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# IMMUNORESTORER ACTIVITY OF PROBIOTIC BACTERIAL DNA: AN *IN VIVO* STUDY

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## ABSTRACT

The increase in the incidence of infectious diseases or immunological disorders raises a need to find new immunomodulators. Probiotics have been shown to be one of the alternative agents which strengthen the immune response of the body. But recently, bacterial DNA is also being explored as an immune enhancer. Present study was conducted to compare the *in vivo* immunomodulating capacity of probiotic strain as live bacteria with their genomic DNA. Swiss albino mice were made immunosuppressed by giving them hydrocortisone (HC) on day 1$^{st}$ and 4$^{th}$ (i.p.) i.e. 5 mg/kg body weight. Probiotic bacteria's ($10^9$ cells $ml^{-1}$) were administered orally whereas their extracted DNA's (75 µg $mL^{-1}$) were injected into the tibialis anterior muscle in 3 doses over a span of 17 days. The animals were sacrificed after the completion of experimental period i.e. 17 days. Immune status of the treated animals was assessed by employing the tests for Humoral Immune Response and Cell Mediated Immune Response as Delayed Type Hypersensitivity, Nitroblue Tetrazolium Reduction test, Inducible Nitric Oxide Synthase and Bactericidal activity was studied in SRBC immunized mice. Levamisole ($25mgkg^{-1}$) was used as the standard drug. Overall, these results demonstrated that a substantial augmentation in immune efficacy was observed in the animals receiving genomic DNA over the group

receiving viable bacteria. It is concluded that genomic DNA of probiotics should be exploited as a potent immune enhancer and as a biotherapeutic agent.

**KEYWORDS:** *Lactobacillus, Bifidobacterium,* Immunomodulatory activity, Bacterial DNA, Humoral Immune Response, Cell Mediated Immune Response.

## INTRODUCTION

Immunosuppression is a reduction of the activation or efficacy of the immune system. Probiotics are considered to be living microorganisms which when administered in adequate amount leads to health benefits.[1] Probiotics are generally regarded as immunomodulators, which stimulate the innate, cellular and humoral immune response. There are different mechanism by which probiotics directly enhance the immune response[2,3,4] such as they mediate the immune effect by secreation of soluble peptides, increases the production of NO and interleukin (IL)-1β ,IL-6,IL-12 and TNF-α.[8] Recently, some bacterial cell components such as peptidoglycans, lipoteichoic acid, secrete soluble substances[9,10] and genomic DNA[11] reportedly play role in immunomodulation responses but primary component is yet to be identified.

The immunostimulatory effect by bacterial DNA were defined to be dependent upon short sequences of CpG dinucleotides which differ from that found in eukaryotic DNA.[8,9] Unmethylated CpG motifs are found in bacterial DNA. In eukaryotic DNA, CpG-containing sequences occur at a much lower frequency than in bacterial DNA[11,12] and they appear to be under represented in eukaryotic genomes; a phenomenon known as "CpG suppression" and when it is present, the cytosine is methylated[13],which prevents their immune stimulatory effects.[14,8]

It has been evidenced that Bacterial DNA and immunostimulatory CpG-ODNs activate Antigen Presenting Cells (APCs) such as macrophages and dendritic cells. Cell activation occurs upon DNA endosomal uptake and within minutes it results in activation of the Stress Kinase pathway and NF-kB. As a consequence, APCs produce cytokines including IL-12, IL-6 and IL-1 and upregulate coreceptor molecules.[15] The purpose of current study was to compare *in vivo,* immunorestorer activity of probiotic viable bacteria's with their isolated genomic DNA.

## MATERIAL AND METHODS

**2.1 Bacterial strain and culture condition:** The strain of *Lactobacillus delbrueckii* 405 (LB 405) was procured from National Dairy Research Institute, Karnal, Haryana. The cultures so obtained were given two revival cyclesin de Man–Rogosa–Sharpe broth (MRS broth) at 37 °C. Bacterial cultures were grown and maintained for further use. For genomic DNA preparation, cells were grown in the corresponding medium containing 1 to 1.5 % glycine to facilitate cell lysis.[16]

