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Effect of lactic acid bacteria isolated from fermented mustard on immunopotentiating activity

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PEER REVIEW

Peer reviewer

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Comments

The present manuscript is well written, with good methodologies and the results are well discussed. The results herein obtained are very interesting and reveal a good potential for supplement to induce immunopotentiating activities.
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ABSTRACT

Objective: To investigate the effect of lactic acid bacteria isolated from fermented mustard on immunopotentiating activity

Methods: One hundred and fifty nine strains of lactic acid bacteria isolated from traditional Taiwan fermented mustard were evaluated for their immunopotentiating activity on a murine macrophage cell line RAW 264.7.

Results: Of the strains, pronounced increases in the levels of nitric oxide (NO), tumor necrosis factor-α and interleukin-6 were observed in strains B0040, B0110 and B0145. Among them, strain B0145 had the highest NO and tumor necrosis factor-α generation in RAW 264.7 cells; strains B0040 and B0110 were also superior to that of *Lactobacillus casei*. These results demonstrated that NO and cytokines were effectively induced when the bacterial stimulants were treated with macrophages. In addition, strains B0040 and B0110 were identified as *Lactobacillus plantarum*, and B0145 as *Weissella cibaria* using 16S rDNA analysis.

Conclusions: The results implicated selected strains may be regarded as a biological response modifier and had a broad application prospects in exploiting new functional food or as a feed additive.

KEYWORDS
Immunopotentiating activity, Interleukin-6, Lactic acid bacteria, Nitric oxide, Tumor necrosis factor-α

1. Introduction

Some lactic acid bacteria (LAB) are believed to play important roles in the development and maintenance of health benefit of host. These possible health effects include assimilation of cholesterol[1], reducing dental caries[2], modulating the immune system, increasing the antibacterial, anticancer and antimutagenic activities and preventing cancer recurrence[3-5]. Recently, a considerable attention has been focused on immunological functions of LAB as a promising strategy for health-promoting

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effects. For instance, some LAB stimulate natural killer cell and modulate systemic inflammation, which contribute to exhibit antitumor and antiinfectious activity[6-8]. In addition, activated macrophages are able to recognize and lyse tumor cells which are resistant to cytostatic drugs and can play a key role in novel immunotherapeutic approaches to the treatment of cancer[9]. Previous studies noted that activated macrophages were able to induce the production of hydrogen peroxide, nitric oxide (NO), and cytokines, such as interferon-γ, tumor necrosis factor (TNF)-α and interleukin (IL)-6 which conducted a pivotal function in a variety of immune response[10-12]. In other words, the intake of LAB which activated macrophages may enhance resistance against infection by pathogenic organism and help in the prevention of cancer[4,5].

In Asia, fermented fruits and vegetables products had a long history in human nutrition from ancient ages and were associated with the several social aspects of different communities. Recent studies were conducted to evaluate traditional fermented vegetables as potential natural sources of probiotic bacteria[13]. Suan-tsai is traditional fermented mustard which is widely used in Taiwan. It is made from green mustard and its production is a spontaneous fermentation process by a mixed microbial population mainly composed of LAB[14]. However, the information related to immunological functions of LAB isolated from traditional Taiwan fermented mustard is limited. The aim of this study was to evaluate the *in vitro* effect of LAB isolated from suan-tsai on the induction of NO and cytokines such as TNF-α and IL-6 in RAW 264.7 macrophage cells and the strains were also identified.

2. Materials and methods

2.1. Microorganism and culture condition

One hundred and fifty nine strains of LAB isolated from suan-tsai were used in the experiments. In addition, the probiotic lactobacilli *Lactobacillus casei* (*L. casei*), isolated from a commercial yogurt of Yakult Co., Ltd. (Taipei, Taiwan), was also included in the study. All strains were maintained at -80 ˚C in 20% (v/v) glycerol. Prior to each of the experiments, all LAB were cultured in de Man, Rogosa and Sharpe broth (MRS broth; Difco Laboratories, Detroit, MI, USA) at 37 ˚C for 18 h two or three times.

2.2. Preparation of bacterial stimulants

After cultivation, the bacteria were collected by centrifugation and washed twice with phosphate-buffered saline (pH 7.2). The spent culture supernatant (SCS) of LAB was collected by centrifugation at 8 500 rcf for 10 min at 4 ˚C and filtered through a 0.22 μm pore filter unit (Millipore, Bedford, MA). For all experiments involving heat-inactivated bacterial preparations, samples of freshly-prepared cultures were enumerated using the appropriate agar, while additional samples of the same cultures were heat-inactivated (95 ˚C/30 min). Successful heat-killing was confirmed by the absence of bacterial growth on MRS agar plates. Subsequently, the concentration of the heat-inactivated preparation was adjusted in lieu of the live plate counts and used for further experimentation. For the treatment of RAW 264.7 cells, the viable and heat-inactivated bacteria were then suspended in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, St. Louis, USA) at 10^9 CFU/mL, respectively. The bacterial preparations were stored at -80 ˚C until use.

