozyme) after treatment with supernatant of *L. fermentum*

DLBSA204-treated cells. The lysozyme levels were measured in units/min.

Effect of DLBSA204 to cytokine expression

Cytokine expression is an inducible marker of macrophage activation. To reveal whether macrophage activation by probiotic is dependent on TNF-á and IL-6 signal transduction, we traced the gene expression of macrophage after

Table 1. Primer Sequences

Primer	Forward sequence	Reverse sequence
TNF-α	AAC TTC GGG GTG ATC GGT CC	CAA ATC GGC TGA CGG TGT GGG
IL-6	CCG GAG AGG AGA CTT CAC AG	ACA GTG CAT CAT CGT TGT TC
GAPDH	TCA CCA CCA TGG AGA AGG C	GCT AAG CAG TTG GTG GTG CA

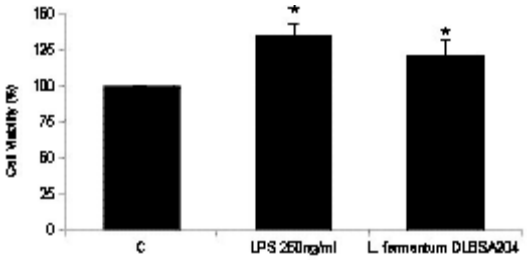

Fig. 1. Cell Viability Analysis of macrophage RAW 264.7 treated with LPS 250 ng/ml and *L. fermentum* DLBSA204 after 24h. The amount of viable cells was determined by MTT assay. The result is presented as percentage of value obtained from the treated compared to untreated control. Values are expressed as means ± standard deviation of two independent experiments. Note: *P<0.05

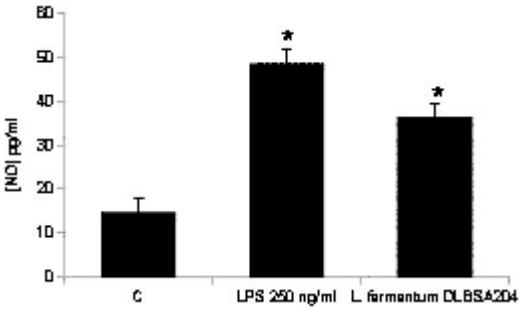

Fig. 2. Nitric Oxide of macrophage RAW 264.7 cells treated with LPS 250 ng/ml and *L. fermentum* DLBS A204 after 24h. Values are expressed as means ± standard deviation of two independent experiments. Note: *P<0.05

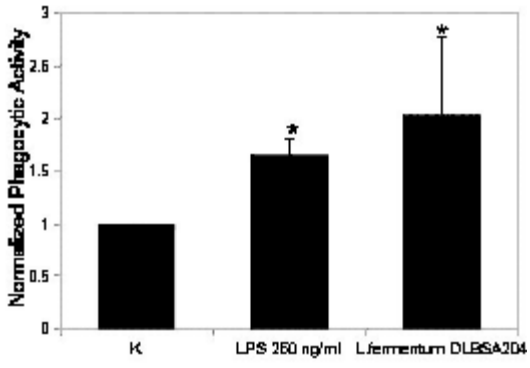

Fig. 3. Phagocytic Activity of RAW 264.7 macrophage on *S. pneumonia* after treatment with LPS or *L. fermentum* DLBSA204. The amount of bacteria growth was determined by colony count. The result is presented as normalized fold value obtained from treated compared to un-treated control cell culture. Results are shown as mean value ± standard deviation of two independent experiments. Note: *P<0.05

