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RESEARCH ARTICLE

ENHANCEMENT OF IMMUNE RESPONSES BY PROBIOTIC PROPERTIES OF *LACTOBACILLUS ACIDOPHILUS* NCDC 195

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ABSTRACT

Probiotics are microbial food supplements with beneficial effects on human health. Along with the dietary supplement, probiotics also have immunomodulatory properties. Present study was carried out using the *in-vivo* utilization of albino mice for the study of immunomodulatory effect of *Lactobacillus acidophilus* NCDC 195. To estimate the increase immune responses **Nitroblue Tetrazolium reduction test**, **(Inducible Nitric Oxide synthase) test** and **Phagocytosis** (% age) have been studied. Results were compared with the control value and. It was observed that feeding the Swiss albino mice for 15 days with Probiotic dosage (Yogurt), causes **1.98** times increase in Nitroblue Tetrazolium reduction test as compared to control, whereas Inducible Nitric Oxide synthase and phagocytosis shows **1.71** and **1.82** times increase in immune responses respectively. **Cell mediated immune** response was also calculated, where there was increase of **1.04**, **1.07** and **1.02** times in **Delayed Type Hypersensitivity** (DTH) as compared to control was observed.

Keywords: Immunomodulation, Nitroblue Tetrazolium (NBT) reduction, Probiotic, Phagocytosis, Cell mediated immune response, Inducible Nitric Oxide synthetase.

INTRODUCTION

Probiotics were defined as "microbially derived factors that stimulate the growth of other microorganisms". Yogurt is one of the most widely used source of Probiotics. Traditionally made Yogurt is one of the good source of Probiotic consortium. It contains *Lactobacilli, Streptococci, Enterococci and Lactococci*. *Bifidobacteria, Bacillus* species and yeasts like *Saccharomyces* too find a place in the long list of Probiotics. They are normal inhabitants of human intestine and colon. Most of the Probiotic bacteria fall in *lactobacilli* group. Lactic Acid Bacteria (LAB), a group of Gram +ve bacteria, consists of several species including the genera *Lactobacillus, Lactococcus, Leuconostac, Pediococcus, Aerococcus* and *Bifidiobacterium*. With the LAB, the genus *Lactobacillus* is the most widely encountered for probiotics. Certain strains of LAB and *Bifidobacteria* are able to induce tumor necrosis factor–α (TNF-α) and interleukin-6 (IL-6) as well as to stimulate other nonspecific immune system (Isolauri *et al.*, 1998)[1]. Microbial probiotics (especially those of LAB) can influence the systemic immune system in various ways [2-3] (Perdigon *et al.*, 1995; Fang *et al.*, 2000). Perdigon[4] *et al.*, 1999 proposed that *Lactobacilli* can directly stimulate the gut immune system *via* localized gastrointestinal (GI) tract lymphoid cell. Perdigon[5] *et al.* (2000) and Valeur[6] *et al.* (2004) examined the local colonization of human gastrointestinal tract after dietary supplementation to determine subsequent response and found increase in local antibody levels, macrophages and number of natural killer cells. Oral ingestion of LAB by rats increases lymphocyte proliferation and Interferon production (Aattouri *et al.*, 2002)[7]. Gut microflora participates in immune exclusion. It prevents other bacteria from adhering by competition for nutrients and places of adhesion, it produces anti-bacterial agents, and it stimulates the production of specific antibodies (Bengtmark and Jeppsson, 1995)[8]. The gastrointestinal tract serves as an interface between the gut and immune system, with the intestinal lining functioning as a barrier, *Lactobacilli* colonization decreases the passage of bacteria from the gut into the bloodstream. *Lactobacilli*, can preferentially occupy a space or form a biofilm on the surface of intestinal lining, that would otherwise be colonized by a pathogen. Thus LAB induces a competitive environment, and follows survival of fittest. So in order to investigate the beneficial effect of Probiotics using experimental animals (*In-vivo* stuides), present study was carried out to determine their effect on immune system of test organism (Albino mice).

