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Antibacterial Activity and Macrophage Activation of Lactic Acid Bacteria

So Hee Park, Yun A Kim, Do Kyung Lee, Sangjin Lee[1], Myung Jun Chung[2], Byung Yong Kang[3], Kyungjae Kim and Nam Joo Ha[※]

Department of Pharmacy, Sahmyook University, Seoul 139-742, Korea
[1]Division of Animal Science, Sahmyook University, Seoul 139-742, Korea
[2]Cellbiotech, Co. Ltd., Gyeonggi-do 157-030, Korea.
[3]Research Institute for Life Science, Sahmyook University, Seoul 139-742, Korea

유산균의 항균효과와 대식세포 활성화

박소희, 김윤아, 이도경, 이상진[1], 정명준[2], 강병용[3], 김경제, 하남주[※]

삼육대학교 약학과, [1]동물과학부, [2](주)셀바이오텍, [3]삼육대학교 생명과학연구소

요 약

유산균(Lactic acid bacteria)은 *Escherichia coli*와 *Salmonella typhimurium*과 같은 병원균에 항균 활성을 지니며 면역 증강효과를 나타내는 등 건강에 이로운 다양한 역할을 한다. 유산균에 의한 항균 효과는 *E. coli*와 *S. typhiumurium*에 대항하는 항균 활성으로 측정되어 졌으며, 면역 증강 효과는 유산균을 처리한 RAW264.7 대식세포의 활성화로 측정하였다. *Lactobacillus acidophilus, Streptococcus thermophilus, Bifidobacterium bifidum*의 *E. coli*와 *S. typhimurium*에 대한 항균활성은 9시간 이상 혼합 배양하였을 때 가장 좋은 항균효과를 나타내었고 *E. coli*와 *S. typhiumrium* 두 균주가 9시간 이후에는 모두 콜로니를 형성하지 않았다. RAW264.7 세포는 유산균에 의한 NO와 TNF-α의 생성과 대식세포의 형태 변화를 알아보기 위한 대식세포로서 이용되었다. NO와 TNF-α의 생성은 유산균을 처리한 RAW264.7세포의 24, 48시간 배양 시 농도 의존적으로 증가하였고 대식세포의 형태 변화 역시 유산균에 의해 영향을 받았음을 확인할 수 있었다. 이를 통하여 (주)셀바이텍으로부터 분양받은 유산균은 항균활성과 대식세포의 활성을 유도하여 면역을 증강시키는 효과를 지니고 있음을 *in vitro* 실험을 통해 확인하였다.

Key words : antibacterial effect, lactic acid bacteria, NO and TNF-α

INTRODUCTION

Lactic acid bacteria (LAB) are nonpathogenic and Gram positive bacteria which inhabit the intestinal tract of humans and animals. LAB can be divided into 5 genera: *Streptococcus, Lactobacilus, Leuconostoc, Bifidobacteria and pediococcus* (Yu *et al.*, 2003). LAB are used in commercial fermented dairy products and have been suggested to exert health promoting effects on the host by maintaining the in-

※ To whom correspondence should be addressed.
Tel: +82-2-3399-1607, Fax: +82-2-3399-1617
E-mail: hanj@syu.ac.kr

testinal microflora balance, improving lactose tolerance, reducing serum-cholesterol levels, increasing synthesis of vitamins, and aiding anti-carcinogenic activity. Certain well-defined strains of LAB have also been shown to be potent modulators of immune function in children, adults and the elderly (Oshima and Bartsch, 1994). LAB-ingestion has been proposed to enhance resistance to infection by pathogenic organisms (Yasui and Ohwaki, 1991) and potentially prevent cancer (Sekine *et al.*, 1995).

LAB ward off disease by suppressing harmful bacteria in the intestines through the propagation of macrophages. LAB contribute greatly in the intestinal regulation due to its specific proteins that can combine strongly to the mucosa and epithelia. LAB also produce antimicrobial substances with the capacity to inhibit the growth of pathogenic and spoilage microorganisms (Herreros *et al.*, 2005) as well as having an inhibitory effect on various food-born pathogens (Nigatu and Gashe, 1994; Idris *et al.*, 2001). A variety of pathogens have been found on fruit and vegetable products, including members of the *Salmonella* and *Shigella* species, enteropathogenic strains of *Escherichia coli* (*E. coli*), *Aeromonas hydrophila, Yersinia enterocolitica*, and *Staphylococcus aureus*. These pathogens grow and cause disease depending on the type of product, conditions of storage (time, temperature and atmosphere), and competitive microflora. Thus, there is a particular interest to introduce additional safety measures for these products (Brackett, 1992).