**2.2 Preparation of genomic DNA of bacterial strain:** Genomic DNA was isolated and purified with several modifications.[16] Briefly, an overnight culture (1.5 ml) was pelleted at 14000 rev $min^{-1}$ (microcentrifuge) 25°C for 5 minutes and resuspended in 500µL EDTA ($50mM^{-1}$). 100 µL of $30mgml^{-1}$ Lyosozyme was added to cell suspension and incubated for 60 minutes at 37°C. Cell lysis was achieved using NaOH/SDS solution (pH 12.5) and incubation 20 min at 37°C followed by 10 min incubation on ice. Protein removal was carried out with phenol followed by chloroform: isoamyl alcohol (24:1) extraction. DNA was precipitated by addition of isopropanol and washed with 70% ethanol to remove residual contamination. DNA was then resuspended in 20-30 µL of TE (Tris 10mM, EDTA 1mM pH 8.0). The concentration and purity of DNA were analyzed spectrophotometricaly (Shimadzu, UV-1650 PC spectrometer) by measuring OD260/OD280. Only the DNA with OD260/OD280 ratio ranging between 1.8 and 2.0 respectively was used. The quality of DNA was further analyzed on 1 % agarose gel (100V for 20-40 min) containing 0.5 $\mu gm^{-1}$ ethidium bromide. The endotoxin level in the DNA preparation were <0.001 $ng\mu g^{-1}$ of DNA according to Limulius amebocyte lysate assay.

**2.3. Mice:** Swiss albino male mice (18-22gm) maintained on standard laboratory diet (Kisan Feeds Ltd., Mumbai, India) and water *ad libitum* were employed in the present study. The animals were divided into respective groups each of minimum six animals, housed individually in the departmental animal house and were exposed to 12 hr cycle of light and dark. The experimental protocol was approved by Institutional Animal Ethical Committee (Registration No: 107/99/CP-CSEA-2010-40) were carried out as per the guidelines of committee for Purpose of Control and Supervision of Experimental on Animals (CPCSEA) Ministry of Environment and Forest, Government of India.

**2.4 Induction of Immunosuppression.**[17]**:** Swiss albino mice (25-27gm) were made immunosuppressed by giving them hydrocortisone (HC) obtained from Wyeth Lederie Limited on day 1st and 4th (i.p.) i.e. 5 mg/kg b.wt. and after that they were divided into following groups and were given respective doses.

**2.5 Experimental design**

Group I: Untreated control i.e.mice fed basal feed.

Group II: Only Hydrocortisone (5 mg/kg b.wt.)

Group III: LB 405($10^9$cells $day^{-1}$ $mouse^{-1}$) i.e. mice which were immunosuppressed were given LB 405.

Group VI: DNA LB 405 (75µg $mL^{-1}$ $mouse^{-1}$) i.e. mice which were immunosuppressed were given DNA of LB 405.

**Follow up**

After making the animals immune suppressed the animals were treated with respective doses for 17 consecutive days. On day 4th of treatment all the groups were immunized with a single dose of SRBC[18] and blood was collected from retero orbital plexus on day 0, 8th and 13th for humoral immune response by direct haemagglutination.[19] All SRBC primed groups were challenged intradermally on day 15 with SRBC and footpad thickness was measured at 0, 24, 48 and 72 h to assess delayed type hypersensitivity response (measure of cell mediated immune response).[20] The animals were sacrificed on day 18th, their spleen was excised in MEM for immunological assays. The numbers of cells were adjusted to $2 \times 10^6$ viable cells/ml. The cells were employed to assess the immune status of the animals employing the various techniques: Nitro blue Tetrazolium Chloride (NBT) reduction test[21], Inducible Nitric Oxide Synthase (iNOS) test[21], Bactericidal activity[21] and Delayed type of hypersensivity (DTH) response.[20]

**2.6. Statistical Analysis**

All the results were expressed as mean ± S.E.M. Data of tests were statistically analyzed using one-way ANOVA followed by Turkey's multiple range test, applied for *post hoc* analysis. The data were considered to be statistically significant if the probability had a value of 0.05 or less.

## 3. RESULTS

### 3.1 Effect on humoral immune reponse

The treatment of animals with hydrocortisone resulted in decrease in anti SRBC antibody titer (Fig. 1) .However, when these immune suppressed animals were treated with DNA LB 405 a significant ($p<0.001$) rise in anti SRBC antibody titer was observed. In untreated animals the titer was 1:8 and 1:32 on day 8$^{th}$ and 13$^{th}$ respectively, whereas it was 1:2 and 1:4 on day 8$^{th}$ and 13$^{th}$ in hydrocortisone treated animals. However, with DNA LB 405 rise in titer was observed to 1:16 and 1:128 on day 8$^{th}$ and 13$^{th}$ respectively.

