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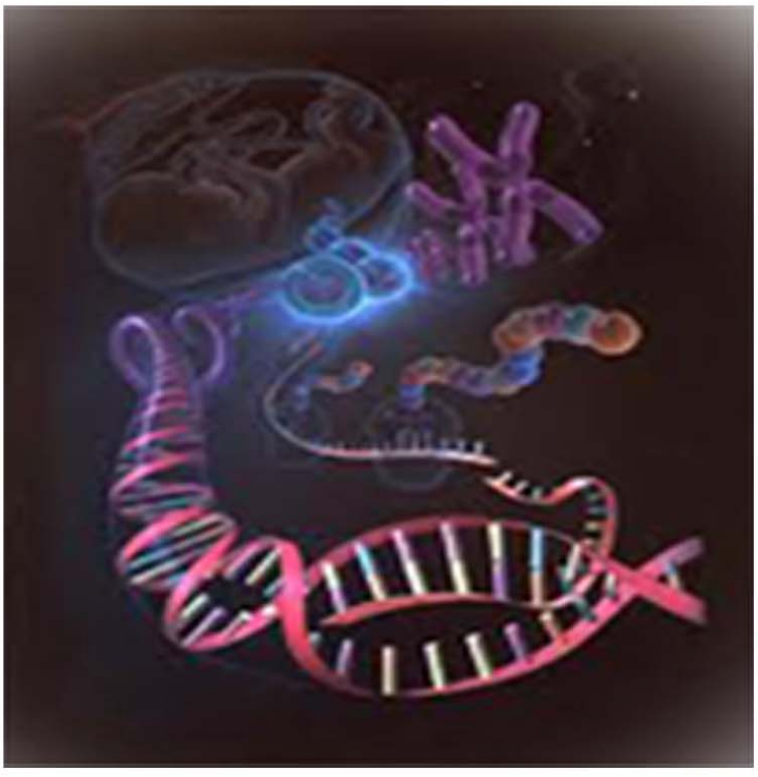

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Research Paper

COMPARISON OF THE IMMUNOMODULATORY PROPERTIES OF FOUR PROBIOTIC STRAINS OF LACTOBACILLUS: PREDICTION FOR *IN VIVO* EFFICACY

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Probiotics, microorganisms that have a favorable influence on physiological and pathological processes of the host by their effect on the intestinal flora, may play a role in improving human health. One of the putative effects is the modulation of immune function. The present study was carried out to evaluate the immune modulatory potential of four strains of *Lactobacillus acidophilus* in swiss albino mice. Immune response was assessed by various methods as the development of antibody titers, the delayed type hypersensitivity test, the nitroblue tetrazolium reduction test, inducible nitric oxide synthase test and the phagocytic activity test. The results showed that immunomodulation by *L. acidophilus* is strain specific and it is asserted that orally supplemented *L. acidophilus* could exert an indirect effect on T-lymphocyte activity through stimulation of other cell types, such as phagocytes.

Keywords: Immune modulation, Probiotics, *Lactobacillus*

INTRODUCTION

Immune modulation by dietary bacteria has continued to be a subject of growing interest. Probiotics can be defined as living microorganisms that upon ingestion in certain numbers exert health effects beyond inherent basic nutrition (Guarner et al.,1998). Naidu et al. 1999 described probiotics as microbial dietary adjuvants that beneficially affect the host's physiology by modulating their mucosal and systemic immunity as well as improving the nutritional and microbial balance in their intestinal tracts. Probiotics have profound effects on potentiating both arms of immune responses i.e., cell mediated immunity and humoral immunity.

Microbes from many different genera are being used as probiotics. Recently, there has been much interest in Lactic acid bacteria due to their 'Generally Recognized As Safe and beneficial effects' (GRAS) in health. The most commonly used Lactic acid bacteria are; *lactobacilli, enterococci and bifidobacteria*. In particular

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lactobacilli form a source of potential modulators of the immune system. This may have historical reasons since Metchnikoff (1908) proposed that the *lactobacilli* present in yoghurt would have a health promoting effect. It has been demonstrated that specific *lactobacillus* strains can modulate host immunity, which positively correlates with enhanced resistance to various viral and bacterial infections (Kwon 2010; Bhatia et al., 2008; Matsuzaki 2000). Moreover, the beneficial effects of the probiotic strains vary not only at the species level, but at the strain level too. Hence, the main objective of this work was to study the immunomodulatory potential of orally induced four strains of *Lactobacillus acidophilus in vivo*.

