Research Article

# Immunomodulatory and Immunorestorative Potential of Intragastrically Fed Viable and Non-viable *l. Acidophilus* in Swiss Albino Mice

**Ritu Pawan*, Aruna Bhatia[1]**
*Assistant Professor, Department of Biotechnology, Baba Farid College, Deon, Bathinda, Punjab, India.
[1]Professor and Dean, Faculty of Life Sciences, Department of Biotechnology, Punjabi University Patiala, Baba Farid College, Deon, Bathinda, Punjab.
***Corresponding author's E-mail:** aruna_bhatia@rediffmail.com

**Accepted on:** 18-04-2013; **Finalized on:** 30-06-2013.

**ABSTRACT**

Though the immunomodulatory effect of probiotics is known but hardly any literature shows its immunorestorative effect. In the present study the effect was immunorestorative capacity of intragastrically administrated live and dead (lysates) probiotic strain of *Lactobacillus acidophilus* was investigated in hydrocortisone-treated Swiss Albino mice. The cell-mediated immune response was assessed by Nitroblue Tetrazolium (NBT) reduction, inducible Nitric Oxide Synthase activity (iNOS), bactericidal activity and Delayed Type Hypersensitivity (DTH) response. The development of anti-BSA antibodies as a measure of humoral immune response was checked by ELISA. The results showed that both the live and dead bacteria feeding potentiated the cell-mediated immune response as well as humoral immune response, however the activity was better ($p<0.05$) in the former. Moreover, the suppressive effect of chemically damage immune system by hydrocortisone in mice was attenuated by live and dead *L. acidophilus* and was restored towards normalcy. Our findings that even the dead probiotic bacteria may exert immunomodulatory effects and improve the immune function damaged by immunosuppressive agents. It is concluded that in immunocompromised host where live bacteria may give harmful effects, the dead bacteria may be given to boost the immune response.

**Keywords:** Immune response, Immunosuppressive agents, iNOS, *Lactobacillus acidophilus*, Probiotics.

## INTRODUCTION

Research is beginning to reveal the possible health benefits associated with probiotic lactic acid bacteria (LAB).[1,2] Probiotics LAB are the 'beneficial microorganisms which upon ingestion in adequate amount beneficially effect the host health beyond the basic nutrition by altering the gastrointestinal tract'. The worldwide emerging trends of functional foods containing probiotics in maintaining gut health has been receiving research attention. It appeared that two paradoxical effects of probiotic feeding are reported according to the initial immunological state of the host: probiotics can stimulate innate immunity (phagocytosis, cytokine release) in individuals, a property likely to help in shortening the course of infectious diseases (gastroenteritis in infants) or in the efficacy of vaccination. On the other hand, an anti-inflammatory effect is reported with selected strains of probiotics, in inflammatory bowel diseases such as ulcerative colitis.

Though the probiotic effect on the gut immunity is well established by affecting the pathogenic bacteria and stimulating the gut immune organs,[3] but only a few reports revealed the effects of probiotics on systemic immune response.[4,5] Hence, it becomes necessary to find out the effect of dead bacteria as immunostimulatory and the immunorestorative potential of both the live and dead probiotics in immunocompromised host. Moreover, LAB-containing functional foods are often included in the diet of immunocompromised patients, and it is known that agents with immunomodulatory activity may induce contrasting effects in normal and immunosuppressed individuals,[6,7] applicable to the assay of immune modulation by antibiotics in normal and immune compromised mice.[8]

The presents study was design to see the immunorestorative potential of a viable and non-viable probiotics *Lactobacillus acidophilus* strain on the immune system of immunocompromised hosts *i.e.* drug induced immunosuppressed mice.

## MATERIALS AND METHODS

### Micro organisms

The lactic acid bacterial strain *Lactobacillus acidophilus* MTCC 447 was obtained from MTCC, IMTECH, Chandigarh. The strain fulfilling the criteria purposed to achieve probiotic status (acid and bile tolerant, antimicrobial activity) grown in MRS broth (Himedia, Mumbai, India) at 37°C for 24 h and used in this study.