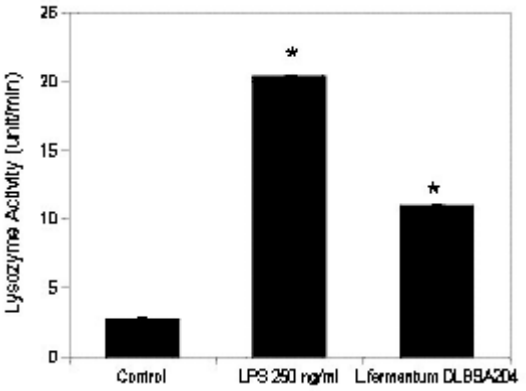

Fig. 4. Lysozyme activity of RAW 264.7 cells macrophage treated with LPS and *L.fermentum* DLBSA204. One activity is equivalent to an absorbance decrease of 0.001 unit per minute. Note: *P<0.05

administration of LPS and DLBSA204. As shown in figure 5, after 24 h treatment, *L. fermentum* DLBSA204 induced higher level of TNF- á and IL-6 compared to control

Effect of *L. fermentum* DLBSA204 on *S. pneumoniae* adherence

In order to examine the adhesion competition between *L. fermentum* DLBSA204 and *S. pneumoniae*, investigation was done using SEM photographs and total plate count method. In the control *S. pneumonia* and *L.fermentum* DLBSA204 samples, it was apparent that separately, the number of adherent *S.pneumoniae* to A549 cells was higher compared to the number of adherent *L. fermentum* DLBSA204 (fig. 6A, 6B, 6C & Table 2). In the co-inoculation samples, pre-incubation of A549 cells with *L. fermentum* DLBSA204 prior to *S. pneumoniae* reduced adhesion of *S. pneumoniae* to 3.19% (fig. 6D). Pre-incubation of A549 cells with *S. pneumoniae* prior to *L. fermentum* DLBSA204 also showed good result. The adhesion of *S. pneumoniae* was reduced to 9.82% (fig. 6E). This result proved that *L. fermentum* DLBSA204 is able to inhibit and remove pathogenic bacteria adhesion on the human lung cells.

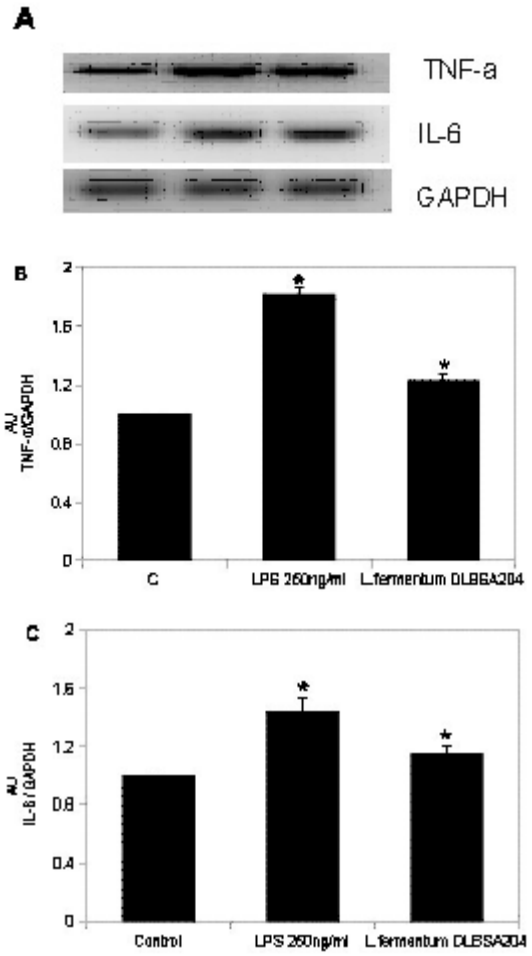

Fig. 5. Expression of several immunomodulatory signals in RAW 264.7 after treatment with LPS 250 ng/ml and *L.fermentum* DLBSA204 for 24h. (A) Shows PCR of TNF-á, IL-6 and GAPDH gene expression. Lane 1 shows gene expression from untreated cells, lane 2 from LPS 250 ng/ml treated-cells, lane 3 from *L. fermentum* DLBSA204 treated-cells. (B) Shows arbitrary unit (AU) of TNF-á gene expression. (C) Shows AU of IL-6 gene expression. Results are expressed as mean value ± standard deviation of two independent experiments.
Note: *P<0.05

Antioxidative Activity of *L. fermentum* DLBSA204

The antioxidative activity of *L. fermentum* DLBSA204 was determined by the percentage of inhibition to DPPH scavenging activity. Inhibition effect of *L. fermentum* DLBSA204 on DPPH radical was observed in a dose-dependent manner (Figure 7). The lowest dose of *L. fermentum* DLBSA204 (1/4 dose) in this experiment was $2x10^8$ CFU/ml with 41.43% DPPH inhibition, while *L. fermentum* DLBSA204 10^9 CFU/ml inhibit 76.48% DPPH.