MATERIALS AND METHODS

Strains of Microorganisms: Strains of *L. acidophilus LA 5, L. acidophilus Russian Strain C, L. acidophilus Strain R, L. acidophilus Chr. Hansen Lab Denmark (C H L Den)* were procured from the NDRI (National Dairy Research Institute), Karnal, Haryana. The cultures which were so obtained were given two revival cycles in deMan-Rogosa- Sharpe broth (MRS) at 37^0C. The bacterial cultures were grown and maintained for further use.

Animal groups and feeding: Swiss albino male mice (weighing 18-22 gm, aged 4-6 weeks) which were maintained on a standard laboratory diet (Kisan feeds Ltd., Mumbai, India) and water ad libitum were employed in the present study. After one week of acclimatization, the mice were divided into respective groups, each containing 6 animals, were housed individually in the departmental animal house and were exposed to 12 hr cycles of light and darkness. The mice were divided into 6 groups: Group- I: Control group (not subjected to any treatment i.e. kept only on normal diet); Group- II: Antigen sensitized control [mice sensitized with sheep red blood cells (SRBC) and kept on normal diet]; Group III: Normal diet fed + *L. acidophilus LA 5;* Group- IV: Normal diet fed + *L. acidophilus Strain R;* Group- V: Normal diet fed + *L. acidophilus C H L Den;* Group- VI: Normal diet fed + *L. acidophilus Russian Strain C.* The animals received an oral dose of 100 μl of either *L. acidophilus LA5, L. acidophilus Strain R, L. acidophilus Russian Strain C*, or *L. acidophilus C H L Den* for 18 days consecutively.

Immunization: Sheep blood was collected in Alsever's solution in the ratio 1:2 and was centrifuged at 400 × g for 10 minutes at 4^0C. The erythrocyte pellet was washed and suspended in PBS (0.1 M, pH 7.2) for further use (Alsever et al. 1941). All mice were antigenically challenged intraperitoneally with a single dose (100μl/ml of 1×10^7 cells/ml) of sheep red blood cells (SRBC).

IMMUNOLOGICAL TESTS EMPLOYED

Humoral Immune Response by Haemagglutinating Antibody (HA) Titer: To assess the humoral immune response, blood was withdrawn from the retro-orbital plexus of all SRBC antigenically challenged animals on day 0 (pre-immunized), 8 and 13 (post-immunization). The serum was separated and assayed by direct haemagglutination (Haghighi et al., 2005). Titer was described as the highest dilution which was capable of visible agglutination.

CELL MEDIATED IMMUNE RESPONSE

Delayed Type of Hypersensitivity (DTH): The delayed type of hypersensitivity was assessed by the footpad swelling method given by Hudson

and Hay 1989. The swelling in footpad was noticed after 24-48 hrs in both the foot pads after carrageenan induced in the left foot pad and normal saline in the right hind foot pad. The swelling in the foot was measured with micro-caliper at 0, 24 and 48 h after the challenge. The difference in paw thickness as compared to control was taken as a measure of DTH. Net Swelling = (T 24/48 - T0) - (C 24/48 - C0); where, T 24/48- footpad thickness 24 and 48 h after carregenan challenge (left foot), T0 - footpad thickness before carregenan challenge (left foot), C 24/48 - footpad thickness 24 and 48 h after normal saline challenge (right foot) and C0 - footpad thickness before normal saline challenge (right foot).

Total Lymphocyte Isolation from the Spleen: The spleen was excised aseptically and the lymphocytes were isolated by teasing the tissue. The cells were centrifuged (400 × g for 10 min at 4^0C) and lysed by using the ACK lyses solution (0.5 M NH_4Cl, 10mM $KHCO_3$ and 0.1 mM disodium EDTA, pH 7.2). The lymphocytes which were obtained, were washed thrice in PBS, counted and adjusted to the desired concentration in RPMI for further use.