### Animals and Ethics

Swiss Albino mice of either sex weighing 25 ± 02 g obtained from Central Research Institute, Kasauli, India, were housed in University Animal House, under pathogen free conditions with 12 h cycle of light and free access to commercial chow (Kisan Feeds Ltd., Mumbai, India)) and sterile water. The experiments and used protocol, approved and supervised by the local Animal Ethic Committee as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forests, Government of India (Reg. No. 107/1999/CPCSEA).

**Feeding Procedure of Live and Bacterial Lysates (Dead) *L. Acidophilus***

***Live Bacteria Feeding***

Freshly cultured probiotic *L. acidophilus* in MRS broth were harvested by centrifugation (2600 rpm, 4°C for 10 min), with 3× washing in sterilized PBS (pH 7.2) and re-suspeneded into low fat milk (Verka, Government undertaking, Punjab, India). After overnight incubation at 37°C cell viability was checked by plating onto MRS agar $2.2 \times 10^9$ cfu/mL were observed and used to fed the animals (approx. @ $10^9$ cells/day/mouse).

***Dead Bacteria Feeding***

Freshly cultured *L. acidophilus* harvested by centrifugation and viable bacterial cell count was checked (1% trypan blue) and lysed by sonication at maximum amplitude (50%) of the instrument in normal saline (0.85%) for 12 min and resulted dead bacteria (lysates) re-suspeneded into low fat milk and used to feed the animals approximately @ $10^9$ cells/ml.

The animals received a daily dose of 100 µL (about $10^9$ cells/mL) prepared live and dead *L. acidophilus* dose by intragastric route with the help of sterile stainless steel needle (Dispovan, HMD, India) and 1 mL syringe for 15 days.

**Grouping of Animals and Study Design**

***I. Immunomodulatory Effect of Live/Dead L. Acidophilus Feeding In Mice***

The animals divided into three groups having ten mice in each: Control C group fed on normal diet, Test LL group I fed on prepared live bacteria feed and Test LD group II fed on prepared dead bacteria feed for consecutive 15 days. The bacteria feeding in animals were given in addition to the normal diet. Animals were bled (on day 7$^{th}$ and 15$^{th}$) from retro-orbito-plexus to check humoral immune response, after that the mice were sacrificed, and their spleen was taken to assess the different immunological parameters.

***II. Immunomodulatory Effect of Live/Dead L. Acidophilus Feeding In Immunosuppressed Mice***

For this experiment the animals were divided into four groups: Control group- untreated; Control Hc group- mice treated with hydrocortisone (Hc) and fed on normal diet; Test LLHc group-I - mice treated with hydrocortisone and fed with live *L. acidophilus* and Test LDHc group-II - mice treated with hydrocortisone and fed on dead bacteria (bacterial lysates) for 15 days. The bacterial feeding in Test group I and II *L. acidophilus* (live and dead) administration was started on day 6$^{th}$ after hydrocortisone treatment and continued or the full experiments period until mice were sacrificed (day 21$^{st}$)

**Treatment with Hydrocortisone (Hc)**

Animals were injected intraperitoneally with 2 doses (day 0 and day 5$^{th}$) of hydrocortisone (Glaxo SmithKline, Pune, India) @ 10mg/kg body weight/dose/mouse.

**Immunization**

The animals were immunized by giving intraperitoneally injection of 1% BSA (Hi-Media, Mumbai, India) in 0.85% NaCl as a source of antigen in 3 doses at weekly interval *i.e.* day 8$^{th}$, 14$^{th}$ and 20$^{th}$ to stimulate the antibody production.

**Follow Up of Study**

The blood samples were collected after 24 h of immunization to assess the humoral response using ELISA. The mice were sacrificed after the completion of test diet and immunization schedule. The spleen was collected in minimal Essential Medium (MEM, Hi-Media, Mumbai, India) and desired spleenocytes suspension were made to assess the immune response by different immunological tests.

**Methods Used to Assess the Immune Status**

**Determination of iNOS Expression (Inducible Nitric Oxide Synthase Test)**

The iNOS activity of splenocytes assessed using arginine and Griess reagent as described by Stuehr and Marletta.[9] The splenocytes were anaerobically incubated with arginine at 37 °C for 24 h in 5% $CO_2$. The purple color developed due to citrulline formation from arginine was determined by monitoring absorbance at 540 nm against control.