MATERIALS AND METHODS

Procurement and revival of microbial Culture:

The strain of *Lactobacillus acidophilus* NCDC 195 was procured in lyophilized form from National Dairy Research Institute (NDRI), Karnal and maintained in the laboratory. Culture was revived by sub-culturing 4-6 times. Strains were activated first on skim milk and then in MRS broth at 37°C for 24h. All strains were checked for specificity by microbiological analysis i.e. Gram staining and negative staining (Nigrosine). After activation, these were maintained on MRS agar slants and were kept under refrigerated condition and were sub-cultured after 10 days from the stock cultures.

Animal for in-vivo studies

Swiss albino mice employed for *in-vivo* study were procured from Central Research Institute (CRI), Kasauli. Each animal weighed 18-20gm. They were housed for acclimatization one week before the experiment

in the animal house of institute. They were maintained on standard diet with free access to water.

Treatment Groups

Group I (Untreated control group):

No treatments were given to mice of this group. They were fed on normal diet for entire period of research i.e. 15 days.

Group II (Control immunized group):

Mice were subjected to immunization with 200µl BSA (1%) on every 5th day (starting from 0 day) and were fed on normal diet.

Group III (Treated group):

The animals were administered with prepared *L. acidophilus* NCDC 195 dosages (inoculated in skim milk/yogurt) for 15 consecutive days.

Immunization

200µl BSA (1%) solution was prepared. All mice were antigenically challenged with a single dose intraperitoneally (ip) after every 5 days.

All the groups were treated and maintained under same atmosphere and all the subgroups were immunized on every 5th day of treatment starting from 0 day with a single dose of BSA intraperitoneally (ip).

Preparation of probiotic dose (inoculum)

Probiotic cultures were inoculated in autoclaved skim milk and incubated at 37°C for 24h. After 24h, final inoculum was made 10^9 cells/ml. These inoculums were then transferred to small autoclaved vials in sterilized conditions under laminar air flow. The Probiotic doses in the form of Probiotic yogurt were then stored in refrigeration at 4°C.

Nitroblue Tetrazolium (NBT) reduction test

NBT reduction test was measured by the method of Hudson and Hay (1989)[9]. For each test sample, two set of test tubes were taken one as control as other as test. 100µl splenocytes were taken in both control and test set. Then 900µl of MEM was added into both test and control sets. Then incubated at 37^{o}C for 20 minutes. After incubation samples were centrifuged at 4000 rpm for 3 minutes. The pellet was taken and 100µl of PBS added in each test tube. 0.5 ml of dioxane was added in each test tube. Test tubes were incubated at 70^{o}C for 20 minutes. O.D. was taken at 520 nm (Shimadzu, UV-1650 PC). The results are expressed as mean ± S.E.M. of percentage dye reduced to formazon using dioxane as standard.

Calculations

$$\text{Percent NBT Reduction} = \frac{O.D.of\ Test\ (T) - O.D.of\ Control\ (C)}{O.D.of\ control\ (C)} \times 100$$

iNOS (Inducible Nitric Oxide synthase) test

The inducible Nitric oxide synthase activity was assayed in lymphocyte suspension according to Stuehr and Marletta (1985)[10] with certain modifications. The lymphocyte suspension was incubated for 2h at 37ºC, 5% CO_2, 98% humidity. Arginine was added to the suspension and further incubated for 24h under similar conditions. The cells were then treated with Griess reagent. The purple color developed (indicating presence of citrulline) was measured spectrophotometrically at 540 nm against Griess reagent as blank and the results are expressed as percentage enzyme produced.

Phagocytosis or Bactericidal activity

Phagocytosis by immunocytes was measured by Raghuramulu *et al.* (1983)[11]. Bactericidal activity was determined as described by Raghuramulu *et al.* (1983)[11]. The lymphocyte suspension was mixed with bacterial suspension (*E. coli*) in the ratio of 1:2 and incubated at 37°C for 60min. 100µl of sterile distilled water was added to lyse the lymphocytes. Suspension so formed (100µl) was spread on nutrient agar plates. The plates were incubated at 37°C for 24h. Bacterial cell suspension was spread in the control plate. Number of colony forming units (CFU) developed in control and test plates were counted and the results are expressed as percentage bactericidal activity.

