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ORIGINAL ARTICLE

Comparison of innate immune activation after prolonged feeding of milk fermented with three species of Lactobacilli

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ABSTRACT

The present investigation aimed at identifying the abilities of three different species of probiotic lactobacilli to modulate cellular immune responses in mouse neutrophils and macrophages *in vivo* over a study period of 60 days. Neutrophil respiratory burst enzymes (cytochrome c reductase and MPO) showed remarkable increased activity ($P \leq 0.01$) after consumption of milks fermented by different species of probiotics over 30 and 60 days of feeding trials. Enzyme activities (β-galactosidase and β-glucuronidase) and nitric oxide production also increased considerably ($P \leq 0.01$) in macrophages, both in peritoneal fluid and in enriched cell cultures. The effects of enhanced enzyme activities were corroborated by simultaneous increases in the phagocytic activities of neutrophils and macrophages. The increases in cellular functions were invariably maximal during the first 30 days of study and were maintained, but did not increase, over the next 30 days. Further, *Lactobacillus helveticus*-fed groups were most effective at modulating neutrophil functions whereas *Lactobacillus paracasei*-fed groups were more potent at enhancing macrophage functions. Together, our results indicate that probiotics have strain specific effects on stimulating cellular functions while not causing excessive stimulation of the immune system over longer feeding periods, thereby resulting in maximum and stable health benefits.

Key words Lactobacilli, macrophages, neutrophils, probiotics.

Probiotics are live micro-organisms which, when administered in adequate amounts, confer health benefits on their hosts. Since the early observations reported by Metchinkoff in 1907 (1), microorganisms belonging to different genera and species have been recognized as probiotics and their multifaceted protective and therapeutic effects on human health have gained global attention. A number of studies have established that probiotics can not only harmonize the intestinal microflora but are also capable of conferring a variety of health benefits to their hosts, including prevention of urogenital diseases, alleviation of constipation and lactose intolerance, protection against diarrhea, reduction of hypercholesterolemia, protection against colon and bladder cancer, and improvement of immunity in old age (2–4). Consequently, there has been a global expansion in marketing probiotic-containing foods, particularly dairy products, as bio-therapeutic agents.

Generation of an effective immune response is a complex, coordinated process that requires many different kinds of cells, including neutrophils, macrophages, natural killers, dendritic cells, and lymphocytes, which act in a specific and coordinated manner to mount a potentially lethal immune response against invading pathogens. The cells of the innate immune system not only provide basic and immediate protection against pathogens but also help direct and provoke pathogen-specific adaptive immune responses. Therefore, activation and responsiveness of the innate immune system is key to mounting an appropriate immune response. Recent

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List of Abbreviations: BD, basal diet; cfu, colony forming unit; DMEM, Dulbeco's modified eagle medium; LA, *Lactobacillus acidophilus*; LC, *Lactobacillus paracasei*; LH, *Lactobacillus helveticus*; LPS, lipopolysaccharide; MPO, myeloperoxidase; MRS, deMan Rogosa Sharpe; NADPH, nicotinamide adenine dinucleotide phosphate; NO, nitric oxide; PFM, probiotic fermented milk; SM, skim milk.

research indicates that most of the purported health benefits of probiotics are based on their ability to stimulate the immune system. Different species of probiotic bacteria are known to enhance immune response in both innate and adaptive arms of the immune system, thereby conferring a variety of health benefits. However, these effects are not necessarily common to all bacterial strains, or even to those within the same species. Further, the purported immunomodulatory effects of probiotics in *in vivo* studies have generally been identified in studies that have run for only 1–2 weeks; hence, these effects may be transient. Thus, further assessment and validation of the effects of probiotics over longer periods is warranted, particularly in the context of the innate immune system. Moreover, to the best of our knowledge, there is no published information regarding effects of probiotics on *in vivo* neutrophil functions. Because they are the most abundant leucocytes, it is imperative to assess the immunomodulatory effects of probiotics on various functional aspects of neutrophils. In response to the above observations, the present study was designed to study the effects of feeding milk fermented by three different probiotic strains of lactobacilli on mouse neutrophil and macrophage functions *in vivo*.