Efficient inhibition of the growth of pathogens on ready-to-use vegetables may be achieved by using strains of LAB that grow and secrete antimicrobial compounds under refrigeration conditions. LAB compete with other microbes by modifying the microenvironment through their metabolic end-products. Beside lactic acid, bacteriocins as low molecular mass compounds (LMMC), are produced by LAB in foods and this contributes to the antimicrobial effects of added LAB cultures (Niku-paavola *et al.*, 1999).

LAB apparently enhance several immune functions, including macrophage and lymphocyte activation (Harcher and Lambrecht, 1993; Sekine *et al.*, 1994), antibody production (Lee *et al.*, 1993; Link-Amster *et al.*, 1994; Yasui *et al.*, 1995), and the proliferate responses in spleen and Peyer's patches. The epithelial lining of the gastrointestinal tract provides an extensive surface area for the absorption of nutrients and presents a barrier to the vast number of extraneous antigens that pass through the gut. The exclusion or elimination of potential foreign antigens is mediated by the gut immune system. The intestinal epithelium also contains a large number of lymphocytes that are able to secrete a variety of cytokines and influence the local immunoregulatory environment. Thus, the possibility that LAB or its products may act directly on these cells, while in the gut lumen, and stimulate an immune reaction also exits. Furthermore, it is also possible that LAB or its products may gain access to the body via non-specific and receptor-mediated mechanisms. The ability of some LAB to adhere to the intestinal epithelial cells *in vitro* and to the intestinal mucosa *in vivo* is well-documented. The interactions between LAB or their products and the immunocompetent cells, such as macrophages and T cells, then result in the secretion of a variety of cytokines that are known to have a multitude of effects on both immune and non-immune cells.

Macrophages play a major role in host defense against infection and other immune functions. Of particular interest, the production of nitric oxide (NO) by macrophages, mediates killing or growth inhibition of tumor cells, bacteria, fungi and parasites (Snyder and Bredt, 1992; Clancy *et al.*, 1998). Macrophages may also regulate immunity via the enhanced production of several mediators such as tumor necrosis factors (TNF-α). While these mediators play vital homeostatic functional roles, they are potentially capable of injuring the host tissue (Vilcek and Lee, 1991). Thus, regulation of these mediators is critical for normal physiological immune status. Characterization of the effects of LAB on the production of macrophage mediators may contribute to a better understanding of how this genus affects im-

mune function at cellular level.

The aim of this study was to investigate the effects of LAB on immune reinforcement and to study the antimicrobial activities of products produced by LAB against, *E. coli* and *S. thyphimurium*. The effect of LAB on immune reinforcement was determined by studying macrophage activation, nitric oxide (NO) production, TNF-α production and morphological changes of cells. The RAW 264.7 cell-line was used for these experiments. The antimicrobial activity of LAB was studied against two other pathogens, *E. coli* ATCC 25922 and *S. typhimurium* ATCC 13311, which may cause gastrointestinal disease.

MATERIALS AND METHODS

1. Bacterial strains and media

LAB (*Lactobacillus acidophilus, Streptococcus thermophilus* and *Bifidobacterium bifidum*) was obtained from Cellbiotech Co., Ltd. (Korea) and *E. coli* ATCC 25922 and *S. typhimurium* ATCC 13311 were used as pathogenic strains. Three LAB species (*L. acidophilus, S. thermophilus* and *B. bifidum*) were grown in General anaerobic medium (GAM) broth (Nissue, Japan) at 37°C for 24 hours and *E. coli* ATCC 25922 and *S. typhimurium* ATCC 13311 were cultured in Nutrient broth (Difco, USA) at 37°C for 24 hours.

2. Antimicrobial activity assay

E. coli ATCC 25922 and *S. typhimurium* ATCC 13311 were used as test strains to evaluate the antimicrobial effects of LAB. *L. acidophilus* and *S. thermophilus* were cultured on Lacto bacilli MRS broth (Difco, USA) and *B. bifidum* was cultured on GAM broth under anaerobic conditions for 18 hours at 37°C *E. coli* ATCC 25922 and *S. typhimurium* ATCC 13311 were incubated in nutrient broth for 18 hours at 37°C. The co-culture of LAB with *E. coli* ATCC 25922 or *S. typhimurium* ATCC 13311 were achieved as follows: culture broth of *E. coli* ATCC 25922 (5×10^5 cfu/mL) and *S. typhimurium* ATCC 13311 (5×10^5 cfu/mL) were diluted to 10^1 and 10^2 and mixed with GAM broth and MRS broth containing LAB (5×10^7 cfu/mL). After 3, 6, 9 and 24 hours of incubation, 100 μL of culture broth was plated onto nutrient agar plates and incubated for 24 hours. After incubation, viable cells were counted.