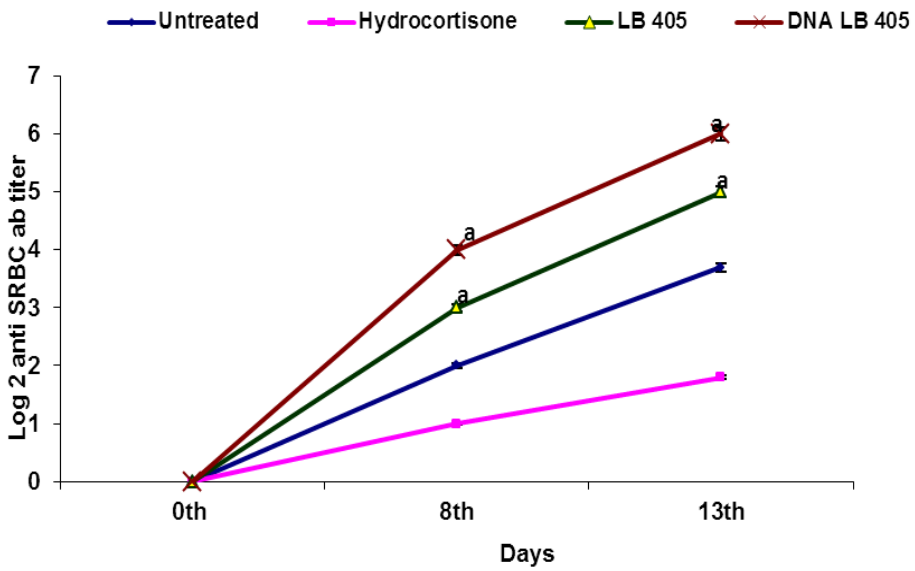


**Fig. 1: Effect of DNA LB 405 on anti SRBC antibody titer in immunosuppressed animals. The results are presented as Mean ± S.E.M (n=6).[a] p<0.001 as compared to control group.**

### 3.2 Effect of DNA LB 405 on T cell function assessed by footpad swelling.

Effect on T cell response was studied by Delayed Type Hypersenitivity. It was observed that the hydrocortisone treated animals did not show any rise in footpad thickness. However, when these immune suppressed animals were treated with DNA LB 405 it resulted in significant ($p<0.001$) rise in footpad thickness as shown in Table 1 & Fig 2. The maximum effect was observed at 48 hr.

**Table 1: Effect of DNA LB 405 on anti SRBC antibody titer in immunosuppressed animals.**

| Animal groups | Footpad thickness (mm) Time period (h) after SRBC challenge | | | |
|---|---|---|---|---|
| | 0 | 24 | 48 | 72 |
| Control | 1.68 ± 0.01 | 1.74 ± 0.02 | 1.70 ± 0.02 | 1.67 ± 0.01 |
| Hydrocortisone | 1.69 ± 0.01 | 1.71 ± 0.01 | 1.70 ± 0.02 | 1.69 ± 0.01 |
| LB 405 | 1.70 ± 0.02 | 1.82 ± 0.01 [a] | 1.85 ± 0.02 [a] | 1.73 ± 0.01 |
| DNA LB 405 | 1.68 ± 0.02 | 1.87 ± 0.02 [a] | 1.95 ± 0.01 [a] | 1.79 ± 0.01 |

