Augmentation of Macrophage Phagocytic Activity by Cell-Free Extracts of Selected Lactic Acid-Producing Bacteria

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ABSTRACT

Oral and intraperitoneal administration of lactic acid-producing bacteria can significantly augment the immune response in murine models; however, the immunopotentiating effects in these studies differ significantly. Murine macrophagelike cell line J774 was cultured in the presence of cell-free extracts of *Lactobacillus acidophilus* and *Bifidobacterium longum*, and the effect on macrophage function was evaluated by measurement of synthesis of selected enzymes and their ability to take up either acrylamide particles or live *Salmonella typhimurium*. Lysozyme activity of J774 cells was significantly decreased by cell-free extracts of *B. longum*, but not of *L. acidophilus*, whereas extracts of both strains induced morphological changes and significantly enhanced phagocytosis of inert particles or viable *Salmonella*. Whole cell extracts of lactic acid-producing bacteria are therefore capable of altering macrophage function in a strain-dependent manner.

(**Key words**: *Lactobacillus*, macrophage, phagocytosis, probiotics)

Abbreviation key: **D-MEM** = Dulbecco's modified Eagle's medium, **LPS** = lipopolysaccharide.

INTRODUCTION

The therapeutic role of dietary lactobacilli (probiotics) has received increasing interest within the past 5 yr. The complex mechanisms by which these bacteria or their products control proliferation of undesirable pathogens are becoming better understood (3, 18). Evidence is mounting that the host immune system appears to be positively influenced by probiotic bacteria. A direct reduction in metastatic tumors in mice and suppression of tumors in intestinal cancer following bacteriotherapy with lactobacilli suggest that certain probiotic bacteria may possess anticarcinogenic properties (4, 6, 9, 10, 16). Furthermore, concurrent administration of ascites tumor cells with oral feeding or injection of whole cell lysates of lactic acid-producing bacteria either prolonged the onset or totally prevented tumor growth (8, 15). Considerable evidence confirms the existence of certain bacterial species that constitute only a minority of the flora of the colon but contribute significantly to the stability of the intestinal ecosystem. Although data from these and similar studies must be interpreted cautiously using appropriate controls and statistical analysis, they support the beneficial effect of probiotics on host immunity (7).

The present study was undertaken to investigate the effects associated with administration of cell-free extracts of lactic acid-producing bacteria on the response of cultured macrophages. The goals of this study were to determine whether 1) crude extracts of lactic acid-producing bacteria could induce a response similar to activation of macrophage cells, 2) the response is specific to the bacterial strain tested and dependent on the quantity of extract used, and 3) in vitro assays measuring macrophage responses could be employed for routine screening of potentially beneficial probiotics.

MATERIALS AND METHODS

J774 Cell Culture

Mouse monocyte-macrophage cells ATCC TIB 67 (J774A.1) were obtained from the Tumor Immunology Bank at the American Type Culture Collection (Rockville, MD). A continuous macrophagelike cell line, derived from a

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reticulum cell sarcoma, was chosen because it is representative of peritoneal macrophages and has been well studied and characterized (12). The cells were cultured and maintained in Dulbecco's modified Eagle's medium (D-MEM) supplemented with L-glutamine (Gibco, Grand Island, NY), 5% fetal bovine serum (Sigma Chemical Co., St. Louis, MO), and 2-mercaptoethanol (Sigma Chemical Co.) at a final medium concentration of 5×10^{-5} *M*. Cell viability was determined by trypan blue dye exclusion.

Bacterial Extracts

Freeze-dried bacterial culture concentrates of *Lactobacillus acidophilus* and *Bifidobacterium longum* used for this study were obtained from commercial stocks (Chr. Hansen's Lab., Inc., Milwaukee, WI). Bacterial extracts were prepared by washing 1.0 g of dry bacterial concentrate in three separate 5-ml aliquots of PBS, followed by centrifugation at $2000 \times g$ at 20°C between each washing. The final suspension of washed bacteria was disrupted as glass beads were vortexed for 3 to 5 min. Following disruption, each extract was centrifuged as before; the supernatant was standardized to 20 μg of protein/μl using the bicinchoninic acid method (Pierce, Rockford, IL) and filter-sterilized (.22-μm pore). The standardized supernatant was aliquoted and stored frozen at –20°C. The limulus amebocyte lysate assay for detection of endotoxin (E-TOXATE® kit; Sigma Chemical Co.) was performed on the extract according to the manufacturer's procedure and did not detect endotoxin at a lower limit of .5 EU (endotoxin units)/ml.