3. Cell culture

The murine macrophage cell line (RAW 264.7) was obtained from the American Type Culture Collection (ATCC). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM), which was supplemented with high glucose, L-glutamine, 110 mg/L sodium pyruvate, 10% fetal bovine serum (FBS), and 1% (v/v) penicillin (10,000 U/mL)/streptomycin (10,000 U/mL) (P/S), LPS (*E. coli*, 0127: B8 Westphal type) was purchased from Sigma Chemical Co. (St. Louis, MO. USA). All cultures were incubated at 37°C in humidified atmosphere with 5% CO_2. Cell number and viability were assessed by trypan blue dye exclusion on a Neubauer hemacytometer (American Optical, Buffalo, NY).

4. Effect of LAB (*L. acidophilus, S. thermophilus* and *B. bifidum*) on macrophage morphology

To determine the effects of LAB strain on the macrophage morphology, RAW 264.7 cells were cultured in sterile glass-slide chambers at a density of 1×10^3 cells/well for 48 hours. The culture medium was removed, and the cells were treated with either LPS (100 ng/mL), or cell-free culture supernatant (1, 3 μL/mL) of three LAB (1×10^8 cfu/mL) strains and incubated for 48 hours. Following the treatment, the culture supernatant was removed. The cells were fixed and stained in Diff quick Solution (Baxter, Houston, TX).

5. Nitric oxide assay

Nitrite accumulation was used as an indication of NO production. This procedure for NO determination was based on the Griess reagent. Flat-bottomed 96

wells, LPS (20 ng/mL), cell-free ssupernatant (3.1, 6.25, 12.5, 25, 50, 100 μL/mL) of three LAB strains (1×10^8 cfu/mL), DMEM-10 media, RAW 264.7 cells, and Griess reagent (N-1-naphthylethylene diamine 0.1% in H_2O, sulfanilamide 1% in 5% H_3PO_4) were used as materials in this study. NO production was carried out according to the method reported by Stuehr and Nathan, 1989. LPS, cells only (1×10^6 cells/mL), cell-free supernatant of three LAB strains (*L. acidophilus, S. thermophilus* and *B. bifidum*: 3.1, 6.25, 12.5, 25, 50, 100 μL/mL), were prepared as the treated groups. 4 wells per group were used and 200 μL of the cells were added to each well. The plates were incubated overnight and 100 μL from the surface of each well was transferred into a new plate. The new plate was then incubated for 10 minutes at room temperature (RT) and was measured by an ELISA reader at 540 nm. Nitrite concentration was determined using sodium nitrite as a standard.

6. TNF-α production

LPS, cells only (1×10^5 cells/mL), and cell-free supernatant (3.1, 6.25, 12.5, 25, 50, 100 μL/mL) of three LAB (1×10^8 cfu/mL) were prepared as the treated groups and were incubated for 48 hours. After incubation, TNF-α quantification was measured by TNF-α Immunoassay Kit (BioSource International. Inc., Camarillo). Briefly, 1×10^5 cells/mL of RAW 264.7 cells were treated with each cell-free supernatant of LAB (*L. acidophilus, S. thermorphilus* and *B. bifidum*: 3.1, 6.25, 12.5, 25, 50, 100 μL/mL) in 5.5% CO_2 humidified air for 48 hours at 37°C. After incubation, 100 μL of culture fluid and standard solution were added to each well respectively. This was followed by the addition of 50 μL of Biotin Conjugate, incubation for 90 minutes at RT, aspiration and four washes 100 μL of Streptavidin HRP working solution was then added and the reaction was incubated for 30 minutes at RT, aspirated and washed thoroughly. 100 μL of Stabilized Chromogen was then incorporated and incubated for 30 minutes at RT. The reaction was stopped by the further addition of 100 μL of stop solution and absorbance values read at 450 nm.

7. Statistical analysis

Nitrite and cytokine production was expressed as means ± SD of 2 to 4 independent experiments. The statistical significance was estimated using Student-t tests.

RESULTS

1. Antimicrobial activity

To test the inhibitory effect of LAB (*L. acidophilus, S. thermorphilus, B. bifidum*) on the growth *E. coli* ATCC 25922 and *S. typhimurium* ATCC 13311, co-cultures of *E. coli* ATCC 25922 or *S. typhimurium* ATCC 13311 with LAB were performed. Antibacterial activities are displayed in Figs. 1 ~ 3.