2.3. Cell culture

The mouse macrophage cell line RAW 264.7 was purchased from Bioresource Collection and Research Center (Hsinchu, Taiwan) and maintained in DMEM complete medium, supplemented with 10% fetal bovine serum, streptomycin (100 μg/mL), and penicillin (100 IU/mL) at 37 ˚C in a 5% CO_2 humidified incubator.

2.4. Determination of NO and cytokines

For the experiments, RAW 264.7 cells were cultured in 24-well tissue culture plates with a density of 5×10^5 cells/mL. To the wells, either viable or heat-inactivated bacteria at representing a bacteria:cell ratio of 25:1 were added. After 24 h incubation (37 ˚C, 5% CO_2), the supernatant were collected and analyzed for NO and cytokines.

The cultured supernatant was collected and analysed for NO production via the Griess reaction[10]. Briefly, the supernatant was mixed with an equal volume of Griess reagent (1% naphthylethylenediamine dihydrochloride and 1% sulfanilamide in 5% phosphoric acid) in 96-well plates. After the mixed solution had reacted for 10 min at room temperature and absorbance was measured at 540 nm, NO concentrations were calculated on the basis of a standard curve prepared using sodium nitrite.

The concentrations of IL-6 and TNF-α were assayed with commercial ELISA kits (Pharmingen, CA, USA) according to the manufacturer's recommendations, and absorbance was measured at 450 nm using the 96-well plate reader.

2.5. Cell viability

The cytotoxicity of the LAB on RAW 264.7 cells was estimated based on the method of 3-(4,5-dimethylthiazol-2-yl)-2,5-

diphenyltetrazolium bromide (MTT, Sigma-Aldrich). The RAW 264.7 cells at a density of 5×10^5 cells/mL were treated with either viable or heat-inactivated bacteria at representing a bacteria:cell ratio of 25:1 for 48 h. After removing the supernatant from the plate, the cells were incubated at 37 ℃ with 0.5 g/L MTT for 60 min. The medium was aspirated and the cells were solubilized in dimethylsulfoxide (250 μL) for at least 2 h in the dark. The extent of the reduction of MTT was quantified by measuring the absorbance at 540 nm. Cell viability was expressed as the optical density of formazan formed in the cells, and was calculated as follows:

Viability % of control = $[(A_{sample} - A_{blank}) / (A_{control} - A_{blank})] \times 100$

Where A_{sample} is the absorbance of the cells treated with various bacterial stimulant of LAB or lipopolysaccharide (LPS from *Escherichia coli* O55:B5, Sigma, USA), A_{blank} is the absorbance of the solution used, and $A_{control}$ is the absorbance of the cells alone.

2.6. Strains identification

LAB isolates with high immunopotentiating activities were identified by API 50 CHL fermentation assays (bioMérieux Inc., Lyon, France) and 16S rDNA sequence analysis. The primarily confirmed by API 50 CHL fermentation assays were following the instruction procedure. In 16S rDNA sequence analysis, the PCR primers designed from the 16S rDNA genes primers 27F/1492F[1]. The amplification products were purified with DNA purification kit (Promega, WI, USA) and sequenced by nucleic acid synthesis and analysis core laboratory (Cheng Kung University, Tainan, Taiwan). Sequence homologies were examined by comparing the obtained sequence with those in the DNA databases (http//www.ncbi.nim.nih.gov/BLAST).

2.7. Statistical analysis

All statistical analyses were performed using SPSS software (ver. 12.0; SPSS Inc., IL, USA), and results were calculated with fold or percentage relative change of control by dividing experimental data by control values and presented as the means ± SD of at least three independent experiments. Data were evaluated with One-way ANOVA and compared using Scheffé multiple tests. Values of $P < 0.05$ were regarded as statistically significant.

3. Results

Based on observations that LAB have the ability to activate macrophages, we assessed the potential effects of LAB isolated from traditional Taiwan fermented mustard on the production of NO, TNF-α and IL-6 in macrophages.