***Bactericidal Activity***

The bactericidal activity as a function of phagocytes was assessed by splenocytes against *E. coli* according to the method of Raghuramulu.[10] Briefly, the splenocytes and bacterial suspension (1:2) were incubated at 37 °C for 60 min followed by plating (100 µl) on nutrient agar plates using a sterile spreader. After 24 h at 37°C, number of colony forming units (cfuml$^{-1}$) was counted. Plates containing bacterial suspension (*E. coli*) used as control. The percent bactericidal activity was calculated as;

$$\text{\% Bactericidal Activity} = \frac{(\text{cfuml}^{-1} \text{ in control} - \text{cfuml}^{-1} \text{ in test})}{\text{cfuml}^{-1} \text{ in control}} \times 100$$

**Determination of Nitroblue Tetrazolium Reduction (NBT)**

NBT reduction, a measure of respiratory burst in the spleenocytes was determined as described by Hudson and Hay[11]. Briefly, splenocyte suspension from each mouse was incubated in the presence (test) or absence of NBT and the formed formazon was measured spectrophotometerically at 520 nm using UV-VIS spectrophotometer (Shimazu, USA), against dioxan as blank. The NBT reduction was calculated as follow;

$$\text{NBT Index} = \frac{\text{Absorbance of test (T) - Absorbance of control (C)}}{\text{Absorbance of control (C)}}$$

### Enzyme Linked Immunosorbant Assay (ELISA)

ELISA was carried out as described by Hudson and Hay.[11] Briefly, 200 µl of previously diluted test sera was incubated with antigen-coated, flat-bottom 96-well microtitre plate (Tarson, India) at room temperature (RT) for 60min. The antibodies from each cell were visualized by application of horse radish peroxidase-anti-Ig conjugate followed by a substrate (OPD) overlapping for 10-30 min in dark and quantified the colour reaction in an ELISA reader (BIO-RAD) set at 492 nm after stopping the reaction with sulfuric acid (12.5%).

### *In vivo* Delayed Type of Hypersensitivity (DTH) Response

The DTH response was assessed by foot pad swelling methods as described by Hudson and Hay[11] as a measure of T- cell activity. The swelling in foot pad was determined after 24-48 hr in the foot pads after carrageenan (30 µl in 0.85% NaCl, 200 mg/kg weight) induction in right foot pad and sterilized normal saline (30 µl) in left hind foot (control).The swelling in the right and left foot was measured with Varnier's caliper at 0, 24 and 48 hrs. The difference between paw thickness by comparing test paw with control was taken as a measure of DTH. The net swelling calculated by following equation:

$$\text{Net Swelling} = (T_{24/48} - T_0) - (C_{24/48} - C_0)$$

Here, $T_{24/48}$- footpad thickness at 24 or 48 h after carrageenan challenge (right foot), $T_0$ - footpad thickness before carrageenan challenge (right foot), $C_{24/48}$ - footpad thickness at 24 or 48 h after normal saline challenge (right foot) and $C_0$ - footpad thickness before normal saline challenge (right foot).

### Statistical Analysis

All the results were expressed as mean±standard error of mean (S.E.M.). Statistical analysis for the results was done using one-way ANOVA followed by Tukey's multiple range test as post hoc analysis. A value of $P < 0.05$ was considered to be statistically significant.

## RESULTS

### I. Immunomodulatory Effect of Live/Dead *L. Acidophilus*

*Effect of Live/Dead Probiotic L. Acidophilus Feeding on Cell-Mediated Immune Response (CMI)*

The results of cell-mediated immune response evaluated by iNOS, NBT, bactericidal activity and DTH response in Swiss Albino mice are shown in Figure 1 and 2. The statistical analysis revealed a significantly higher (p<0.05) NBT reduction, iNO's and bactericidal activity in Test group LL and a non-significant (p ≥ 0.05) high activity in LD group animals as compared to control group animals. The NBT reduction, iNOS and bactericidal activity were 41.6%, 19.35±0.769 and 20.5% higher (p < 0.05) in test animals fed on live bacteria, and 15.9%, 7.8% and 10.1% higher (p ≥ 0.05) in test LD animals respectively than control group (Fig. 1). Moreover, non-significant difference existed between both the test groups.