Two strains of LAB (*L. acidophilus* and *S. thermophilus*) inhibited both of the tested strains (*E. coli* ATCC 259222 and *S. typhimurium* ATCC 13311). Viable colonies of *E. coli* ATCC 259222 and *S. typhimurium* ATCC 13311 were undetected when these bacteria were co-cultured with *L. acidophilus* or *S. thermophilus* for 24 hours. *B. bifidum*, however, showed no antibacterial activity against *E. coli* ATCC 259222 or *S. typhimurium* ATCC 13311.

2. Effect of LAB (*L. acidophilus, S. thermophilus* and *B. bifidum*) on macrophage morphology

Normal RAW 264.7 cells tend to be round in morphology when cultured in medium alone (Fig. 4a). The gradual change inmorphology of different groups of cells, which were treated with LPS (10 ng/mL; Fig. 4b) alone, supernatants of *B. bifidum* (25 μL/mL; Fig. 4c), *L. acidophilus* (12.5 μL/mL; Fig. 4d) and *S. thermophilus* (6.25 μL/mL: Fig 4e), *B. bifidum* (12.5 μL/mL; Fig. 4f) with LPS (10 ng/mL) were examined. Those treated with the supernatants of LAB were larger and rougher than those that were exposed only to the medium or LPS; these morphological changes

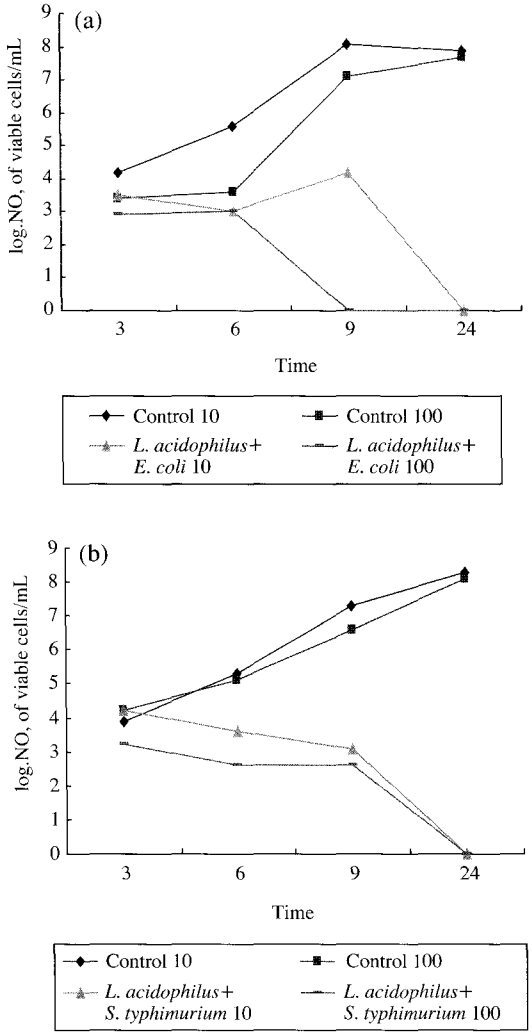

Fig. 1. Growth inhibition of *E. coli* ATCC 25922 and *S. typhimurium* ATCC 13311 by *L. acidophilus*. *E. coli* ATCC 25922 (10: 5×10^4 or 100: 5×10^3 cfu/mL) and *S. typhimurium* ATCC 13311 (10: 5×10^4 or 100: 5×10^3 cfu/mL) were co-cultured with *L. acidophilus* (5×10^7 cfu/mL) culture broth and growth was measured by viable cell count. (a) Growth inhibition of *E. coli* ATCC 25922 by *L. acidophilus*; (b) Growth inhibition of *S. typhimurium* ATCC 13311 by *L. acidophilus*.