Mitogen

Macrophages are activated by numerous factors that are often referred to as biological response modifiers. Because bacterial lipopolysaccharide (LPS) is a strong activator of macrophages, it served as a positive control for cell stimulation. The LPS [Bacto-LPS W (*Salmonella typhosa*); Difco, Detroit, MI] was prepared; 200 μg of LPS powder were dissolved in 100 ml of D-MEM and frozen at –20°C in 1.5-ml aliquots until needed. For experiments, the concentrate was diluted 1:10 (vol/vol) in fresh culture medium to yield a final concentration of .02 g/100 ml of D-MEM.

Enzyme Assays

Lysozyme Production by J774 Cells. Lysozyme is a constitutively secreted enzyme of all macrophages and hydrolyses the *N*-acetylmuramic-β(1–4)-*N*-acetylglucoseamine linkages of peptidoglycan in Gram-positive bacterial cell walls. A quantitative assay based on the lysis of a suspension of *Micrococcus lysodeikticus* immobilized in agarose was used to measure lysozyme activity (20). Briefly, sample wells (2 mm) were punched approximately 20 mm apart in 1% agar containing 30 mg of freeze-dried cells/100 ml, 50 m*M* Tris·HCl, and .02% sodium azide. Ten microliters of tissue culture medium or standard lysozyme solution were added to each well. Plates were incubated at 22°C for 20 h until zones of lysis developed. The area of the lysis zone was proportional to the $\log_{10}$ of the egg white lysozyme concentration. Zone diameters were measured in millimeters and quantified using a curve constructed from standards containing .03 to 10 μg of egg white lysozyme/ml.

Leucine Aminopeptidase Production by J774 Cells. Leucine aminopeptidase, a widely distributed enzyme in many animal tissues, has been well studied in the mouse, in which it appears to be restricted to monocytes and tissue macrophages. The enzyme is classified as an ectoenzyme because of its sensitivity to papain under conditions in which cell integrity is preserved. Leucine aminopeptidase activity was measured in cell lysates by following the hydrolysis of leucine *p*-nitroanilide to *p*-nitroaniline (5). Briefly, 10 m*M* leucine *p*-nitroanilide substrate was prepared by dissolving 28.8 mg of the substrate in 10 ml of absolute methanol. The aminopeptidase standard was prepared by hydration of the freeze-dried enzyme in 1.0 ml of sterile distilled water. The working solution was prepared by diluting the enzyme 1:100 (vol/vol) in PBS containing .1% BSA as a protein carrier. For each sample, 25 μl of cell lysate were added to 75 μl of .05% Triton X-100 in 12- × 75-mm glass tubes. To each tube, 800 μl of PBS were added, and the tubes were incubated at 37°C for 10 min. Substrate (100 μl) was

added to each tube to provide a final concentration of 1 m*M*, and each tube was reincubated for 15 min. The reaction was stopped when the tubes were placed on ice. Concentrations of *p*-nitroaniline were read spectrophotometrically at 405 nm within 1 h. At the 1 m*M* substrate concentration, the reaction was linear for up to 20 min of incubation; the expected absorbance range was .1 to 1.7. Specific activity was determined by calculating nanomoles per milligram of protein per minute at 37°C using a molar extinction coefficient of 9600 for *P*-nitroaniline at 405 nm [i.e., specific activity = optical density × 1000/(milligrams of protein in lysate × time of reaction (minutes) × 9.6)].

Production of Alkaline Phosphodiesterase I by J774 Cells. Increased alkaline phosphodiesterase concentration is reported to be a nonspecific inflammatory event associated with activated macrophages. Phosphodiesterase I hydrolyses phosphoribonucleotides or oligodeoxyribonucleotides stepwise, starting at the free 3′-OH end of the chain and liberating 5′-nucleotides. The assay method chosen can detect activity in 10^6 resident peritoneal macrophages (5). Substrate was prepared by dissolving 8.11 mg of TMP-*p*-NP (thymidine-5′-phosphate-*p*-nitrophenol) in 10 ml of Sorensen's glycine II-zinc acetate buffer at pH 9.6 (Calbiochem-Novabiochem Corp., La Jolla, CA). Assays were performed in 12- × 75-mm glass tubes to which 50-μl aliquots of cell lysate and .5 ml of prewarmed substrate were added, followed by incubation at 37°C for 30 min. At the end of incubation, the tubes were placed on ice, and the reaction was stopped with 1.0 ml of .1 *M* NaOH. Absorbance at 400 nm was read within 30 min. One unit of activity is defined as the amount of enzyme that hydrolyzes 1 μmol of substrate under defined conditions as described.