A total of 159 microbial strains were obtained from traditional Taiwan fermented mustard as raw material. They were selected based on criteria such as morphological shape, catalysis negativity, Gram positive, and lactic acid formation, consequently, they were considered as LAB. In order to understand whether the selected strains displayed potential immunopotentiating activities, the NO, TNF-α and IL-6 were determined.

RAW 264.7 cells were cultured with either viable, the heat-inactivated bacteria or LAB-SCS from LAB strains, and the amount of NO in the culture supernatant was measured using a Griess assay. RAW 264.7 cells in DMEM alone without either bacterial stimulants or LPS treatment was contributed as a control. When the cells were co-cultured with various bacterial stimulants, the production of NO generally increased. Of the 159 strains, 3 strains indicated in Table 1 exerted marked increase in the production of NO in viable LAB-induced cells. NO production in heat-inactivated LAB-induced cells, strains B0040, B0110 and B0145 appeared to be more profound than *L. casei*. NO production by the selected strains was almost two to three times as the production by *L. casei* treatment. Identically, NO production induced by *L. casei* cell-free supernatant was found to be lower than the production induced by the selected strains. It is interesting to note that the level of NO at viable bacteria of strain B0145 showed the highest concentration.

Table 1

NO production by RAW 264.7 cells with various bacterial stimulants.

Strains	NO relative change (fold of control)		
	Viable LAB	Heat-inactivated LAB	LAB-SCS
L. casei	6.39 ± 0.54	3.84 ± 0.81	3.07 ± 0.47
B0040	9.72 ± 0.99*	8.82 ± 0.48*	5.28 ± 0.02*
B0110	8.34 ± 1.49*	8.56 ± 0.44*	5.69 ± 0.90*
B0145	11.58 ± 0.82*	8.06 ± 0.30*	8.24 ± 1.54*
LPS (5 μg/mL)	26.39 ± 4.89*		

The results were calculated with fold of control by dividing experimental data by control values. Each value in the table represents the mean value ± SD from at least 3 trials. Values marked with asterisk differ significantly from *L. casei* values ($P < 0.05$).

To evaluate the effect of LAB strains on TNF-α and IL-6 in RAW 264.7 macrophages, cells were incubated with either viable, the heat-inactivated bacteria or LAB-SCS, and the induction of cytokines in the culture supernatant was subsequently monitored by ELISA. In the production of TNF-α, the RAW 264.7 cell alone was contributed as a control, whereas the level of TNF-α in the supernatant from cells co-stimulated with LPS (5 μg/mL), which is known to be a strong activator of macrophages, was measured at 4.61-fold of control. The production of TNF-α in macrophages treated with various bacterial stimulants was markedly induced in all selected strains but strain B0110, which was also superior to that of *L. casei* (Table 2). However, it is worth noting the level

of TNF-α at strains B0040 and B0145 were similar to that of LPS. As shown in Table 3, IL-6 production in the macrophages treated with various bacterial stimulants was markedly induced in all selected strains, which was superior to that of *L. casei*. Interestingly, the induction of NO and cytokine such as TNF-α, in the case of viable bacteria, was higher than that of the heat-inactivated bacteria and LAB-SCS, however, less induction on the production of IL-6. These observations demonstrated that viable bacteria, heat-inactivated bacteria or LAB-SCS, which were isolated from suan-tsai, were able to stimulate macrophages and exhibit immunopotentiating activities, including the induction of several cytokines and NO.

Table 2

TNF-α production by RAW 264.7 cells with various bacterial stimulants.

Strains	TNF-α (fold of control)		
	Viable LAB	Heat-inactivated LAB	LAB-SCS
L. casei	1.82 ± 0.04	1.21 ± 0.13	2.12 ± 0.07
B0040	4.95 ± 0.86*	5.60 ± 0.41*	3.48 ± 0.06
B0110	2.47 ± 0.18	3.15 ± 0.16*	3.53 ± 0.11
B0145	5.89 ± 0.16*	4.80 ± 0.49*	4.21 ± 0.01*
LPS (5 μg/mL)	4.61 ± 0.17*		

The results were calculated with fold of control by dividing experimental data by control values. Each value in the table represents the mean value ± SD from at least 3 trials. Values marked with asterisk differ significantly from *L. casei* values ($P < 0.05$).

Table 3

IL-6 production by RAW 264.7 cells with various bacterial stimulants.