Nitroblue Tetrazolium Reduction Test (NBT): NBT reduction test, a measure of burst in the leucocytes was carried out by method given in the practical book by Hudson & Hay 1989. Briefly the splenocytes suspension was incubated with NBT and the formazon which was formed, was extracted in dioxan. The reduction in NBT was measured spectrophotometrically at 520nm (Shimadzu, UV-1650 PC) against dioxan as blank. Percentage NBT reduction was calculated as (O.D. of test – O.D. of control) × 100 / (O.D. of control).

inducible Nitric Oxide Synthase (iNOS) Activity: The iNOS activity of leukocytes was assessed spectrophotometrically by using arginine by employing the method mentioned by Mishra 2010. Briefly the splenocytes were incubated with arginine at 37^0C for 24 hours in CO_2 chamber. The citruline formation from arginine was read by taking O.D. at 540nm using MEM + GRIESS reagent (1:1) as blank.

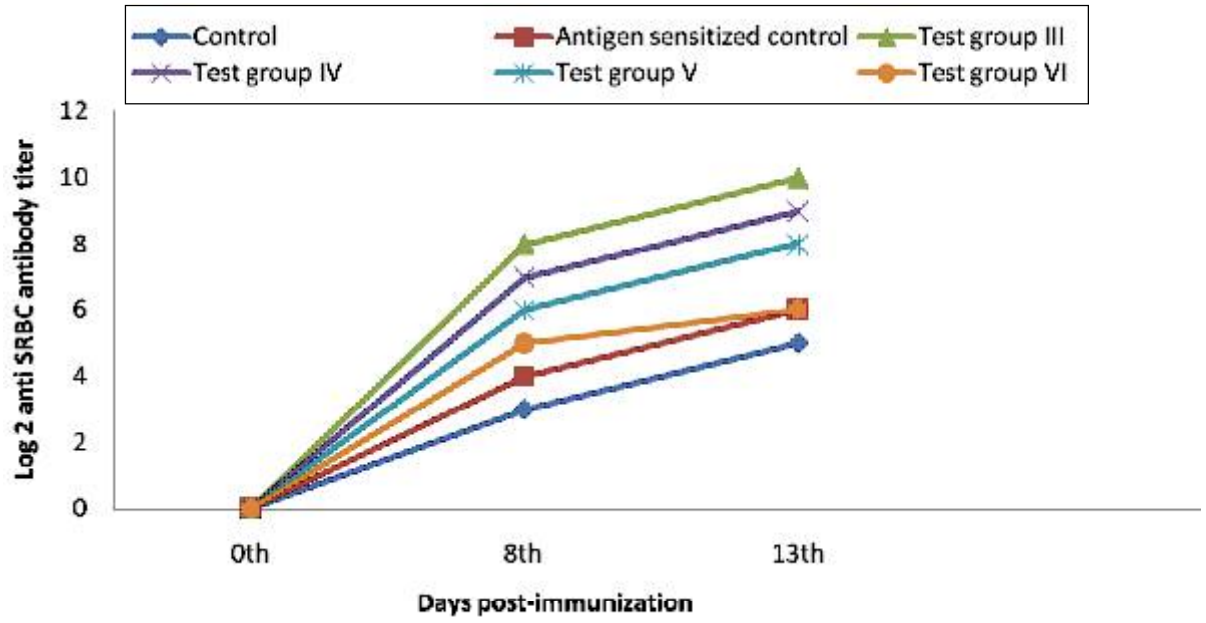
Phagocytic Activity: The phagocytic function was assessed by phagocytosis of microorganism using the method given by Raghuramulu, 1983. In short lymphocytes and *E.coli* were incubated for 1 hour at 37^0C followed by the suspension plated on the nutrient agar plate at 37^0C and checking the growth of bacteria. After 24 hours colonies were counted on each plate. Only *E.coli*. spread plate was taken as control. Percentage bactericidal activity = (cfu/ml in control – cfu/ml in test) × 100 / (cfu/ml in control)

Statistical Analysis: All the results were expressed as mean ± S.E.M. The data of the tests were statistically analyzed by using one – way ANOVA, followed by Turkey's Multiple Range Test which was applied for post hoc analysis. The data was considered to be statistically significant if the probability had a value of 0.05 or less.