Phagocytic Assays

Phagocytosis of Polyacrylamide Particles. The J774 cells harvested from 7-d-old cultures were standardized to 1.5×10^6 viable cells/ml, and 200 μl were dispensed into each well of a 24-well plate and incubated for 24 h at 37°C under 5% CO_2 in the presence of 1) no stimulant (negative control), 2) LPS, or 3) either *L. acidophilus* or *B. longum* extract (500 or 1500 μg of protein/ml). After 24 h of incubation, 10 μl of polyacrylamide particles (Immunobead® Reagent; Bio-Rad, Richmond, CA) were added. These carboxylic acid beads were 5 to 10 μm in diameter and, after rehydration in distilled water, produced a suspension of 10 mg/ml. All plates were subsequently incubated at 37°C for 15 min in 5% CO_2 to allow for particle uptake. The plates were removed to a freezer (–20°C) for 5 min to cool prior to being counted; while one plate was being counted, the other was kept at 0°C. Prior to the determination of the number of acrylamide particles taken up by the cells in culture, 10 μl of trypan blue dye were added to each well. This addition provided the necessary differentiation between beads that were unattached or surface attached and phagocytosed under phase contrast microscopy. The average number of beads taken up by 200 cells was determined by manual counting.

Phagocytosis of Viable Salmonella Organisms. Salmonella typhimurium ATCC 13311 was obtained from the Clinical Laboratory Sciences Program culture collection at the University of Wisconsin-Milwaukee. *Salmonella* suspensions were prepared by transferring single colonies into PBS and standardizing to a .5 McFarland turbidity standard. The standardized suspension (10^8 bacteria/ml) was diluted 1:10 (vol/vol) with sterile PBS, and 100 μl were added to each well, providing approximately a 20:1 ratio of *Salmonella* to J774 cells. Mitogen (LPS) or probiotic extract was added to J774 cells 24 h prior to the addition of *Salmonella*. Following 2 h of exposure to *Salmonella*, the cells were washed twice with 2-ml portions of Hanks balanced salt solution, followed by 1.0 ml of fresh Hanks balanced salt solution, which was dispensed into each well. The cells were lysed with .2 ml of a 2% SDS solution in PBS. The number of viable bacteria (colony-forming units per milliliter) was determined by a standard pour plate counting method. Because all *Salmonella* could not be removed from the test cultures by two washing with buffer, each series included control wells, which contained only culture medium to which *Salmonella* was added. Bacterial counts from these controls were deducted from the total count in each group.

Statistical Analysis

Numerical results were analyzed using RS/1 statistical software (BBN Software Corporation, Cambridge, MA) by one-way ANOVA with Duncan's multiple range test for evaluation of sample means (14). All experiments were carried out in triplicate determinations and repeated at least twice.

RESULTS

Lysozyme production by J774 cells in control wells was .1 μg/ml and was not influenced following exposure to *L. acidophilus* extract; however, lysozyme production was significantly less ($P < .05$) in the presence of *B. longum* extract or LPS (Figure 1).

Resident macrophages can synthesize DNA and proliferate (19), but activated macrophages lose this ability (1). The photomicrographs in Figure 2 depict the morphological characteristics consistently observed during culture. The degree in which cell morphology was influenced by the various treatments ranged from mostly rounded and unvacuolized control cells (Figure 2A) to highly active and vacuolized cells stimulated with LPS (Figure 2D).

Cell lysates from *Salmonella* phagocytosis assays were also analyzed for leucine aminopeptidase activity. Macrophages were incubated with cell-free extracts of either *L. acidophilus* or *B. longum* or with LPS for 8 h before addition of *S. typhimurium*. Leucine aminopeptidase specific activity per 10^6 cells was determined at 2 h following exposure to *Salmonella* (Table 1). Results demonstrate no difference in enzyme activity when bacterial extracts were added at 500 μg of protein/ml. With addition of 1500 μg of protein/ml of bacterial extract, the aminopeptidase concentration approached that expressed by J774 cells stimulated with LPS.