The results of DTH response showed maximum foot pad thickness at 24 h in all the groups. At 48 h a statistical significant enhancement (2.04mm±0.0535, p < 0.05) was observed in test LL animals fed on live bacteria as compared to control C animals whereas a non-significant difference (1.78mm±0.0313, p ≥ 0.05) in test group LD animals fed on dead bacteria as compared to test LL animals fed on live bacteria (Figure 2).

*Effect of Live/Dead Probiotic L. Acidophilus Feeding on Humoral Immune Response (Figure 3)*

The results show significantly higher (p < 0.05) anti-BSA antibody titres in test animals fed on live bacteria and non-significantly higher (p ≥ 0.05) in test group II animals fed on dead bacteria (bacterial lysates) as compared to the control group.

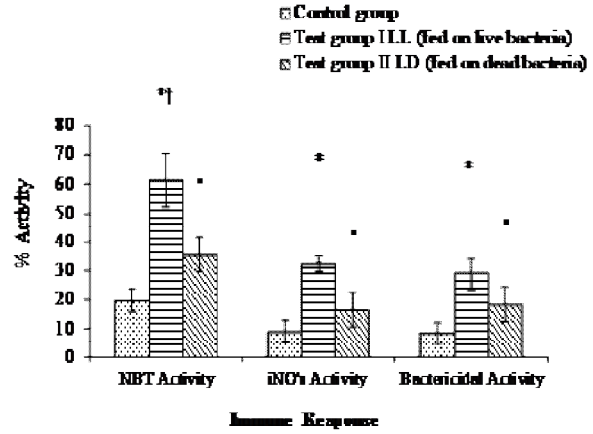


**Figure 1:** Effect of live and dead *L. acidophilus* feeding on iNOS, NBT reduction and Bactericidal activity in Swiss Albino mice. [Values are mean%±SEM], *=*P*<0.05 vs. control animals,†=*P*≥0.05 vs. Test group II LD.

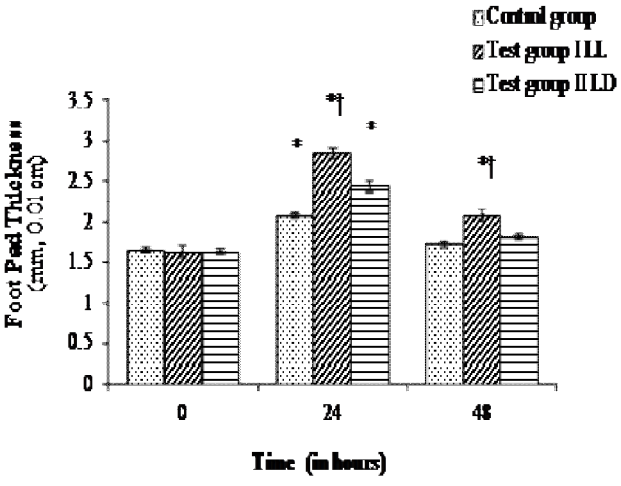


**Figure 2:** Effect of live and dead *L. acidophilus* feeding on DTH response (mm, $10^{-2}$ cm) in Swiss Albino mice. [Values are mean%±SEM] *=p<0.05 vs. 0 h, †=p<0.05 vs. control group.

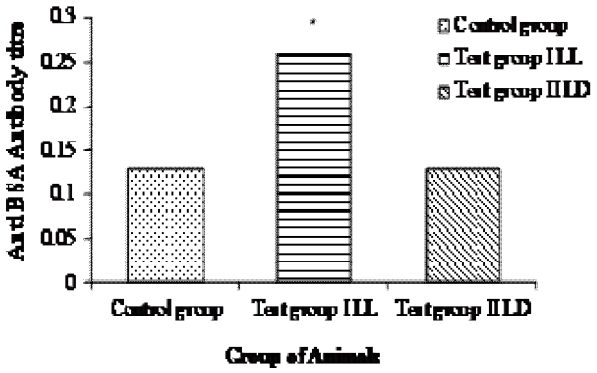


**Figure 3:** Effect of live and dead *L. acidophilus* feeding on Humoral immune response in Swiss Albino mice. *= p<0.05 vs. control C group.