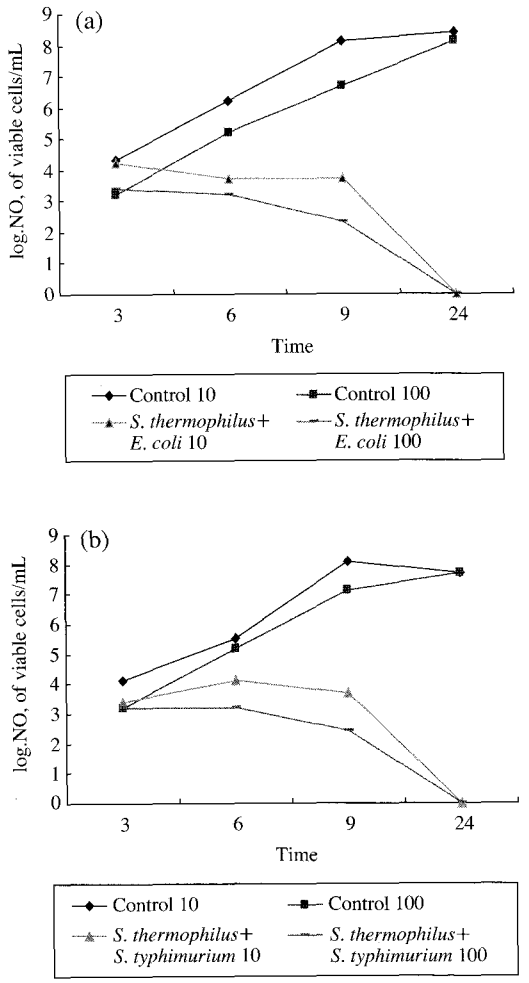

Fig. 2. Growth inhibition of *E. coli* ATCC 25922 and *S. typhimurium* ATCC 13311 by *S. thermophilus*. *E. coli* ATCC 25922 (10: 5×10^4 or 100: 5×10^3 cfu/mL) and *S. typhimurium* ATCC 13311 (10: 5×10^4 or 100: 5×10^3 cfu/mL) were co-cultured with *S. thermophilus* (5×10^7 cfu/mL) culture broth and growth was measured by viable cell count. (a) Growth inhibition of *E. coli* ATCC 25922 by *S. thermophilus*; (b) Growth inhibition of *S. typhimurium* ATCC 13311 by *S. thermophilus*.

were dose dependent. These results suggested that macrophages treated with the supernatants of LAB showed greater activation than those that were exposed to either media or LPS alone.

3. Nitric oxide assay

NO is an important macrophage-mediator because it acts as a reactive oxygen and nitrogen intermediate during oxygen-dependent phagocytosis. The effects of LAB exposure on NO production in RAW cells

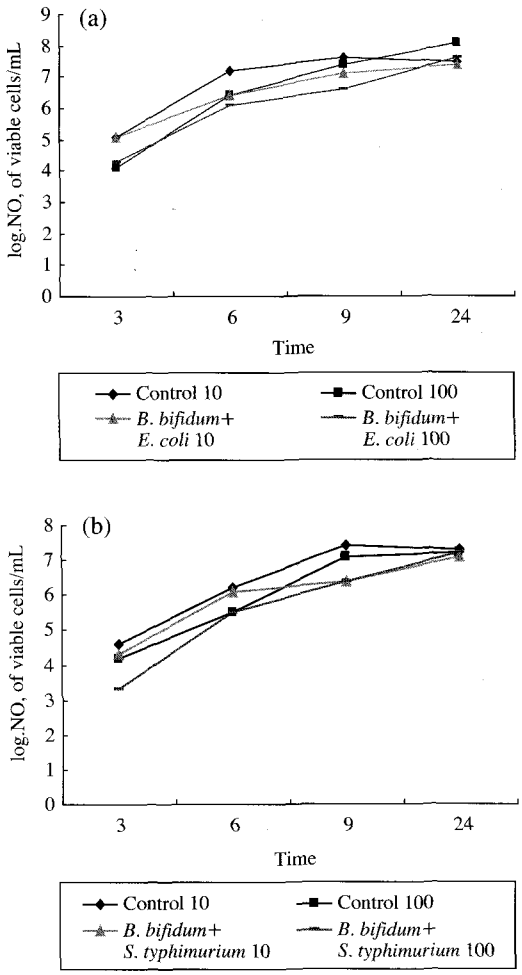

Fig. 3. Growth inhibition of *E. coli* ATCC 25922 and *S. typhimurium* ATCC 13311 by *B. bifium*. *E. coli* ATCC 25922 (10: 5×10^4 or 100: 5×10^3 cfu/mL) and *S. typhimurium* ATCC 13311 (10: 5×10^4 or 100: 5×10^3 cfu/mL) were co-cultured with *B. bifium* (5×10^7 cfu/mL) culture broth and growth was measured by viable cell count. (a) Growth inhibition of *E. coli* ATCC 25922 by *B. bifium*; (b) Growth inhibition of *S. typhimurium* ATCC 13311 by *B. bifium*.

were evaluated using Griess assay (Fig. 5). As shown in Fig. 5, the macrophages did not release NO in response to the medium only. In this study, LPS was used as a positive control for macrophage-activation. LAB exposure in the range of 3.1 to 100 μL amounts of supernatants markedly increased the production of NO. LPS at a concentration of 20 ng/mL alone induced production significantly. Furthermore, when co-stimulated with LAB and LPS, the level of NO was significantly higher than LAB alone. The production of NO by *B. bifidum* in particular, was significantly increased. Production of nitric oxide by *B. bifidum* (20 μM) was compared to the control group of LPS-treated (20 ng/mL) cultures (19 μM) at a concentration of 25 μL and increased in a dose dependent manner up to 50 μL of supernatant of *B. bifidum* which gave 22 μM of nitrite products.