MATERIALS AND METHODS

Microorganisms and culture conditions

Lactobacillus acidophilus NCDC 15 (LA) and *Lactobacillus helveticus* NCDC 292 (LH) used in the present study were procured from the National Dairy Research Institute, Karnal, India, whereas *Lactobacillus paracasei* (LC) was isolated from fermented milk. These cultures were selected on the basis of known probiotic attributes and immunomodulatory activities (5). The cultures were stored at −80°C in MRS broth supplemented with 20% (v/v) glycerol and activated prior to use by sub-culturing twice in MRS broth for 18 hr at 37°C. Fermented milks were prepared by inoculating aliquots of sterile skim milk individually with each bacterial strain and incubating for 18 hr at 37°C. The number of bacteria in the fermented milk was determined by plate counting on MRS agar plates after aerobic incubation at 37°C for 24–48 hr.

Animals and feeding procedure

Male swiss albino mice weighing 18–20 g were procured from the small animal house, National Dairy Research Institute, Karnal and randomized into five groups, each of eight animals. All animal experiments were conducted according to the guidelines of the Institutional Animal Ethics Committee. For studying innate immune responses, three groups of animals were fed BDs (protein 12% and fat 10%) along with PFM from three different *Lactobacilli* cultures (LA, LC, and LH) at final concentrations of 1×10^9 cells/mL. The remaining two groups served as controls; one was maintained on BD and the other on BD supplemented with SM. The animals were fed continuously for periods of 30 or 60 days depending on their study group, after which they were killed by cervical dislocation to assess various cellular immune functions.

Enzymatic indicators of neutrophil functions

Neutrophil functions were assessed in terms of respiratory burst activity by evaluating the enzymes cytochrome c-reductase and MPO. Neutrophils were isolated from whole blood by density gradient centrifugation using Histopaque (Sigma, St Louis, MO, USA) solutions 1077 and 1119 by the method of Costa *et al.* (6). The resulting cell suspensions contained more than 99% of neutrophils with overall viability greater than 95% as determined by the trypan blue exclusion method. Neutrophil cytochrome c reductase activity was evaluated using a cytochrome c reductase (NADPH) assay kit (Sigma, CY0100) according to the manufacturer's protocol. Briefly, neutrophil cell suspensions (1×10^6 neutrophils/mL) were sonicated in enzyme dilution buffer (300 mM, pH 7.8) containing 0.05% Triton X-100. The suspensions were centrifuged at 12,000 *g* for 10 min and the supernatants analyzed for cytochrome c reductase activity. Neutrophil MPO activity was analyzed according to the method of Bradley *et al.* (7) by monitoring changes in absorbance at 460 nm in the presence of *o*-dianisidine dihydrochloride (0.167 mg/mL) and hydrogen peroxide (0.0005%).

Biochemical indicators of macrophage functions

Macrophage functions were assessed in terms of release of lysosomal enzymes (β-galactosidase, β-glucuronidase), NO production and phagocytic activity. Macrophages were collected from the peritoneal cavities of mice with 6 mL of DMEM Ham's F12 (without phenol red) following gentle massage of the abdomen. The cell suspensions were incubated in a humidified atmosphere at 5% CO_2 in air at 37°C for 2 hr to allow attachment of adherent cells (8). Non-adherent cells were removed after washing and the adherent macrophages cultured in DMEM Ham's F-12 medium for 18 hr. The activities of the lysosomal enzymes (β-galactosidase and β-glucuronidase) were assayed in peritoneal fluid as well as in macrophage culture supernatants. β-galactosidase activity was assayed by the method of Conchie *et al.* (9) and β-glucuronidase activity by the method of Stossel *et al.* (10). One fraction of the cultured macrophages (5×10^5 cells/mL) was further suspended in DMEM Ham's F-12 medium supplemented with heat-inactivated FCS (10%) and dispensed at 300 μL/well in 24-well cell culture plates in the presence of *Escherichia coli* (serotype 055:B5) LPS (1 μg/mL final concentration) and incubated in a CO_2 incubator perfused with 5% CO_2 in air at 37°C for 24 hr. The cell free supernatants collected at 24 hr

were analyzed for NO production according to the method of Miranda *et al.* (11).