### II. Immunomodulatory Effect of Live/Dead *L. Acidophilus* Immunosuppressed Mice

*Effect of live/dead L. acidophilus feeding on cell-mediated immune response*

The results showed significantly higher (p<0.05) NBT reduction, iNOS and bactericidal activities in both HC treated test groups fed with bacteria (live/dead) *i.e.* LLHc and LDHc as compared to treated control Hc group non-bacteria fed animals as shown in Fig. 4. The NBT reduction, iNOS and bactericidal activities were 23.57%, 10.84% and 11.5% higher (p < 0.05) in Test LLHc, and 18.52%, 6.98% and 4.7%% higher (p < 0.05) in LDHc fed on bacterial lysates (dead) respectively as compared to control HC animals (Figure 4).

The results of DTH showed a significant enhancement (p < 0.05) in DTH response as observed in both of the test groups, hydrocortisone treated and bacteria fed animals *i.e.* test LLHc animals fed on live bacteria (2.36mm±0.0120) and test LDHc animals fed on dead bacteria (1.95mm±0.0368) as compared to treated control Hc animal. Similar to 24 h at 48 hours also a statistically significant (p<0.05) enhanced DTH response in Test LLHC animals was observed as compared to control Hc animals as shown in Figure 5.

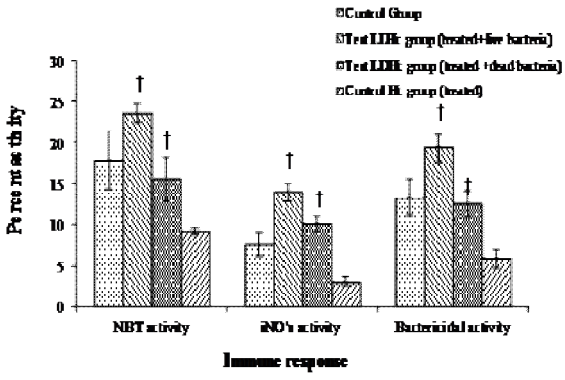


**Figure 4:** Effect of live and dead L. acidophilus feeding on iNOS, NBT reduction and Bactericidal activity in immunosuppressed mice. [Values are mean ± SEM]; †=p<0.05 vs. control Hc group (treated).

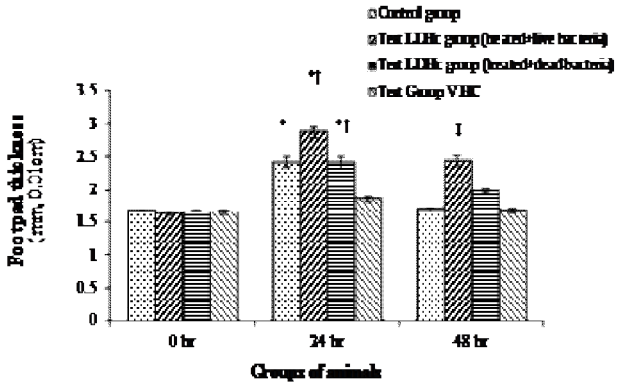


**Figure 5:** Effect of live and dead bacteria feeding on DTH response in immunosuppressed mice. [Values are mean±SEM]; *****=p<0.05 vs. control Hc group, †=p<0.05 vs. control HC group V, ‡= (p<0.05) vs. Control group, Test LDHc and control Hc group.

*Effect of live/dead L. acidophilus feeding on humoral immune response*

The results of ELISA showed a higher anti-BSA antibody titers in Test group LLHc fed on live bacteria whereas non-significant (p≥0.05) high titres in Test LDHc animals fed on dead bacteria as compared HC treated control animals as shown in Figure 6.

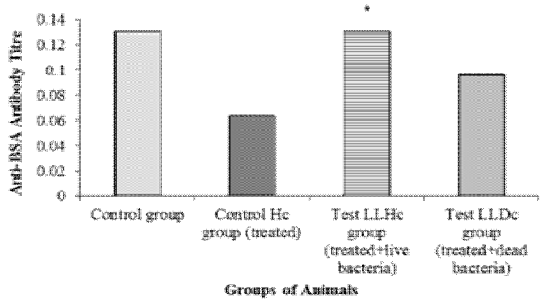


**Figure 6:** Effect of live and dead *L. acidophilus* feeding on Humoral immune response in immunosuppressed mice. *= p<0.05 vs. control Hc group.