4. TNF-α production

To assess the effects of LAB on TNF-α production by macrophages, RAW 264.7 cells were incubated with a range of bacterial concentrations in the absence or presence of LPS. The cytokine secretion in the culture supernatant was monitored by ELISA. The patterns observed for LAB on TNF-α stimulation, are displayed in Fig. 6. In both the LPS-treated, as well as the untreated cells, exposure to LAB supernatant affected TNF-α production in a dose-dependent manner. When co-stimulated with LPS (10 ng/mL) and a lower concentration of LAB, TNF-α levels were higher than LPS (10 ng/mL) alone. Generally, When co-stimulated with LPS (10 ng/mL) the production of TNF-α increased to a much greater lever than either LPS or LAB alone.

DISCUSSION

LAB are live microorganisms belonging to the natural flora with no pathogenicity, but with functions of importance to the health and well being of the host. Since they are normal components of the human intestinal flora, they are commonly used as starter cultures in dairy products, and can produce resistance to infectious diseases, alleviate food allergies and suppress cancer development. It is thus increasingly accepted that these bacteria might represent effective tools for controlling overgrowth of pathogens and thereby control or prevent infections.

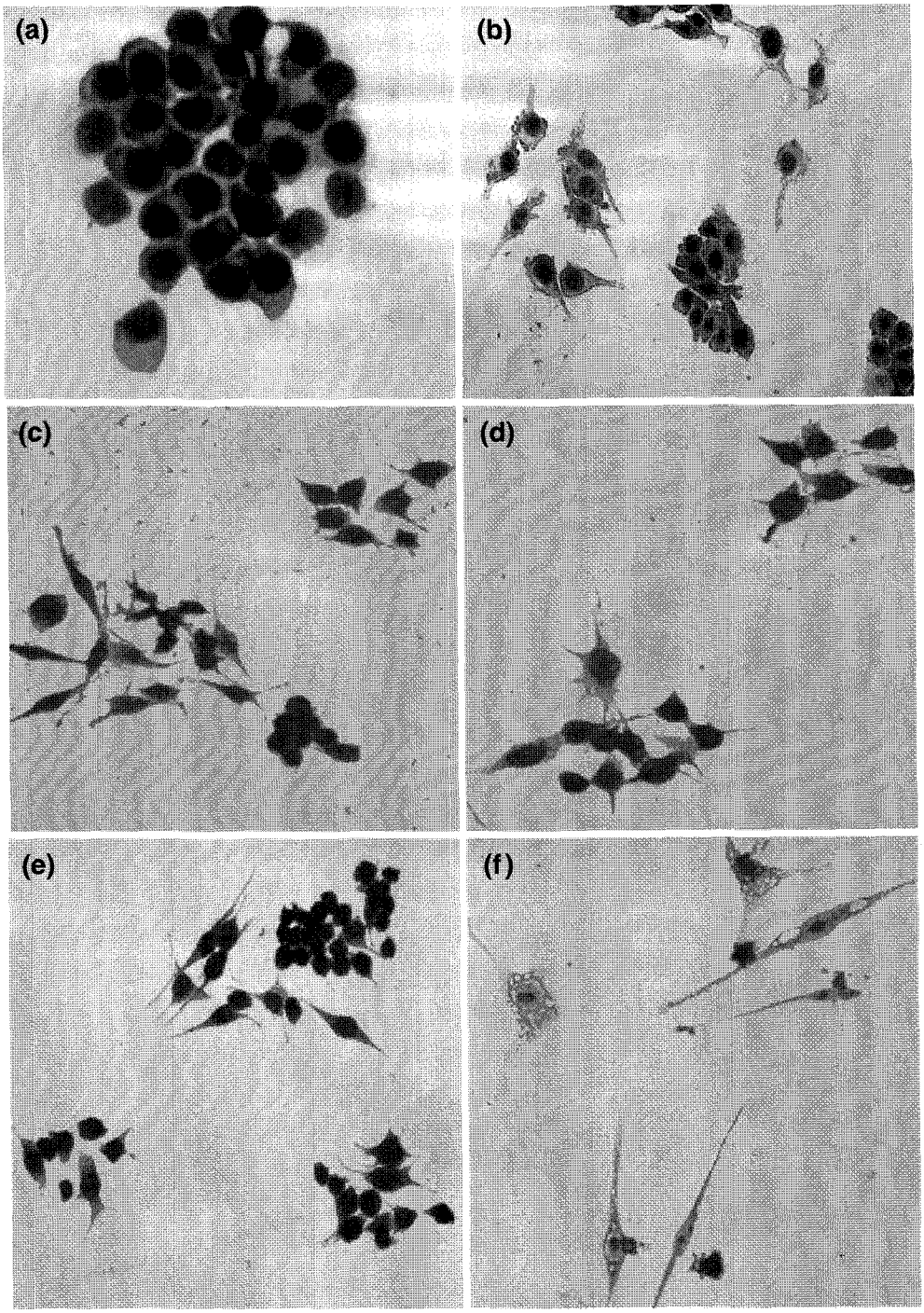