Phagocytic activity of neutrophils and macrophages

Neutrophil suspensions (1×10^6 cells/mL) and peritoneal exudate containing macrophages (1×10^5 cells/mL) were used to assess phagocytic activity according to the method of Hay and Westwood (12). Phagocytosis was observed at $1000 \times$ magnification under oil immersion (Olympus Optical, Tokyo, Japan) and the percentage phagocytosis calculated according to the following formula:

$$\text{Percentage phagocytosis} = \text{Number of neutrophils/macrophages with yeast cells internalized per 100 neutrophils/macrophages}$$

Statistical analysis

Results are presented as means ± SEM. ANOVA was performed using GraphPad PRISM version 5.0 statistical software package and the differences among groups were tested using the Tukey–Kramer post-hoc test. Statistically significant difference was set at $P \leq 0.05$.

RESULTS

Neutrophil functions

Cytochrome c reductase activity in peripheral blood neutrophils of animals fed on the various experimental diets is given in Figure 1a. After 30 days of feeding the experimental diets, neutrophil cytochrome c reductase activities were significantly greater (80.8% in LH; 76.09% in LC; and 69.55% in LA; all $P \leq 0.05$) in all groups fed with their respective PFMs than in the control diet-fed animals. Similarly, after 60 days of feeding the enzyme activities were significantly greater (80.33% in LH; 76.65% in LC; and 66.81% in LA groups) in the PFM-fed groups than the BD group. No comparable differences among the PFM-fed groups were observed for either the 30 or 60 day trial periods.

The effects of feeding PFMs on MPO activity of neutrophils in the various experimental groups is depicted in Figure 1b. MPO activity was also remarkably greater ($P \leq 0.01$) in groups fed with PFM than in that receiving BD. As compared with the respective control (basal) groups, the activities of MPO increased by 151.18%, 158.49% and 127.41% in the LH, LC and LA groups, respectively, after 30 days of feeding and by 134.55%, 115.39% and 89.22% in the LH, LC and LA groups after 60 days of feeding. However, no significant differences were observed among the PFM-fed groups during 30 and 60 days of feeding, except for the mice fed with LH fermented milk. The mice fed with SM also showed significantly greater increases in MPO activities (of 31.6% and 20.94%) than did the BD group after 30 and 60 days of feeding, respectively (both $P \leq 0.05$). These activities, however, were considerably less than those found in the PFM-fed groups ($P \leq 0.05$).

Macrophage functions

The effects of feeding three types of PFMs on β-galactosidase activity in peritoneal fluid and enriched macrophage culture supernatants are presented in Figure 2a,b, respectively. After 30 days of feeding, β-galactosidase activity in peritoneal macrophages was significantly greater in the PFM-fed groups (209.24% in LC, 145.8% in LA and 36.12% in LH) than in the control groups. Enrichment of macrophages also resulted in remarkably greater increases in β-galactosidase activities in the PFM-fed groups (236.55% in LH, 249.24% in LC, and 141.37% in LA) than in the control groups. Similarly, feeding of animals with the various PFMs for 60 days caused much greater increases in β-galactosidase activities in both peritoneal fluid and enriched macrophages (both $P \leq 0.01$) than was found in the control groups. In all these feeding trials, all groups except the LA group showed significantly greater enzyme activities in peritoneal fluid after 60 days of feeding than after 30 days. However, there was no comparable difference in enzyme activity in the enriched macrophages after 30 versus 60 days of feeding except in the LA group ($P \leq 0.05$).

The effects of feeding PFM on β-glucuronidase activity in peritoneal fluid and in enriched cell culture supernatants of macrophages are shown in Figure 2c,d, respectively. Only the LC group showed a significantly greater increase (610%; $P \leq 0.001$) in β-glucuronidase activity in peritoneal fluid than was found in the control BD group after 30 days of feeding. However, at the end of 60 days, all PFM fed groups had significantly greater increases in β-glucuronidase activity than did the control BD group. Similarly, the increases in release of β-glucuronidase by enriched macrophages were considerably greater ($P \leq 0.01$) in the PFM-fed groups (LH, 190.16% and 149.57%; LA, 81.14% and 112.82%; and LC, 370.49% and 430.76%) than in the BD group for feeding of duration 30 and 60 days, respectively. Feeding PFM containing LC for 30 and 60 days resulted in the highest and most statistically significant increases in β-glucuronidase levels in both peritoneal fluid and enriched macrophages of all the other test groups.