DISCUSSION

The present study was performed to evaluate the potential of *L.fermentum* DLBSA204 as a mode of prevention and treatment of bacterial respiratory tract infection through immune response modulation. The study was conducted based on observations on three critical parameters; ability to activate macrophage, compete for adherence with *S.pneumoniae* and antioxidative activity (fig. 8).

Stimulation of innate immune system in initiating adaptive immune response is done by macrophage. Macrophages are tissue-based phagocytes which are derived from monocyte. Ability of macrophage to respond to environment stimuli by changing its form and physiological activity is referred to as “activation” response which is pivotal to the host immune function[24]. Macrophage activation by *L. fermentum* DLBSA204 was investigated by measuring the proliferation, gene expression and biochemical

activity of macrophage; which affect the innate and adaptive immune response. Different stimulus leads to different responses by the macrophage. In the present study, we found that *L. fermentum* DLBSA204 increased the number of macrophage. The increment was lower compared to those in the LPS- treatment, which was derived from *E. coli* (fig. 1). Stimulation by microorganism will result in Toll-like receptor (TLR) activation that induces expression of cytokine, such as TNF- á. Level of macrophage proliferation by *L. fermentum* DLBSA204 was in line with the level of upregulation of TNF-á gene expression (fig. 5A, fig. 5B). Upregulation of TNF-á gene is known to be a central key in immune response modulation because it is among the first cytokine produced by phagocytic cells, such as macrophage[25]. This result suggests that macrophage proliferation was initiated by the upregulation of TNF-á gene expression[16].

Increment of TNF- á level will induce NF-ÊB pathway that produces NO as a marker for