## DISCUSSION

The present study shows that probiotic feeding in normal animals in live form does augment the immune response. The results revealed the enhancement in both cell-mediated and humoral immune response of the test animals fed on live and dead *L. acidophilus*. Our results are similar to those observed earlier by other authors.[12,13] The mechanism behind this could be that the probiotics in live form act as a part of normal flora and keep on boosting the immune response by activating the functions of T cells and T cell mediated B cells immunity as has been observed in our experiments by the results of NBT reduction, iNOS, bactericidal activity, DTH response and ELISA tests that the expression of T cells cytokines augmented as well as antibody titres were higher. The production of cytokines (IL-6, IL-10, and IFNγ) and increased $IgA^+$ cells was reported with the administration of *L. fermentum* PL9005 in mice.[14] Moreover in our

experiments dead bacteria also showed enhancement in immune response. This could be due to the cell wall, cell proliferation, bacterial production of bioactive metabolites such as antimicrobial peptides, $H_2O_2$, organic acids, exopolysaccharides etc. which are recognized by Toll-like receptors expressed in intestinal and immune cells as reported production of these beneficial metabolites.[15-17] Earlier Kato et al. reported that dead bacteria may act as adjuvants or immunoenhancers.[18]

In addition to this, our results showed that both the live and dead bacteria feeding in immune suppressed mice showed the restoration of chemically suppressed immune response. Recently, immune response stimulation by heat-killed *L. acidophilus* in mice challenged with *Salmonella typhimurium* has been reported by Lin et al.[19]

The demonstration that many probiotic effects are mediated through modification of the immune functions has been provided by investigations on immunologically intact animals or humans, with the exception of some studies on elderly subjects[20] and malnourished mice.[21,22] The present study shows, that a probiotic strain is able to exert stimulatory effects on the chemically damaged immune system of immune suppressed mice. Recently similar to our study Bujalance, et al., found improved immune response in cyclophosphamide treated mice was reported by live *L. plantarum.* We found *L. acidophilus* even in dead form when given to normal mice or immune suppressed mice, boost their immune function of the body, though to lesser extent than live bacteria.[23] Similar to our study heat-killed lactobacilli can induce some protection against candidiasis in mice were reported by Wagner.[24]

## CONCLUSION

It was concluded that both alive and dead probiotic potential bacteria may be given to boost the immune response and can be applied as immunotherapeutic agents in various infected or non-infected immunological disorders. Also in immune compromised host where live bacteria may give harmful effects dead bacteria may be given to boost immune response and as immunotherapeutic agents in immunological disorders or immune related diseases.