**The results are presented as Mean ± S.E.M (n=6). [a] p<0.001 as compared to control group.**

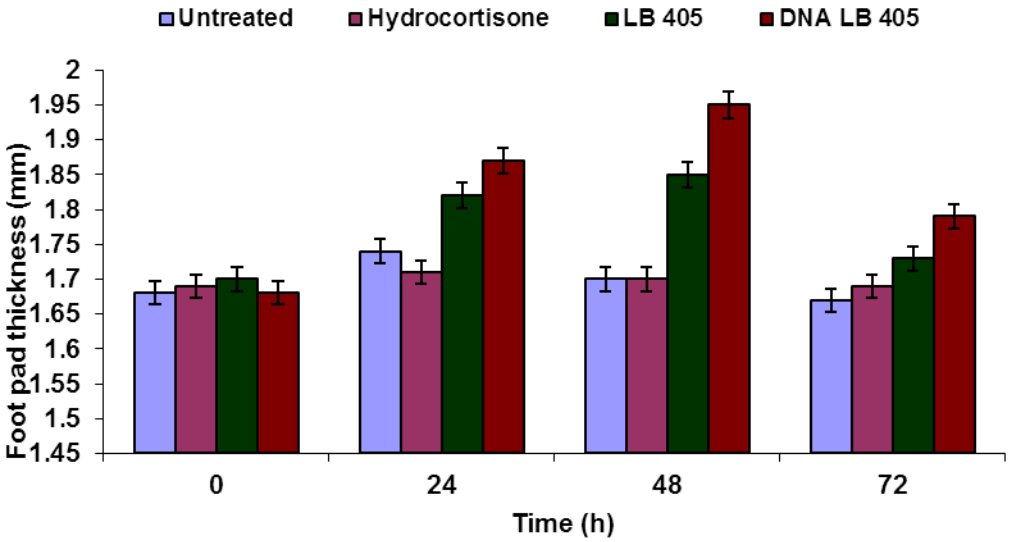


**Fig. 2: Effect of DNA LB 405 on footpad thickness in immunosuppressed animals.**

The results are presented as Mean ± S.E.M (n=6).

### 3.3. Effect of DNA LB 405 on macrophage functions

The effect of DNA LB 405 in immunesuppressed animals on macrophage function was assessed by NBT, iNOS and Phagocytic activity and is shown in Table 2 & Fig.3. It was observed that the treatment of immunosuppressed animals with DNA LB 405 resulted in significant ($p<0.001$) increase in NBT activity as compared to only hydrocortisone treated animals. NBT reduction activity of DNA LB 405 treated group was 32.41 % as compared to LB 405 treated groups (9.2 %). The DNA LB 405 boosted up the suppressed macrophage function as was assessed by enhanced iNOS (44.07%) and bactericidal activity (31.29%).

**Table 2: Effect of DNA LB 405 on macrophage function in immune suppressed Swiss albino mice.**

| Animal Groups | % NBT reduction | % iNOS activity | % Phagocytotic activity |
|---|---|---|---|
| Untreated control | 29.8 ± 1.28 | 25.98 ± 1.41 | 32.1 ± 1.13 |
| Hydrocortisone | 11.25 ± 1.21 | 12.18 ± 1.46 | 14.07 ± 1.12 |
| LB 405 | 32.56 ±1.76 [a,b] | 30.34 ±1.89 [a,b] | 34.76 ± 1.29 [a,b] |
| DNA LB 405 | 39.46 ± 2.76 [a,b] | 37.43 ± 2.34 [a,b] | 42.12 ± 3.45 [a,b] |

**The results are presented as Mean ± S.E.M (n=6) [a] $p<0.001$ as compared with untreated control [b] $p<0.05$ as compared to only hydrocortisone group.**