Strains	IL-6 (fold of control)		
	Viable LAB	Heat-inactivated LAB	LAB-SCS
L. casei	2.84 ± 0.51	2.92 ± 0.26	2.01 ± 0.21
B0040	3.97 ± 0.11*	4.37 ± 0.55*	4.18 ± 0.01*
B0110	3.61 ± 0.11*	4.57 ± 0.46*	4.22 ± 1.01*
B0145	4.12 ± 0.15*	3.52 ± 0.17	5.05 ± 1.11*
LPS (5 μg/mL)	6.28 ± 0.05*		

The results were calculated with fold of control by dividing experimental data by control values. Each value in the table represents the mean value ± SD from at least 3 trials. Values marked with asterisk differ significantly from *L. casei* values ($P < 0.05$).

In addition, the effect of selected strains on cell viability was determined. Clearly, treatment with LPS significantly inhibited cells' proliferation. However, there were no differences when cells were co-cultured with selected strains, indicating the selected strains showed no cytotoxic effect to macrophages (Figure 1). This observation was also shown in Figure 2. When the cells were cultured alone, the majority of cells showed circular morphology and normal proliferation (Figure 2A), whereas the cells treated with LPS, the proliferation of cells was suppressed and a change in the cellular morphology was observed (Figure 2B). However, when the cells were co-cultured with selected strains, the morphology was not changed (Figures 2C, 2D and 2E).

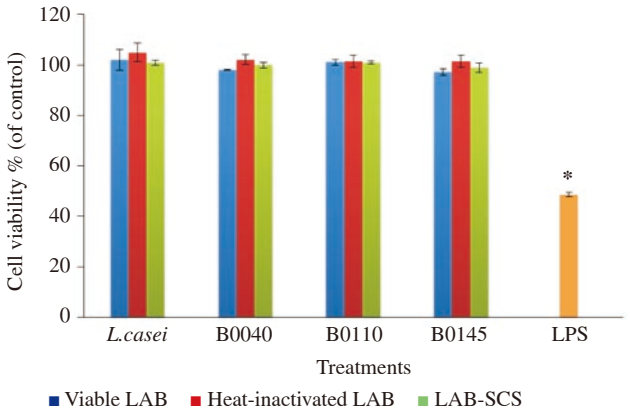

Figure 1. Cell cytotoxicity of various bacterial stimulants on RAW 264.7 cells.

Proliferation of macrophage cells was determined by MTT method. The results were calculated with percentage relative change of control by dividing experimental data by control values and mean values were expressed with SD of at least three independent experiments. Values marked with asterisk differ significantly from *L. casei* values ($P < 0.05$).

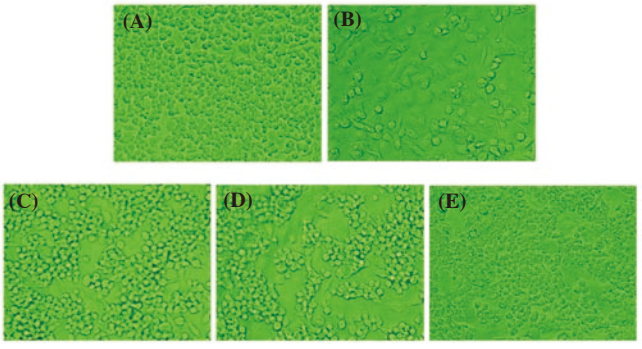

Figure 2. Morphology of RAW 264.7 cells (cultured for 48 h) after different addition.

(A): Dulbecco's modified Eagle's medium only; (B): LPS (5 μg/mL), and heat-inactivated LAB; (C): *Lactobacillus plantarum* (B0040); (D): *Lactobacillus plantarum* (B0110); (E): *Weissella cibaria* (B0145). Magnification A to E: 200×.

According to the results obtained, strains B0040 and B0110 were identified as *Lactobacillus plantarum*, and B0145 as *Weissella cibaria* using API 50 CHL analysis, respectively. To further confirm these findings, the 16S rDNA nucleotide sequences of strains B0040, B0110, and B0145 were analyzed. The data obtained indicated that 16S rDNA sequence of strains B0040, B0110 and B0145 matched well with that of API 50 CHL, and 16S rDNA nucleotide sequence of strains B0040, B0110 and B0145 were 99 % similar with that in the Genebank.