## REFERENCES


1. Shah NP, Functional cultures and health benefits, International Dairy Journal, 17(11), 2007, 1262-1277.
2. Das L, Bhaumik E, Raychaudhuri U, Chakraborty R, Role of Nurtraceuticals in human health, Journal of Food Science and Technology, 49(2), 2013, 179-183.
3. Ljungh A, Wadstrom T, Lactic acid bacteria as probiotics, Current Issues Intestinal Microbiology, 7(2), 2006, 73-89.
4. Cross ML, Microbes versus microbes: immune signals generated by probiotic lactobacilli and their role in protection against microbial pathogens, FEMS Immunology and Medical Microbiology, 34, 2002, 245-253.
5. Souza MM, Aguilar-Nascimento JE, Dock-Nascimento DB, Effects of budesonide and probiotics enemas on the systemic inflammatory response of rats with experimental colitis, Acta Cirurgica Brasileira, 22, 2007, 40-45.
6. Kundu N, Beaty TL, Jackson MJ, Fulton AM, Antimetastatic and antitumor activities of interleukin 10 in a murine model of breast cancer. Journal of the National Cancer Institute, 88, 1996, 536–541.
7. Labro MT, Interference of antibacterial agents with phagocyte functions: immunomodulation or "immuno-fairy tales"? *Clinical Microbiology Review,* 13, 2000, 615-650.
8. Jimenez-Valera M, Moreno E, Amat MA, Ruiz-Bravo A, Modification of mitogen-driven lymphoproliferation by ceftriaxone in normal and immunocompromised mice, International Journal of Antimicrobial Agents, 22, 2003, 607–612.
9. Stuehr DJ, Marletta MA, Synthesis of nitrite and nitrate in murine macrophage cell lines, Cancer Research, 47, 1987, 590-594.
10. Raghuramulu N, Nair KM, Kalayansundhram S, A Manual of Laboratory Techniques, Silver prints, ICMR, Hyderabad, India, 1983, 76.
11. Hudson L, Hay FC, *Handbook of Practical Immunology*, (III[rd] Ed.), Blackwell Scientific Publications, Oxford, England, 1989, 173.
12. Vinderola G, Perdigon G, Duarte J, Farnworth E, Mata C, Effects of the oral administration of the exopolysaccharide produced by *Lactobacillus kefiranofaciens* on the gut mucosal immunity, Cytokine, 36 (5-6), 2006, 254-260.
13. Seal M, Naito Y, Barreto R, Lorenzetti A, Safran P, Marotta FR, Experimental radiotherapy-induced enteritis: a probiotic interventional study, Journal of Digestive Diseases 8(3), 2007, 143-147.
14. Park J-H, Lee Y, Moon E, Immunoenhancing effects of a new probiotic strain, *Lactobacillus fermentum* PL9005, Journal Food Protection, 68(3), 2005, 571-576.
15. Tejada-Simon MV, Pestka JJ, Pro-inflammatory cytokine and nitric oxide induction in murine macrophages by cell wall and cytoplasmic extracts of lactic acid bacteria. Journal of Food Protection, 62(12), 1999, 1435-1544.
16. Stanton C, Ross RP, Fitzgerald GF, Sinderen DV, Fermented functional foods based on probiotics and their biogenic metabolites, Current Opinion Biotechnology, 16, 2005, 198-203.
17. Amrouchea T, Boutinb Y, Prioulta G, Fliss I, Effects of *Bifidobacterial* cytoplasm, cell wall and exopolysaccharide on mouse lymphocyte proliferation and cytokine production, International Dairy Journal, 16(1), 2006, 70–80.
18. Kato I, Endo K, Yokokura T, Effects of oral administration of *Lactobacillus casei* on antitumor responses induced by tumor resection in mice, International Journal of Immunopharmacology, 16, 1994, 29-36.
19. Lin WH, Yu B, Lin CK, Hwang WZ, Tsen HY, Immune effect of heat-killed multistrain of *Lactobacillus acidophilus* against *Salmonella typhimurium* invasion to mice, Journal of Applied Microbiology, 102(1), 2007, 22-31.

20. Gill HS, Rutherfurd KJ, Viability and dose-response studies on the effects of the immunoenhancing lactic acid bacterium *Lactobacillus rhamnosus* in mice, British Journal of Nutrition, 86, 2001, 285-289.

21. Cano PG, Perdigon G, Probiotics induce resistance to enteropathogens in a re-nourished mouse model, Journal of Dairy Research, 70, 2013, 433–440.

22. Hidemura A, Saito H, Fukatsu K, Matsuda T, Kitayama J, Ikeda S, Kang W, Nagawa H, Oral administration of *Bifidobacterium longum* culture condensate in a diet-restricted murine peritonitis model enhances polymorphonuclear neutrophil recruitment into the local inflammatory site, Nutrition, 19(3), 2003, 270–274.

23. Bujalance C, Moreno E, Jimenez-Valera M, Ruiz-Bravo A, A probiotic strain of *Lactobacillus plantarum* stimulates lymphocyte responses in immunologically intact and immunocompromised mice. International Journal of Food Microbiology, 113(1), 2007, 28-34.

24. Wagner RD, Pierson C, Warner T, Dohnalek M, Hilty M, Balish E, Probiotic effects of feeding heat-killed *Lactobacillus acidophilus* and *Lactobacillus casei* to *Candida albicans*-colonized immunodeficient mice, Journal of Food Protection, 63(5), 2000, 638–644.

**Source of Support:** Nil, **Conflict of Interest:** None.