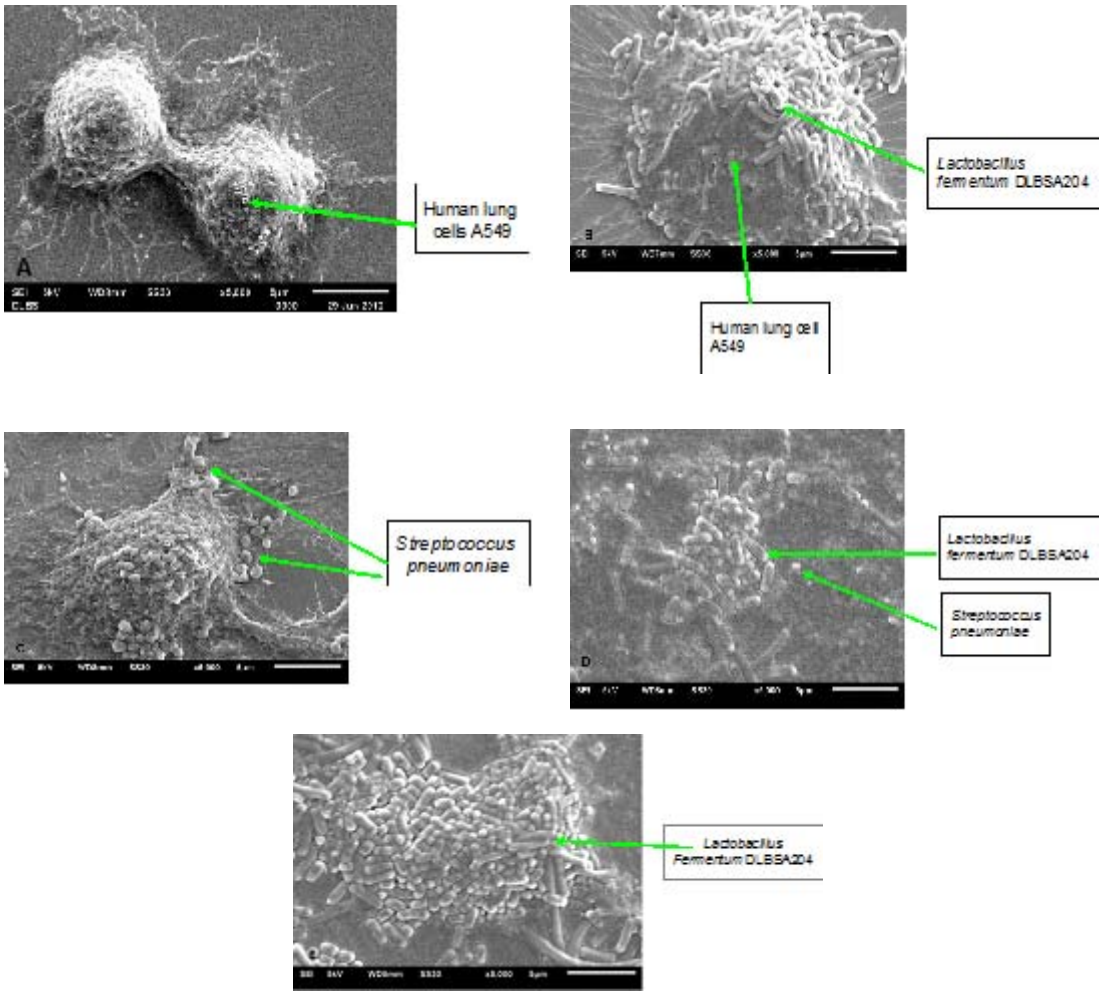

Fig. 6. SEM observation on *L.fermentum* DLBSA204 and *S.pneumoniae* adherence to human lung cell. (A) A549 cells without bacteria adhesion. (B) A549 cells with adherent *L.fermentum* DLBSA204 for 24 hours. (C) A549 cells with adherent *S.pneumoniae*. (D) A549 cells incubated with *L. fermentum* DLBSA204 for 2 hours, followed by addition of *S. pneumoniae* for 24 hours. (E) A549 cells incubated with *S. pneumoniae* for 2 hours, then addition of *L. fermentum* DLBSA204 for 24 hours. Bar = 5 µm

macrophage activation [24]. NO production will increase in response to bacterial lipopolysaccharide. *L. fermentum* DLBSA204 treatment also increased NO production in macrophage although not as high as LPS treatment (fig. 2). Activated macrophage is indicated by its

Table 2. Adhesion of *L.fermentum* DLBSA204 and *S.pneumoniae* on human lung cells

Treatment	% Adhesion to human lung cells	
	L. fermentum DLBSA204	*S. penumoniae*
L. fermentum DLBSA204 only	28.01 %	-
S. pneumonia only	-	68.66 %
L. fermentum DLBSA204 then *S. penumoniae*	30.81 %	3.19 %
S. pneumonia then *L. fermentum* DLBSA204	67.79 %	9.82 %