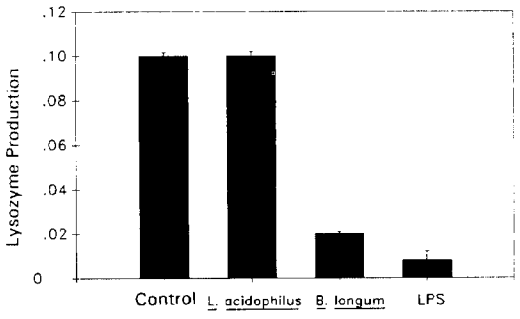

Figure 1. Lysozyme production by J774 cells 72 h following addition of the following: sterile medium only (control), cell-free extract of *Lactobacillus acidophilus* or *Bifidobacterium longum* (1000 μg of protein/ml), or lipopolysaccharide (LPS) (20 μg/ml). Pooled standard error of the means = .004.

To determine alkaline phosphodiesterase I concentration, J774 cells in culture were challenged, as before, with bacterial extracts and LPS. The cells always exhibited morphological characteristics of nonspecific inflammatory events, such as increased size, increased rate of spreading, and increased vacuolization. Analysis of cell lysates for phosphodiesterase I demonstrated no enzyme activity on repeated attempts.

Prior to determination of the number of polyacrylamide particles phagocytosed (Figure 3), trypan blue dye was added to the J774 cell cultures, which facilitated differentiation of phagocytosed and free floating or attached particles. Beads that were taken up by the cells before addition of the dye were colorless, but externally attached or free floating particles were viewed as blue-grey spheres under phase contrast microscopy, which allowed internalized colorless spheres to be counted. Statistical analysis using one-way ANOVA and Duncan's analysis of sample means indicated a significant ($P < .05$) increase in phagocytosis of beads by cells stimulated with LPS or bacterial extract compared with unstimulated cells. Addition of more extract (1500 μg) produced a greater phagocytic response. No significant difference in the number of phagocytosed beads occurred between cells pretreated with 1500 μg/ml of bacterial extract or LPS. The number of beads per cell ranged from 0 to 17; however, few cells had more than 8 beads, and many cells contained no beads at all.

Data from *Salmonella* phagocytosis experiments are shown in Figure 4. Similar to the bead uptake experiments, uptake of *Salmonella* by each of the experimental treatments was significantly greater ($P < .05$) than that of the control. The bacterial extracts stimulated J774 cells to phagocytose *Salmonella* as well as or greater than did LPS.

DISCUSSION

Macrophages play a central role in cell-mediated immunity as antigen-presenting cells and as inflammatory, tumoricidal, and microbicidal cells. For this reason, we examined the effect of lactic acid-producing bacteria on macrophage function. The ATCC J774 cells are characterized as macrophagelike cells in which growth is inhibited by dextran sulfate, purified protein derivative, and LPS and in which large amounts of lysozyme are synthesized. In addition, J774 cells are capable of antibody-dependent phagocytosis. We confirmed lysozyme production by J774 cells and further demonstrated that lysozyme production correlates with observed morphological change consistent with an activated state. The more activated that cells appear, the less lysozyme is produced.

Certain bacteria or their extracts can have an immunostimulatory effect, and several animal models have established that oral application of selected Gram-positive bacteria or their crude extracts can have a priming effect on the immune response to a challenge with pathogenic microorganisms (17). Contrary to some previously reported observations (10, 21), the beneficial effects of probiotic administration