4. Discussion

Many studies noted that LAB were one of the main constituents of the normal indigenous flora of human, which contributed several health benefits including improvement of intestinal tract health, enhancing the immune system, reducing risk of certain cancers, lowering serum cholesterol concentrations, reducing blood pressure

in hypertensives, reducing dental caries, and improving female urogenital infections and *Helicobacter pylori* infections[1,2,15]. Evidence from clinical and animal studies has supported that some LAB can modify immune response of the host[16,17]. In order to evaluate whether selected strains are able to induce the production of inflammatory responses, we examined the ability of the selected strains to induce cytokines secretion by macrophages. Macrophages, taking part in both innate and adaptive immune responses, are derived from monocytes[18]. Once macrophages are activated, they can phagocytose microorganism, secrete proinflammatory cytokines, and present antigens to helper T cells. Many of these activities are medicated through the release of different cytokines[18,19]. For instance, macrophages in the presence of pathogen can produce NO to kill the antigen, and secrete cytokines such as IL-6 and TNF-α that activate specific immune[20]. In the present work, strains B0040, B0110 and B0145 induced significant NO production of macrophages. These findings suggested that the possible way of NO production of macrophages treated with strains B0040, B0110 and B0145 may be through the L-arginine pathway[21]. The role of NO in intestinal inflammation is controversial. In physiological amount, it plays a crucial and protective role in human defense systems, whereas overexpression of NO during inflammation may lead to cytotoxity[21]. The result shows that the NO production of macrophages induced by the selected strains B0040, B0110, and B0145 is less than that of LPS treatment and higher than *L. casei.* Clearly, the NO production of macrophages induced by the selected strains appears to induce positive immune response. In addition, small amounts of NO may stimulate mitochondrial biogenesis and boost the supply amount of oxygen and respiratory substrates to mitochondria. Thus, the use of the selected strains that are able to produce appropriate levels of NO may potentially enhance immune response. Production of TNF-α in macrophage cells that were stimulated with the selected strains was induced in amounts even greater than those treated with LPS, indicating that strains B0040 and B0145 were capable of inducing RAW 264.7 cells to secrete higher levels of TNF-α production. As for IL-6, it is critical to mucosal immunity based on its effects on the differentiation of IgA-committed B cells and its production in the gut by macrophage, T cells and other cells[10,22]. In this work, we demonstrated the ability of the selected strains to induce the production of IL-6 in RAW 264.7 cells, in modulation of the immune response of the host. Moreover, IL-6 production in the cells stimulated with the selected strains gave rise to much higher levels than *L. casei.* These observations suggest that IgA in the cells induced by the selected strains may increase, thereby facilitating the modulation of the immune response of the host.

Among the strains tested, viable cells were better inducers than heat-inactivated strains or LAB-SCS in production of NO and TNF-α. Similarly, the heat-inactivated bacteria and LAB-SCS could induce NO and cytokines production, such as IL-6 and TNF-α, in macrophage cell line RAW 264.7, indicating that viable, nonviable (heat-inactivated) or LAB-SCS can induce the immunopotentiating activities. Cell-free cultures of LAB with probiotic potentials demonstrated to exert antimicrobial and immunomodulatory activities, suggesting the use of probiotic in nonviable forms[23]. Increasing evidence showed that cell wall fragments of bacteria, their metabolites and dead bacteria could elicit immune responses. Chiang *et al.* have reported immunomodulation effects of dead lactobacilli, whole cells and gastrointestinal enzymatic hydrolysates of supernatants and precipitates from *Lactobacillus paracasei* subsp. *paracasei* NTU 101, on RAW 264.7 macrophages and splenocytes[24]. We suggested that the induction of heat-inactivated cells or LAB-SCS on macrophages may result from the intact bacterial cells components or metabolites in LAB such as peptidoglycan, teichoic acid and exopolysaccharide[25,26]. However, further *in vivo* verification is needed.

In conclusion, the present study indicated that strains B0040, B0110 and B0145 did activate macrophage RAW 264.7 to stimulate NO and pro-inflammatory cytokines production. We suggest that strains B0040 and B0110, identified as *Lactobacillus plantarum*, and B0145, as *Weissella cibaria*, may be potential candidates for supplement to induce immunopotentiating activities. However, further *in vivo* verification is necessary.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

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Comments

Background

The LAB have several important roles in the development and maintenance of health benefits of host, like antibacterial, anticancer, and antimutagenic among others.

Research frontiers

The present study evaluates the *in vitro* effect of LAB isolated from suan-tsai on the induction of NO and cytokines such as TNF-α and IL-6 in RAW 264.7 macrophage cell.

Related reports

Several authors report the LAB as probiotic bacteria and few reports are existed about the immunological functions of LAB.

Innovations and breakthroughs

The potential of LAB in immunological functions may be studied concerning their potential to be used in food and pharmaceutical industries.

Applications

The LAB (*Lactobacillus plantarum* and *Weissella cibaria*) isolated from fermented mustard may be used for supplement to induce immunopotentiating activities.

Peer review

The present manuscript is well written, with good methodologies and the results are well discussed. The results herein obtained are very interesting and reveal a good potential for supplement to induce immunopotentiating activities.

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