RESULTS

Humoral Immune Response by Haemagglutinating Antibody (HA) Titer: Group III (*L. acidophilus LA* 5) and group IV (*L. acidophilus Strain R)* had a significantly higher antibody titer as compared to other groups I (untreated control), II (Antigen sensitized control), V (*L. acidophilus C H L Den*) and VI (*L. acidophilus Russian strain C*) (Figure 1). The antibody titer of group III and IV was double than group VI.

Figure 1: Effect of Effect of Different Groups of *Lactobacillus* on Production of Anti-SRBC Antibody Titer on Pre-Immunization (0th Day) and Post Immunization 8th and 13th Day)



CELL MEDIATED IMMUNE RESPONSE

Delayed Type of Hypersensitivity (DTH): The T-cell response was studied by using the delayed type hypersensitivity test. In the untreated control group I, no rise in the footpad thickness was observed. However, group III and group IV showed a significant ($p<0.05$) elicitation of the T-cell response, as was evident by an increase in the footpad thickness as compared to those in the antigen sensitized control group II and for group V and VI. The maximum effect was observed after 48 hrs. in all the groups (Table 1).

Nitroblue Tetrazolium Reduction Test (NBT): The NBT reduction test is an indirect marker of the oxygen dependent bactericidal activity of the phagocytes and the metabolic activity of the granulocytes or the monocytes. All the strains of *L.* acidophilus significantly increased the NBT reduction as compared to control group I (Figure 2). Group III and IV showed maximum NBT reduction, group V, and group VI showed moderate and control group I and II showed minimum NBT reduction.

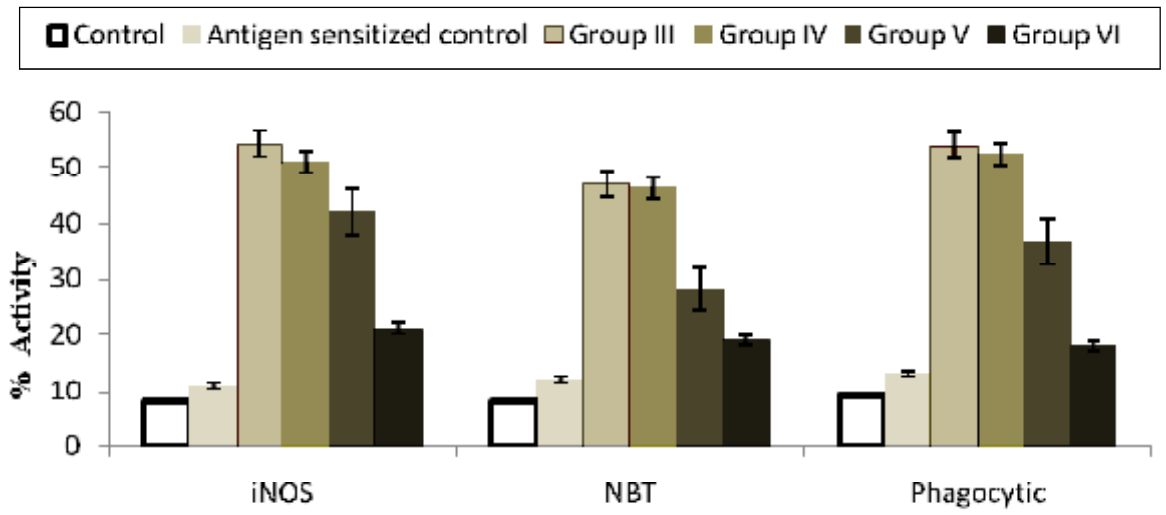
inducible Nitric Oxide Synthase (iNOS) activity: Figure 2 indicates the *Lactobacilli* modulated cell mediated immune response. *Lactobacilli* significantly increased the iNOS activity as compared to the controls .Group III and IV showed comparatively same iNOS activity and for group III, the iNOS activity was found to be 13% higher than that of Group V and 39% higher than group VI.