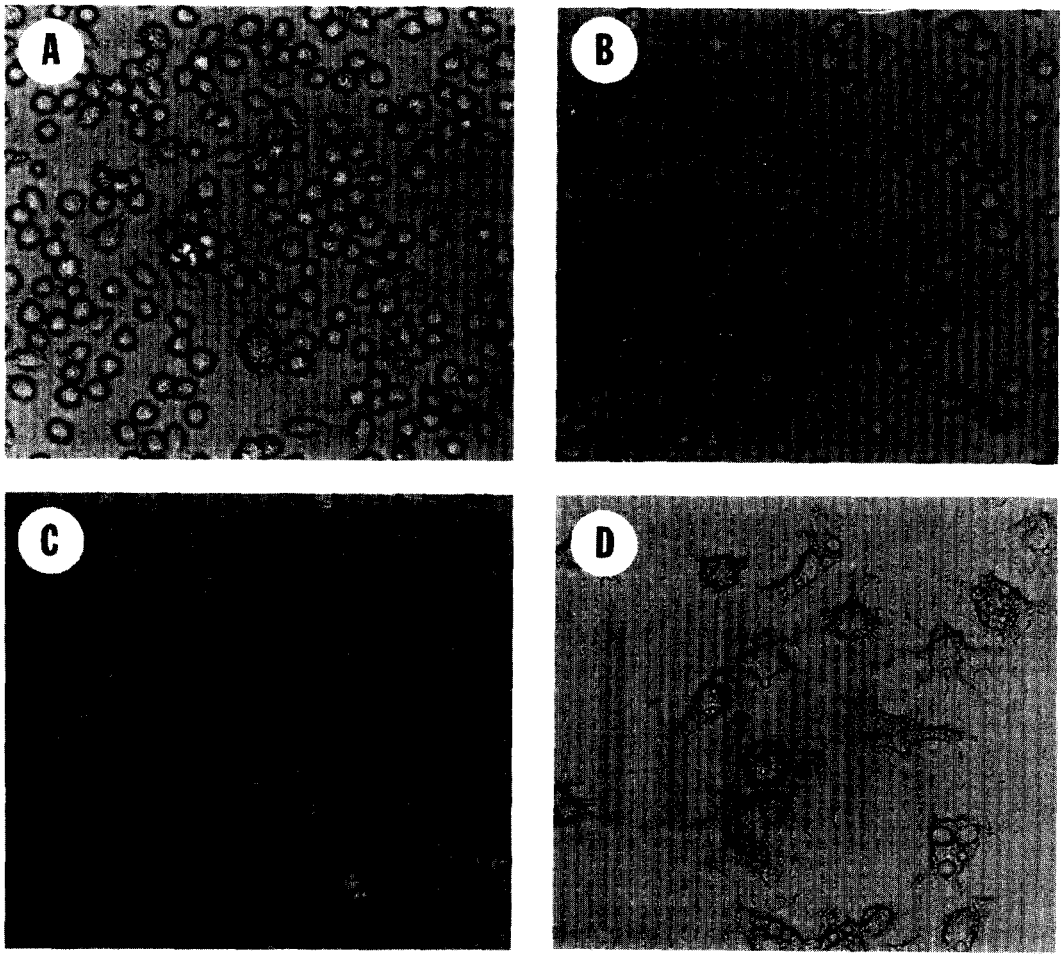

Figure 2. Morphology of J774 cells 72 h following addition of A) Dulbecco's modified Eagle's medium only, B) 1000 μg/ml of *Lactobacillus acidophilus* cell-free extract, C) 1000 μg/ml of *Bifidobacterium longum* cell-free extract, and D) 20 μg/ml of lipopolysaccharide.

TABLE 1. Leucine aminopeptidase activity in J774 cultures following exposure to lipopolysaccharide (LPS) or bacterial extract.

Treatment	Concentration added[1]	Specific activity[2]
	(μg/ml)	$\bar{X}$
Control	0	1.84
LPS	20	2.42[3]
Lactobacillus acidophilus	500	1.93[4]
	1500	2.15[3]
Bifidobacterium longum	500	1.69[4]
	1500	2.10[3]

[1]Based on protein determinations for *L. acidophilus* and *B. longum*.

[2]Specific activity calculated per 10^6 J774 cells (nanomoles of *p*-nitroanaline per milligram of protein per minute at 37°C). Pooled SEM = .090 (Duncan's multiple range test).

[3]Mean specific activity is significantly different from control ($P < .05$).

[4]Mean specific activity is not significantly different from control ($P > .05$).

(ectoenzyme expression and phagocytosis assays) were associated with extremely high (75-fold) doses of probiotic extracts compared with LPS controls. This observation suggests that, not only is the selection of the lactic acid-producing bacterial strain important, but the dose is also important if consistent results are to be expected. Many reported clinical trials, testing the probiotic concept, clearly rely on measures of immunological response to prove efficacy. Apparently, reported inconsistencies may be traceable to either improper probiotic strain selection or improper quantity. High probiotic stimulation would be practical and easy to provide using freeze-dried concentrates at 10^{10} organisms/g.