The effects of feeding three types of PFM on NO production in peritoneal fluid and LPS stimulated enriched macrophage culture supernatant are presented in Figure 2e,f, respectively. Increases in production of NO in peritoneal fluid of the PFM-fed groups was significantly greater ($P \leq 0.01$) at both 30 days (LH, 316%; LA, 314%; LC, 311%) and 60 days (LH, 136%; LA, 113%; LC, 168%) than in the control BD group. However, only the LC-fed group

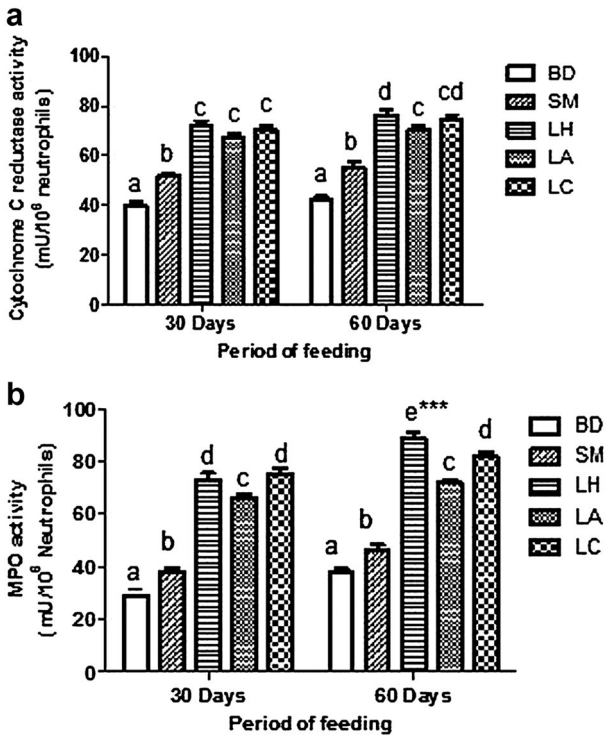

Fig. 1. Effects of feeding probiotic-fermented milk on enzymes involved in respiratory burst activity in neutrophils. (a) Cytochrome c reductase. (b) Myeloperoxidase. Values are expressed as mean ± SEM. Values with different letters are significantly different within the group at $P \leq 0.01$. $^{***}P \leq 0.001$ for treatments fed for 30 versus 60 days.

showed a significant difference ($P \leq 0.05$) between 30 and 60 days of the experimental feeding. Further, there were no statistically significant variations in PFM-fed groups between the two study periods. Similarly, irrespective of duration of feeding, animals fed with the three PFMs showed significantly greater increases ($P \leq 0.01$) in NO production in LPS-stimulated enriched cell supernatants of macrophages than did controls. The LC group had significantly greater increases at both 30 and 60 days of feeding than did the other PFM groups. However, no significant differences were observed when comparing between groups at 30 and 60 days.

In vitro phagocytosis

The effects of feeding PFM on phagocytic activity of neutrophils and macrophages results are depicted in Figure 3a,b, respectively. Increases in percentage of phagocytosis by neutrophils was significantly greater in the groups fed the different PFMs (LC, 63.7% and 44.53%; LH, 58.8% and 43.81%; LA, 54.4% and 40.01%) than in the control groups at 30 and 60 days of feeding, respectively. No comparable differences were observed in the SM and BD fed groups. At 30 days, increases in phagocytic activity of peritoneal macrophages were also significantly greater ($P \leq 0.01$) by 32.76%, 30.59%, and 57.75% in groups fed PFM containing LH, LA, and LC, respectively, than in mice fed on BD. Similarly, after 60 days of receiving experimental feeding significantly greater increases were observed (50.46% in LH, 51.86% in LA and 92.99% in LC) in the PFM-fed groups than in the control groups. In both cases, the LC-fed group showed significantly greater increases than did all the remaining groups; this was also significant according to duration of study. Figures 3c,d depict representative photographs of phagocytosis of yeast cells by neutrophils and macrophages, respectively.