Fig. 4. Characterization of RAW 264.7 cell-morphology changes in response to cell-free supernatants of *L. acidophilus*, *S. thermophilus* and *B. bifidum*. The RAW 264.7 cells were cultured on cover slips in the presence of different concentration of cell-free supernatants of *L. acidophilus*, *S. thermophilus* and *B. bifidum*. (a) Untreated cells, (b) with LPS (10 ng/mL), (c) *B. bifidum* (25 μL/mL), (d) *L. acidophilus* (12.5 μL/mL), (e) *S. thermophilus* (6.25 μL/mL), (f) *B. bifidum* (12.5 μL/mL) with LPS (10 ng/mL).

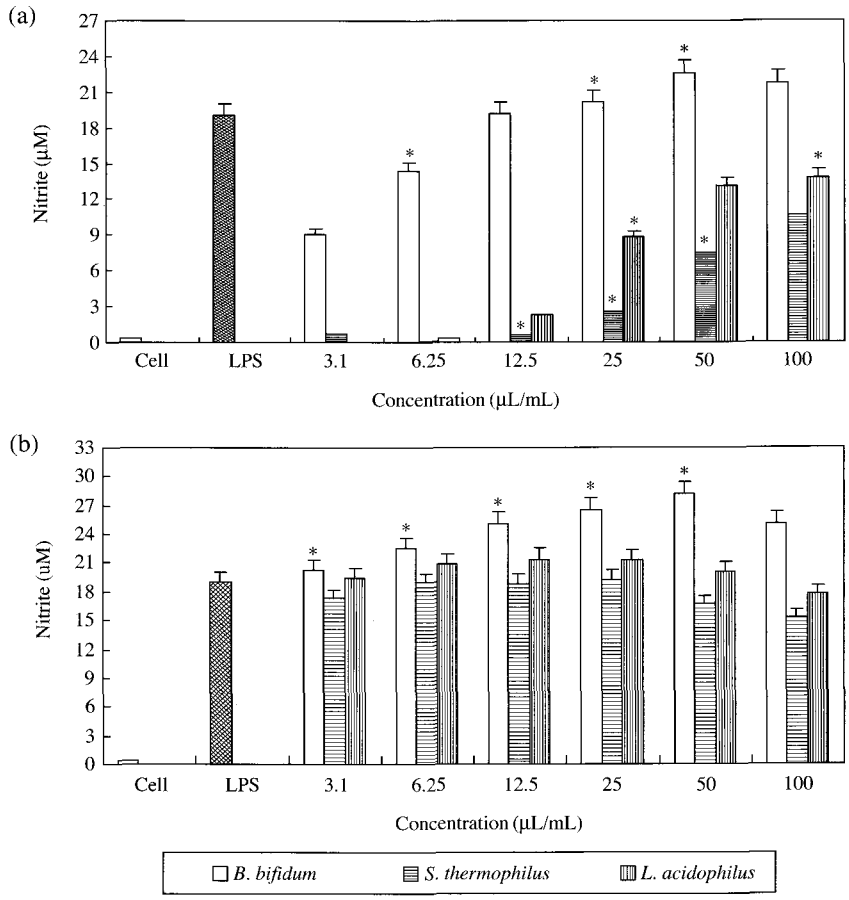

Fig. 5. Effect of cell-free supernatants of *L. acidophilus*, *S. thermophilus*, *B. bifidum* on NO production in the LPS (lipopolysaccharide) stimulated RAW 264.7 cells. The cultures were incubated with 20 ng/mL of LPS in the presence of supernatants of *L. acidophilus*, *S. thermophilus*, *B. bifidum*. NO production by supernatants of *L. acidophilus*, *S. thermophilus*, *B. bifidum* were assessed by Griess reaction. (a) without LPS *$p<0.05$, compared with cells, (b) with LPS *$p<0.05$, compared with LPS.

Indeed, numerous *in vitro* and *in vivo* studies performed with LAB have displayed the capacity of these bacteria to interfere with both growth and virulence properties of various pathogens (Vignolo *et al.*, 1993; Velraeds *et al.*, 1996; Coconnier *et al.*, 1998; Koga *et al.*, 1998; Wang *et al.*, 2005). In the present study, we tested the inhibitory effect of LAB on the growth of *E. coli* ATCC 25922 and *S. typhimurium* ATCC 13311.