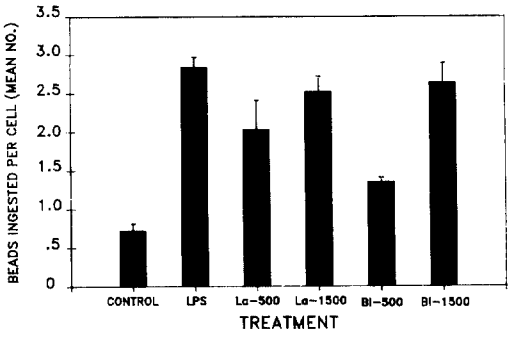

Figure 3. Phagocytosis of polyacrylamide beads by J774 cells 24 h after addition of the following: sterile medium only (control), lipopolysaccharide (LPS) (20 μg/ml), or cell-free extract of *Lactobacillus acidophilus* (La) or *Bifidobacterium longum* (Bl) (resulting in 500 and 1500 μg of protein/ml). Pooled standard error of the means = .42.

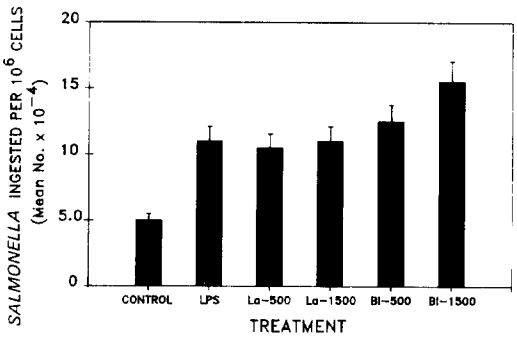

Figure 4. Phagocytosis of viable *Salmonella typhimurium* by J774 cells 2 h following 24-h exposure to the following: Dulbecco's modified Eagle medium only (control), lipopolysaccharide (LPS) (20 μg/ml), or cell-free extract of *Lactobacillus acidophilus* (La) or *Bifidobacterium longum* (Bl) (500 and 1500 μg of protein/ml, respectively). Pooled standard error of the means = .024.

Rook (13) generalized that immunity derived from Gram-positive bacteria is due to peptidoglycan. Our data demonstrated that whole cell, soluble extracts of lactic acid-producing bacteria activated J774 cells in vitro. Extracts from *L. acidophilus* and *B. longum* produced activation in the absence of either viable organisms or cell wall-enriched fractions. At the amounts tested (1500 to 9000 μg of protein), this activation is equivalent to that produced by 20 to 90 μg of LPS (not all data shown). Small amounts (≤20 μg) of LPS were used because optimal mitogenic responses have been previously reported with 2 to 25 μg/ml of LPS. *Bifidobacterium longum* extracts repeatedly provided a greater response, at an equal protein concentration, than did extracts of *L. acidophilus*. The observed effect of LPS on cells in culture agrees with the recently published work by Amano and Akamatsu (2) on LPS-resistant mutants of J774 cells. Cells cultured in the presence of 20 to 90 μg of LPS/

ml of culture medium produced growth and morphological changes indicative of cell activation.

Production of lysozyme by J774 cells was reduced below control cell concentrations when extracts of lactic acid-producing bacteria were added to cells in culture. Production of lysozyme was dependent on the strain and quantity of extract used. *Bifidobacterium longum* extract was similar to LPS in reducing lysozyme production. The use of lysozyme production as a screening aid, however, would not be a reliable analytical tool because poor cell growth could yield a similar result. Leucine aminopeptidase and alkaline phosphodiesterase I, because of their expression at very low to nondetectable concentrations, would also be unsatisfactory screening tools for evaluating probiotic activity. Perdigon et al. (9, 11) used β-glucuronidase as an indicator of macrophage activation. Our experience with other enzyme tests (API-ZYM®; Analytab Products, Inc., Plainview, NY) on selected culture supernatants indicated that the β-glucuronidase expression by J774 cells warranted future analytical evaluation (data not shown).

CONCLUSIONS

The ability of a simple particle phagocytosis model to measure quantitatively the effect of probiotic treatment on macrophage function is novel and has potentially useful application for future routine testing of bacterial strains for probiotic use. Efforts are now focused to determine which components of the bacterial extracts are responsible for the response by J774 cells and to examine other macrophage functions, such as cytokine production. The observation that extracts from lactic acid-producing bacteria activate macrophages in vitro may correlate with the ability of those extracts to induce a response in vivo.

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