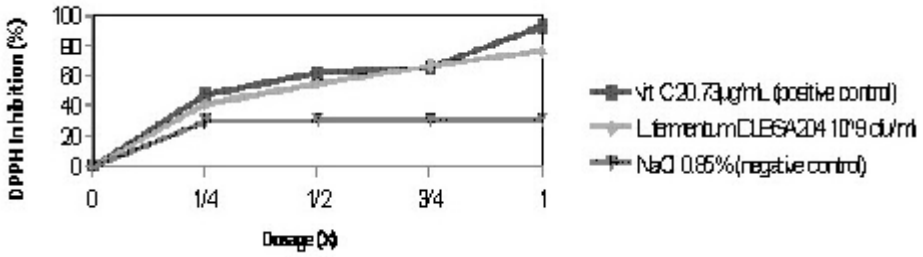

Fig. 7. DPPH radical scavenging activity of *L. fermentum* DLBSA204

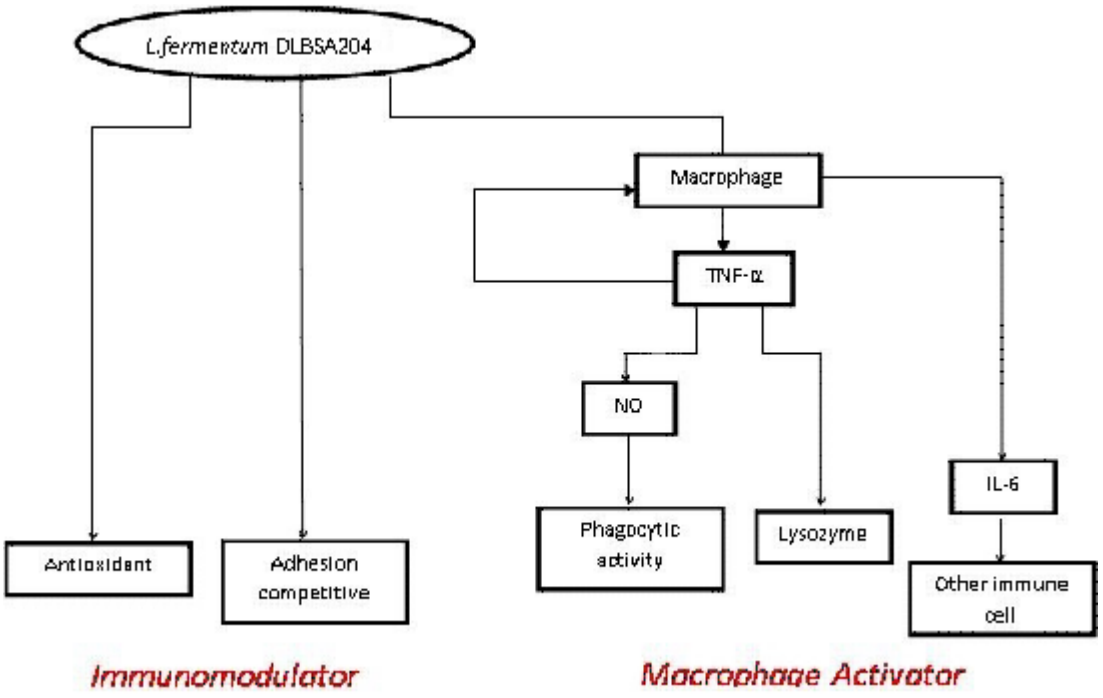

Fig. 8. Proposed Mechanism of Action of *Lactobacillus fermentum* DLBSA204 on *S.pneumoniae* infection

phagocytic activity[24]. Phagocytosis is the primary means of removing foreign microorganism that is mediated by polymorphonuclear neutrophils and macrophages. In the lung, macrophage serves as effector cells which protect the alveolar surface from harmful microorganisms by phagocytosis (26). *L. fermentum* DLBSA204 was able to enhance the ability of macrophage to phagocyte *S. pneumoniae* (fig. 3).

Upregulation of TNF-á expression also increase lysozyme level[23]. Lysozyme is a cationic enzyme that affects the â-1, 4 glycosidic bond between N-acetylmuramic acid and N-acetylglucosamine in the peptidoglycan of bacterial cell wall. Lysozyme works by lysing the cell wall of Gram positive bacteria because it has thicker peptidoglycan layer compared to Gram negative bacteria[27]. Pathogenic bacteria in the respiratory tract is not only limited to Gram positive bacteria, but also Gram negative bacteria. Lysis of other invasive Gram negative respiratory bacteria needs interaction of lysozyme with other defense protein, for example lactoferin[28]. In this study, it was found that *L. fermentum* DLBSA204 enhanced secreted lysozyme level from macrophage (fig. 4). This result is similar to a study by Sun *et al* (2010) which showed that probiotic diet for 60 days increased level of lysozyme in rats[29].