Phagocytic Activity: The effect of the *Lactobacilli* on phagocytic activity was studied in terms of the number of colony forming units (CFU). The treatment of the animals with *L. acidophilus LA5, L. acidophilus strain R* reduced the number of colonies and thus enhanced phagocytic activity as compared to *L. acidophilus C H L Den, L. acidophilus Russian strain* and control group I and II (Figure 2).

Table 1: Delayed Type of Hypersensitivity (DTH)

Animal groups	Footpad Thickness (mm)			
	Time Period (hrs) After Carrageenan Challenge			
	0	24	48	72
Control1.67±0.01	1.67±0.01	1.67±0.01	1.67±0.01	
Antigen sensitized control	1.67±0.01	1.70±0.01	1.71±0.02	1.67±0.01
Test group III	1.69±0.01	1.75±0.01	1.93±0.02	1.74±0.03
Test group IV	1.68±0.01	1.73±0.01	1.80±0.01	1.72±0.02
Test group V	1.67±0.01	1.72±0.01	1.76±0.01	1.71±0.01
Test group VI	1.67±0.01	1.72±0.01	1.74±0.01	1.68±0.03

Figure 2: Influence of *Lactobacillus* on iNOS Activity NBT Reduction and Phagocytic Activity



DISCUSSION

In the present study, the effect of four strains of *L. acidophilus* on the immune response was studied by the direct haemagglutination test, the delayed type hypersensitivity test, the nitroblue tetrazolium reduction test, the inducible nitric oxide synthase test and the phagocytic activity test. Out of the four strains of *L. acidophilus* studied, it was found that *L. acidophilus LA* 5 shows the maximum and *L. acidophilus Russian strain* shows the minimum immunomodulation in mice as compared to *L. acidophilus Strain R* and *L. acidophilus C H L Den.* It is found significant from the results that immunomodulatory effects are strain specific.

Probiotic bacteria have been shown to influence immune responses by enhancing phagocytosis of pathogens as well as modifying cytokine production (Jennifer et al., 2010; Megan *et al*., 2010). In one study, a strain of *L. acidophilus* isolated from human newborn was inoculated into germ free and conventional mice

and phagocytosis of *E. coli* was assessed *in vivo* (Newmann et al., 1998). The monoassociation of germ free mice with these lactic acid bacteria for 7 days improved macrophage phagocytosis capacity as demonstrated by the clearance of *E. coli* inoculated intravenously and similar to our previous studies (Randhawa et al., 2011; Bhatia et al., 2007; Pawan et al., 2007) which observed immunomodulation by different probiotics such as *L. Delbrueckii, L. casei, Streptococcus thermophilus*. The present work done agreed that *Lactobacillus* not only shows immunomodulatory activity but also showed that the immunomodulatory effect varies from strain to strain i.e. strain specific.

From these studies, and based on the described immune cell populations involved in DTH responses, it was asserted that orally supplemented *L. acidophilus* could exert a direct effect on T- helper-1-cell immunity by modulating the amount and activity of antigen-specific T lymphocytes and /or could exert an indirect effect on T-lymphocyte activity through stimulation of other cell types, such as phagocytes. The results suggest that *L. acidophilus* has a genuine effect on Th-1-cell mediated immunological response. Many other studies with mice increasingly confirmed the involvement of T memory cells in the enhanced DTH response after *Lactobacillus* application (Warrd et al., 2003).

CONCLUSION

The immune system can be optimized through oral supplementation of specific *L. acidophilus*. From these results it was suggested that oral administration of *L.acidophuilus* leads to a prominent state of humoral immunity and cellular innate immunity via phagocyte activation, which subsequently enhances Th 1 cell activity. Still, the nature of the cells, in terms of cytokine profiles and cell surface markers, in the observed effects remains to be established.

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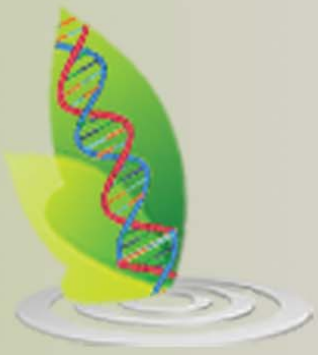

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