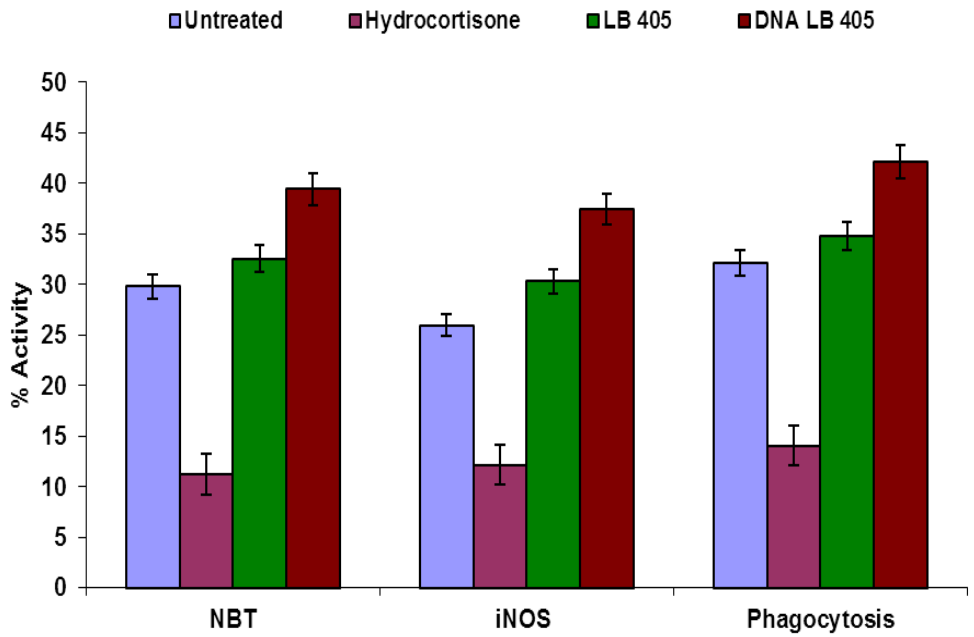


**Fig. 3 Effect of DNA LB 405 on macrophage function in immunosuppressed animals.**

**The results are presented as Mean ± S.E.M (n=6).**

Corticosteroids are steroid hormones produced naturally in adrenal glands. These are involved in wide range of physiologic systems such as stress response, immune response, regulation of inflammation, carbohydrate metabolism, protein metabolism and behaviour. For our study, we have chosen hydrocortisone which is a glucocorticoid. In our study, the treatment of immunosuppressed animals with DNA of LB 405 boosted up the suppressed immune response, as it was assessed by enhanced NBT (32.41 %), iNOS (44.07%) and bactericidal activity (31.29%). Corticosteriods are powerful immunesuppressors that inhibit macrophage activation, antibody production and T cell activity. Although, corticosteroids are generally anti-inflammatory at normal endogenous levels, adrenal steroids appear to function as immunoregulator rather than simply immunosuppressor.[22] But excess of even endogenous

corticosteroids results in Cushing's syndrome which is linked to changes in leukocytes, natural killer cells, T cell response and B cell response.[23] The effect of corticosteroid in murine B cells was studied and it was found that endogenous/exogenous corticosteroid are able to influence the immune system.[24] Similar to this, it was studied that the administration of the probiotics mixture to mice induced both T-cell and B-cell hyporesponsiveness and down-regulated Th2 cytokines without apoptosis induction.[25] They proved the therapeutic effect of the probiotics was associated with enrichment of Tregs ($CD4^+Foxp3^+$ regulatory T cells) in the inflamed regions that represented an applicable treatment for inflammatory immune disorders. It has been reported that probiotic cheese attenuates exercise induced immune suppression in Wistar rats.[26] It was observed that monocyte counts were unaltered in the rats fed with probiotic cheese as compared to significant decrease in the rats which were fed with regular cheese. Most importantly, ingestion of the probiotic cheese resulted in a >100% increase in serum high-density lipoprotein cholesterol and a 50% decrease in triacylglycerols. They concluded that probiotic cheese may be a viable alternative to enhance the immune system and could be used to prevent infections, particularly those related to the physical overexertion of athletes.

However, it has been proved that bacterial DNA containing unmethylated CpG DNA acts as immune enhancer.[27,28,29,30,31,32,33,34,35,36,37] in immunotherapy of immune-suppressed individuals having cancer and act as adjuvant for cancer vaccines such as in breast cancer, melanoma lymphoma, fibrosarcoma and lung carcinoma. Several CpG DNA drug candidates are currently being evaluated, either as monotherapies or as adjuvants (with vaccines, antibodies, antigens and allergens), in preclinical and clinical trials against cancers, viral and bacterial infections, allergies and asthma.[38]

CpG DNA has direct stimulatory effects on APCs including monocytes, macrophages and Dendritic cells (DCs). It induces the monocyte and macrophages to produce inflammatory cytokines such as IL-6, IL-12, IFN-α, IFN-β, TNF-α, IL-1β, and IL-18 , they mediate antibody dependent cellular cytotoxicity (ADCC), express inducible nitric oxide synthase and promotes lytic activity of NK cells and the secretion of IFN-γ.[39,40] When CpG DNA is endocytosed into a cellular compartment, it is exposed to Toll-like receptor 9 (TLR9).TLR9 stimulated plasmacytoid dendritic cells (pDC) migrate to the T-cell zones of lymph nodes and other secondary lymphoid tissues which express increased levels of co-stimulatory molecules that enhance their capacity to activate naive and memory T cells and have increased capacity

to cross present soluble protein antigens to CD8 T cells. As a consequence, CpG DNA promotes strong $T_H1$ CD4 and CD8 T-cell responses.[41] Moreover, CpG DNA increases the development of innate and acquired immune responses and act as immunorestorer in the immune suppressed individuals.

Overall our study highlights that to get the immune effects, it is not necessary to give whole bacterial cell in the host. Instead, bacterial DNA of immunoactive probiotic can be used as a safe immunobiotherapeutic agent (immunorestorer) even in immunocompromised host.

## 4. CONCLUSION

It is concluded that genomic DNA of probiotics should be exploited as a potent immune enhancer.

## 5. ACKNOWLEDGEMENT

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