DISCUSSION

In resting neutrophils, the individual components of NADPH-dependent oxidase are separated in cytosol and membrane compartments, which assemble together in response to endogenous or exogenous shock. During phagocytosis, the oxidase transfers electrons from cytosolic NADPH to intra-phagosomal molecular oxygen, thereby producing superoxide and hydrogen peroxide that are further utilized by MPO to generate hypochlorous acid as a secondary product. The combination of assembly of NADPH oxidase and MPO in phagosomes generates large amounts of lethal antimicrobial agents, including superoxides, singlet oxygen, hypochlorous acid and hydroxyl radicals (13). Our findings indicate that, irrespective of strain type, oral administration of PFMs results in significantly greater increases in the activities of enzymes associated with respiratory bursts in neutrophils than occurs in control groups. However, increases in cytochrome c reductase activity were more pronounced in groups fed with LH fermented milk than in LC- and LA-fed groups at both 30 and 60 days. Similarly, increases in MPO activity were more prominent in the LH-fermented milk group than in the LC and LA groups, in that order, at both 30 and 60 days. This suggests that LH-fermented milk more effectively stimulates neutrophils than do other lactobacilli strains.

While comparing activities of neutrophil enzymes over the 2-month feeding trials, it became evident that probiotic treatment not only enhances the activities of these enzymes but also maintains these increases over the longer duration of 2 months. Feeding probiotics resulted in maximum activation of immune responses in the first 30 days and this degree of activation was maintained over the next 30 days (60 days overall). This suggests that, although addition of dietary probiotics increases immune response in neutrophils; prolonged treatment does not result in any further stimulation of the innate immune system. Thus, the increase became stable over a longer duration of feeding, which is of extreme importance in studies involving immunity activation because hyper-activation of the immune system, particularly of the innate immune system, can lead to grave consequences. Very few earlier studies have

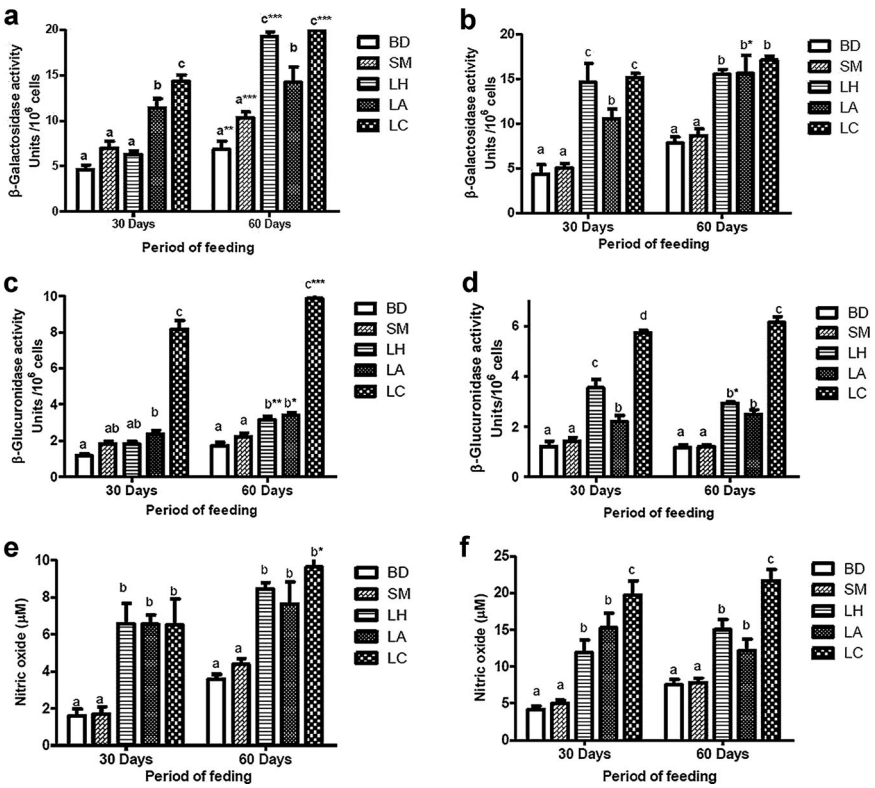

Fig. 2. Effects of feeding probiotic-fermented milk on macrophage functions. β-galactosidase in (a) peritoneal fluid and (b) enriched macrophages; β-glucuronidase in (c) peritoneal fluid and (d) enriched macrophages; nitric oxide production in (e) peritoneal fluid and (f) enriched macrophages. Values are expressed as mean ± SEM; values with different letters are significantly different within the group at $P \leq 0.01$. $^{***}P \leq 0.001$, $^{**}P \leq 0.01$, $^{*}P \leq 0.05$ for treatments fed for 30 versus 60 days.