The co-culturing of *E. coli* ATCC 25922 and *S. typhimurium* ATCC 13311 with the LAB strains *L. acidophilus* and *S. thermophilus*, showed both the bacteriostatic and the bacteriocidal activity of these particular LAB strains on the growth of *E. coli* ATCC 25922 and *S. typhimurium* ATCC 1331. The LAB strain *B. bifidum*, however, did not show any antibacterial and/or antibactericidal activity on *E. coli* ATCC 25922 and *S. typhimurium* ATCC 13311.

LAB have previously been shown to stimulate immune function (Gomez *et al.*, 1988; Lee *et al.*, 1993; Link-Amster *et al.*, 1994). It has been suggested that these activities may arise from their ability to stimulate macrophages and T cells (Harcher and Lambrecht, 1993). Macrophages are important regu-

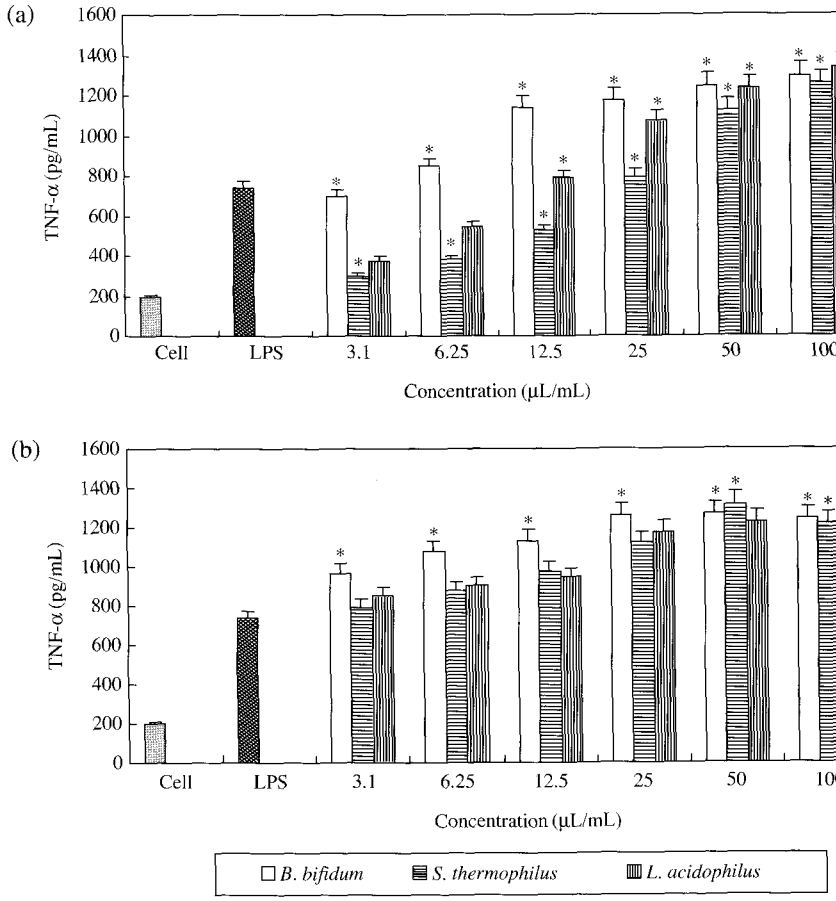

Fig. 6. Effect of cell-free supernatants of *L. acidophilus, S. thermophilus, B. bifidum* on TNF-α production in the LPS (lipopolysaccharide) stimulatd RAW 264.7 cells. (a) without LPS *$p<0.05$, compared with cells, (b) with LPS *$p<0.05$, compared with LPS.

latory and effector cells that play a central role in cell-mediated immunity because they present the antigen and mediate inflammatory, tumoricidal and microbicidal activity. These functions can be altered by a variety of stimulatory or suppressive signals and are influenced by many environmental factors. Numerous macrophage functions are mediated through the release of different cytokines. It is believed that these cytokines may play pivotal roles in the host defense inflammatory response, and autoimmune diseases. Cytokine production is therefore likely to be a good indicator of the degree of macrophage activation. In the present study, exposure of RAW 264.7 cell line to LAB isolates resulted in marked increase of NO and TNF-α production. The results of this study also suggest that other beneficial effects of the intake of LAB may include the reinforcement of immune function.

Furthermore, the present results imply that both human and commercial isolates of LAB increase the secretion of several macrophage mediators and thus could potentially modulate the host immune response, and produce antimicrobial substances with the capacity to inhibit the growth of pathogenic microorganisms.

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