Cell-mediated immune response (Delayed type hypersensitivity; DTH)

Delayed type hypersensitivity response (DTH) was monitored as described by Titus and Chiller, (1981)[12]. All BSA treated groups were challenged intradermally on day 15 with 200µl BSA (1%) in the hind foot-pad. The control lateral paw was given an equal volume of saline. Paw thickness was measured with micro caliper at 24h interval upto 72h. The difference in paw thickness compared to control was taken as a measure of DTH and expressed in mm. Results are expressed as footpad thickness in mm up to 72h.

The swelling was calculated according to following equation:

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

Where

$T_{24/48}$ = Footpad thickness 24 and 48h after bacterial suspension challenge (left foot),

T_0 = Footpad thickness before bacterial suspension challenge (left foot),

$C_{24/48}$ = Footpad thickness 24 and 48h after normal saline challenge (right foot),

C_0 = Footpad thickness before normal saline challenge (right foot).

RESULTS

Probiotics posses Immunomodulatory properties as *L. acidophilus* has a significant effect on the intestine colonized organisms and immune system of an organism. 15 days feeding of *L. acidophilus* to swiss albino enhances the immune responses significantly.

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. The results of Probiotic consumption on NBT reduction are shown in **Table. 1.1.**

Table 1.1: Effect of Lactobacillus acidophilus NCDC 195 on NBT reduction values

Treatment Name	NBT reduction (%)	Increase in NBT reduction
Untreated Control	30[a]	-
BSA Treated	42[b]	1.4 (↑)
NCDC 195	59.40[c]	1.98 (↑)

The figure in parenthesis ( ) shows number of times increase (↑) or decrease (↓) in % NBT reduction activity as compared to control. The values denoted by different small letters in column differ significantly ($p<0.05$)

There was a significant increase ($p \leq 0.5$) of NBT reduction i.e. 1.98 times, in *L. acidophilus* NCDC 195 treated group as compared to the control group, whereas 1.42 times increase in the NBT reduction was found in the BSA treated control group as compared to the untreated control group.

iNOS has been shown to play an important role in modulating macrophage cellular function during inflammation, and its level increases after stimulation with cytokines such as IL-1, TNF-α, and interferon-γ (Nathan, 1992 and Alican *et al.,* 1996)[13-14]. iNOS activity was analyzed in control and tissue extracts before and after probiotic treatment by measuring arginine to citrulline conversion. The results of iNOS activity in control and treated group are listed in **Table 1.2** as shown below.

Table 1.2: Effect of *Lactobacillus acidophilus* NCDC 195 on iNOS Activity values

Treatment Name	iNOS Activity (%)	Increase in iNOS activity
Untreated Control	24.65[a]	-
BSA Treated	23.32[a]	1.0 (↓)
NCDC 195	42.30[b]	1.71(↑)

The figure in parenthesis ( ) shows number of times increase (↑) or decrease (↓) in % iNOS activity as compared to control. The values denoted by different small letters in column differ significantly ($p<0.05$).

The result shows an increase of 1.71 times in iNOS activity in *L. acidophilus* NCDC 195 treated group as compared to the control group. The results are in agreement with results as studied by Ulisse *et al.* (2001)[15]. The iNOS activity is correlated to the bactericidal activity of the macrophages and has been documented as a measure of the immunomodulatory potential (Park *et al.,* 2001)[16].

These results for iNOS and NBT corroborate the earlier report by Pawan & Bhatia (2007)[17], who observed the increase in the iNOS and NBT activity of macrophages, when the test organism were administered to yogurt.

Phagocytosis activity was observed to be 1.82 times in *L. acidophilus* NCDC 195 treated group as compared to the control group as shown in **Table 1.3**.

Table 1.3: Effect of *Lactobacillus acidophilus* NCDC 195 on Phagocytosis Activity values

Treatment Name	Phagocytosis Activity (%)	Increase in iNOS activity
Untreated Control	35[a]	-
BSA Treated	51[b]	1.4 (↑)
NCDC 195	63.70[c]	1.82 (↑)

The figure in parenthesis ( ) shows number of times increase (↑) or decrease (↓) in % phagocytosis activity as compared to control. The values denoted by different small letters in column differ significantly ($p<0.05$)

These results support the observations as observed by Perdigon *et al.* (1988)[18], that *L. acidophilus* and *L. casei* performed a systemic immunostimulation by increasing the phagocytosis capacity of murine peritoneal macrophages. Perdigon et al. (1986)[19] and Gill *et al.* (1999)[20] also observed the immune-enhancing response due to increased non-specific phagocytic activity against bacteria. Donnet-Hughes *et al,* (1999)[21] and Schiffrin *et al.* (1997)[22], observed the increase in the phagocytosis capacity of leucocytes isolated from the blood of humans who had consumed Probiotics (*Lactobacillus* La1), which is consistent with the adhesion potential of this bacterium.