investigated the role of probiotics in modulating neutrophil functions and there are even fewer reports regarding modulation of enzymes associated with neutrophil respiratory bursts. Mikes *et al.* have reported that dietary consumption of *Enterococcus faecium* for 6 weeks causes a significant increase in the ability of neutrophils from human subjects to produce oxygen radicals following incubation with zymosan or phorbol myristate (14). It has also been demonstrated that administration of $\sim 10^7$ cfu/mL of the lactic acid bacterium *Lactobacillus johnsonii* La 1 stimulates respiratory bursts of peripheral blood leukocytes in healthy adult volunteers (15). Similarly, studies of tilapia fish (*Oreochromis niloticus*) have reported that administration of the probiotics *Lactococcus lactis* RQ516 and *Enterococcus faecium* ZJ4 causes increased serum MPO activity in aquaria water at a final concentration of 1×10^7 cfu/mL (16, 17). The increased activities of respiratory burst enzymes in the present study therefore corroborate these findings and suggest a possible mechanism of action of probiotics in stimulating neutrophil immune functions.

The evident robust increase in activities of respiratory burst enzymes in neutrophils caused by PFM administration was further validated by assessing phagocytic activities. All the PFM-fed groups showed significantly greater increases in neutrophil phagocytic activity than did the control groups. Further, stimulation of cells to phagocytose seemed to occur equally in all PFM-fed groups. The increased activity of neutrophil respiratory burst enzymes in the present study would correlate with increased pathogen killing potential of phagocytes. These results on stimulation of phagocytic activity by feeding probiotic lactobacilli are in agreement with other studies that reported significant increases in granulocyte phagocytosis after administration of probiotics (18, 19). These observations are also supported by a clinical study in which treatment with *L. casei* Shirota (6.5×10^9) three times daily for 4 weeks resulted in greater phagocytic capacity of neutrophils in alcoholic cirrhotic patients than in healthy controls (20). Together, our results on neutrophil respiratory burst enzymes and phagocytosis after probiotic supplementation suggest that the enhanced activity of these enzymes is responsible for the increased phagocytosis by neutrophils that persisted throughout the study period.

Previous studies on short-term feeding of probiotics have reported an increase in the release of lysosomal enzymes in macrophages. Perdigon *et al.* reported that oral administration of *L. casei* CRL431 and *Lactobacillus bulgaricus* CRL 423 to