Both pathogenic and probiotic bacteria adhere to epithelial cells of the host to survive and to further colonize the host. In this study, competition between both bacteria was evaluated to determine the ability of *L.fermentum* DLBSA204 in inhibiting respiratory tract infection by *S.pneumoniae*. The inhibition study was done by *in vitro* assay with human lung A549 cell as a model of human respiratory tract. The ability of *L. fermentum* DLBSA204 to inhibit pathogenic bacteria infection on human respiratory tract was examined in adhesion competition assay against *S. pneumonia*. *S. pneumonia* is a Gram-positive bacteria that induces nasopharyngeal and lung infection. It is a major pathogenic bacteria in human which has the ability to autolysis[30]. This bacteria normally lives in mucosal surface of the upper respiratory tract. Infection will occur if the bacteria colonized sterile parts of the respiratory tract[11]. The adhesion of *L. fermentum* DLBSA204 to A549 cell shows that *L. fermentum* DLBSA204 adhered to human lung cells with higher percentage compared to *S. pneumoniae* (Table 2). These data suggest that DLBSA204 was able to inhibit *S.pneumonia* from adhering to the human lung cells and replace adherent *S. pneumoniae* on the human lung cells. An *in vivo* experiment has been done and demonstrated adhesion competition between probiotic and pathogenic bacteria on mice's respiratory tract[31]. The result showed that *Lactobacillus lactis* NZ9000 was able to reduce colonization of *S. pnuemoniae* on the lung and prevent this pathogenic bacteria from entering the blood. The reduced number of pathogenic bacteria adherence is a result of competition for adhesion sites and nutritional sources, low pH effect from lactic acid, and secretion of antimicrobial substances[32]. The ability to inhibit *S. pneumonia* from adhering to human epithelial cells was also demonstrated by *Lactobacillus rhamnosus* GG (LGG)[31]. LGG was effective in inhibiting adherence in the pre- addition assay but less efficient in post-addition assay. The *L. fermentum* DLBSA204 works in a similar way as LGG. Pre-addition assay demonstrated prevention strategy, while post-addition assay demonstrated curative strategy. Therefore, it can be inferred that both LAB isolates work more effectively as preventive agent.

A double-blind, placebo-controlled study with 326 eligible children that consumed probiotics via oral route demonstrated that probiotic could reduce symptoms of upper respiratory tract infection (URTI)[9]. Reduced occurrence of cough symptoms was explained by enhanced immune response of the host. The probiotics improve local immunity (maintaining the integrity of gut wall) and enhance systemic immunity (enhancing non-specific immune system)[10]. It was shown that administration of probiotic via oral route, rather than via nasal route also enhance immune response of the host.

One of several cytokines that is released by activated macrophage to induce other immune cells is IL-6. IL-6 gene expression was found to be higher after treatment with DLBSA204 (fig. 5A, 5C). IL-6 gene expression by macrophage initiated the activation of other immune cell responses, such as differentiation of B cells and T cells, and production of IgA[25]. Elevated level of IL-6 gene expression observed in the present study further suggests

that IL-6 was the key factor that caused IgA increment in animal after oral administration of probiotic.

Immune system is dependent to the level of antioxidant and oxidant in the body where antioxidant helps maintain cell membrane integrity and functionality, cellular proteins, and nucleic acids. Furthermore, it also helps control signal transduction and gene expression in the cells[33]. Antioxidative activity of *L. fermentum* DLBSA204 was observed and it was found that *L. fermentum* DLBSA204 was able to increase the free radical scavenging activity in a dose-dependent manner (fig. 7). The antioxidative activity of DLBSA204 was measured by DPPH free radical scavenging method which shows the percentage of remaining DPPH when the kinetics reached a steady state[34]. Several studies have shown that other probiotics, such as *Lactobacillus paracasei*[22], *Lactobacillus plantarum*[35] and *Lactobacillus fermentum* ME-3 (LfME-3)[36, 37] also have antioxidative activity ,.

CONCLUSION

Lactobacillus fermentum DLBSA204 has the potential to be used as an immunomodulator and macrophage activator. However, further study is needed to ensure the safety of this bacterium before it can be applied for human consumption.

ACKNOWLEDGMENT

The authors would like to thank Dr. Siswa Setyahadi and Hanna C. Rouli for their assistance in editing the manuscript. This research is supported by Dexa Medica.

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