The *L. acidophilus* NCDC 195 treated group also showed an enhanced DTH response. The results are shown in **Table 1.4**.

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Table 1.4: Effect of *Lactobacillus acidophilus* NCDC 195 on Delayed Type Hypersensitivity

(Time)	Untreated Control (mm)	BSA immunized control (mm)	10^9 cells/ ml (*L. acidophilus* NCDC 195)
0 h	1.67[a]	1.68 [a]	**1.68 [a] (1.00↑)**
24 h	1.67[a]	1.72[b]	**1.75[b] (1.04↑)**
48 h	1.68[a]	1.71[b]	**1.80[c] (1.07↑)**
72 h	**1.67[a]**	**1.67 [a]**	**1.71[b] (1.02↑)**

The figure in parenthesis ( ) shows number of times increase (↑) in DTH response as compared to control. The values denoted by different small letters in row differ significantly ($p<0.05$).

There was an increase of 1.04, 1.07 and 1.02 times in DTH as compared to control. The increase in footpad thickness is also shown graphically in **Figure 1.1**.

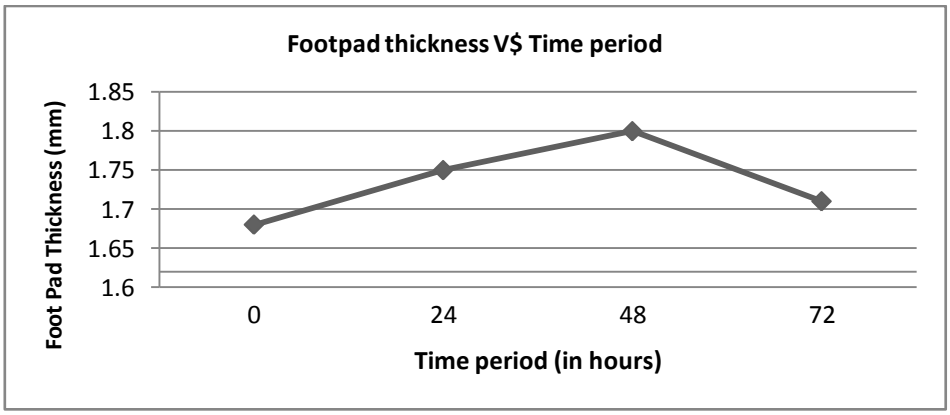

Figure 1.1: Effect of *Lactobacillus acidophilus* NCDC 195 on Foot pad thickness (Cell Mediate Immune Response)

The results corroborates the studies as conducted by Shu *et al.* (2000)[23], who, observes that dietary consumption of probiotic LAB has the potential to offer significant immune-mediate improvement in health.

There is remarkable increase in the % Immunomodulatory activity i.e. Nitroblue Tetrazolium (NBT) reduction (NBT), Inducible Nitric Oxide synthetase Activity (iNOS), Phagocytosis and Delayed Type Hypersensitivity i.e. Cell mediate immune response which suggests the potent immune enhancement effect of *L. acidophilus*.

DISCUSSION:

Probiotics posses Immunomodulatory properties, as Lactobacillus acidophilus has significant effect on the intestine colonized organisms and immune system of an organism. Only 15 days feeding of L. acidophilus to swiss albino mice enhances the immune responses significantly. There is remarkable increase in the Immunomodulatory activity i.e. Nitroblue Tetrazolium reduction (NBT), inducible Nitric Oxide synthetase (iNOS) activity, Phagocytosis and Delayed Type Hypersensitivity i.e. cell mediate immune response, which suggest the potent immune enhancement effect of *L.acidophilus.*

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