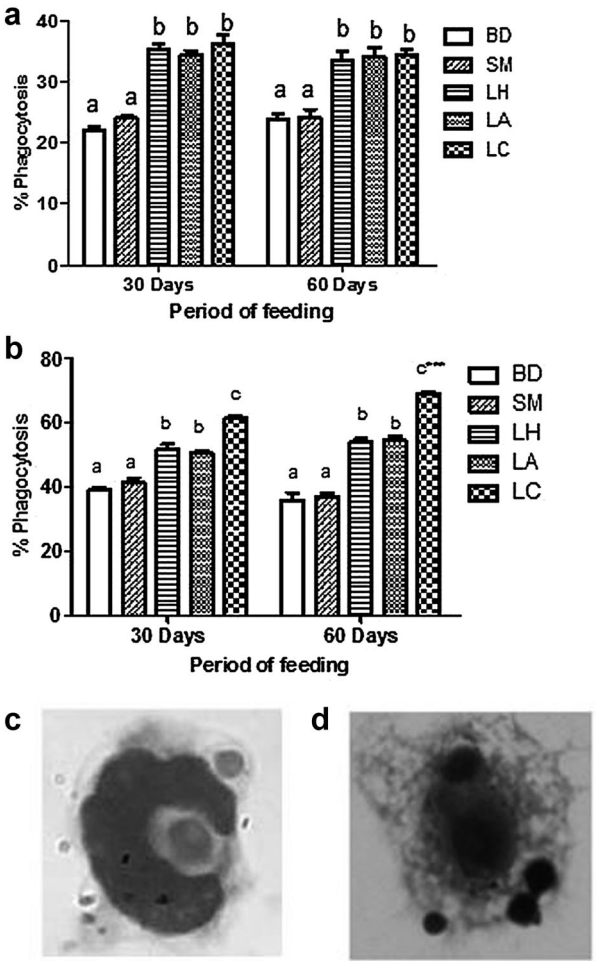

Fig. 3. Effects of feeding probiotic-fermented milk on neutrophil and macrophage phagocytosis. Percentage phagocytosis (a) neutrophils and (b) macrophages. Microphotographs showing yeast cells internalized by (c) a neutrophil and (d) a macrophage. Magnification: 1000 × Values are expressed as mean ± SEM; values with different letters are significantly different within the group at $P \leq 0.01$. $^{***}P \leq 0.001$ for treatments fed for 30 versus 60 days.

mice for 8 days significantly increased release of lysosomal enzymes (β-galactosidase and β-glucuronidase) from peritoneal macrophages as well as in the supernatant of cultured peritoneal macrophages (21). In another short term study, Singh and Kansal reported a profound increase in β-galactosidase activity in peritoneal fluid after oral administration of milk fermented by a mixed culture of *Leuconostoc citrovorum* and three species of *Lactococcus* (*lactis, diacetylactis and cremoris*) (22). However, these researchers found no effects on β-glucuronidase activity. Similarly, previous results in our laboratory have also demonstrated increased activities of peritoneal β-galactosidase and β-glucuronidase enzymes during 8 days of feeding of *L. casei* fermented milk (23).

These observations are further corroborated in the present study, which showed gradual increases in β-galactosidase activity during longer durations of study. The LC-fed group recorded significantly higher levels of β-galactosidase in both peritoneal fluid and enriched macrophages at 30 days than did the other PFM groups. This difference however, disappeared over the next time period of 30 days, during which all PFM groups showed comparable β-galactosidase activities. Similarly, administration of PFM also increased β-glucuronidase activity significantly more than in the control groups. However, the LC-fed group showed the greatest and most significant increase in β-glucuronidase activity not only when compared with control groups but also with other PFM-fed groups. Together with our observations of β-galactosidase activity, it appears that LC fermented milk more potently stimulates macrophages to release lysosomal enzymes than does milk fermented with the other PFMs.

Our findings concerning the effects of prolonged feeding of PFMs on NO production by LPS-stimulated peritoneal macrophages are consistent with those of other reports (24–26). Among the PFM-fed groups, the LC group registered the most significant increase in NO production by LPS-stimulated enriched macrophages, further indicating the macrophage-stimulating ability of LC. Similar to our observations in neutrophils, administration of probiotics enhanced phagocytosis in macrophages in all the PFM-fed groups; however, the LC-fed group recorded a significantly greater increase than the LA- and LH-fed groups. The observed increase in phagocytic activities of neutrophils and macrophages may be attributable to increased lysosomal enzyme activities and NO production. This increase in phagocytic activity again appeared to stabilize over the 2 months of study.

In conclusion, all the probiotic lactobacilli assessed in the present study are able to enhance functions of innate immune cells. However, the efficacy of different probiotic species in stimulating different immune system cells varied, reiterating the fact that probiotic effects are highly selective (27, 28). Whereas LH was most effective at stimulating neutrophils, the LC-fed groups registered the maximum increases in macrophage activity. Structural differences in the cell wall composition of different strains of lactic acid bacteria are purportedly responsible for differences in their observed efficacy. In addition, strains that are able to survive in the gastro-intestinal tract, adhere to gut mucosa and persist above a critical level also stimulate phagocytic cells more efficiently (29, 30). Another important conclusion of our study is that feeding of probiotics for longer periods did not result in any over-stimulation of cellular activities. Probiotic feeding thus enhances cellular activities to a stable maximum. Therefore, dietary supplementation with these probiotic strains could provide an effective way of improving innate immune response and resisting infections. However, further studies involving both innate and humoral components of

the immune system, particularly in the context of a pathogen challenge, are required to fully comprehend the long term effects of probiotics.

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DISCLOSURE

No authors have any conflicts of interest to disclose.

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