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Advances in Applied Science Research, 2012, 3 (2):826-832

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ISSN: 0976-8610
CODEN (USA): AASRFC

In vivo comparison of probiotic bacteria's with their DNA: As augmenter of immune efficacy

Mansimran Kaur Randhawa[a], Aruna Bhatia[*a], Sheikh Abid Ali[b] and Praveen Pal Balgir[c]

[a] *Immunology and Immunotechnology Laboratory, Department of Biotechnology, Punjabi University, Patiala, Punjab, India*
[b] *Indian Institute of Integrative Medicine-CSIR, Srinagar, Kashmir, India*
[c] *Genetic Engineering Laboratory, Department of Biotechnology, Punjabi University, Patiala, Punjab, India*

ABSTRACT

The recent 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 three probiotic strains as live bacteria with their genomic DNA. 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

Probiotics are the live microorganisms that confer health benefits to the host animal when administered in adequate amounts [1]. Probiotics have nutritional benefits, they improve lactose utilization, have anti-cholesterol, anti-carcinogenic activities, anti-mutagenic, anti-infectious, immunomodulating activities, assists in preservation and in single cell protein production [2-7].

The immune system is known to be involved in the etiology as well as pathophysiologic mechanism of many diseases. Modulation of immune response to alleviate the disease has been interest for many years [8]. The function and efficacy of immune system may be influenced by many exogenous factors like food and pharmaceuticals, physical and psychological stress and hormones etc. resulting in either immune stimulation or immunosupression

[8]. Some bacterial cell components such as peptidoglycans, lipoteichoic acid, secreted soluble substances [9,10] and genomic DNA [11] reportedly play role in immunomodulation responses but primary component is yet to be identified.

Structural difference between bacterial and eukaryotic DNA apparently account for the ability of bacterial DNA to serve as an immune activating agent. Specifically, bacterial DNA is thought to activate inflammatory cells because of its high content of short sequences with unmethylated CpG dinucleotides [12].In mammalian DNA, CpG containing sequences occur at a much lower frequency than in bacterial DNA, and the cytosine present in CpG dinucleotide of mammalian DNA is usually methylated [13,14]. However, unmethyalted bacterial DNA motifs comprising cytosine linked to guanine by a phosphate bond (CpG motifs), also known as immunostimulatory sequence (ISS) oligodeoxynucleotides (ODNs), are reportedly mitogenic for murine B cells [12]. Bacterial DNA and immunostimulatory CpG-ODNs activate Antigen Presenting Cells (APCs) such as macrophages and dendritic cells. Cell activation occurs upon DNA endosomal uptake, resulting within minutes 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 importance of present study lies in the fact that probiotic bacteria are used as immunomodulators but a literature survey revealed that DNA of probiotics have not been studied for immune response. The purpose of current study was to compare *in vivo*, immunomodulatory activity of probiotic viable bacteria with its isolated genomic DNA.

MATERIALS AND METHODS

2.1 Bacterial strain and culture condition

The strain of *Lactobacillus delbrueckii* 405 (LB 405) , *Lactobacillus brevis 403* (LB 403), *Bifidobacterium bifidium* BD4 234 (Bif 234) was procured from National Dairy Research Institute, Karnal, Haryana. The cultures so obtained were given two revival cycles in 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 OD_{260}/OD_{280}.Only the DNA with OD_{260}/OD_{280} 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 μgm^{-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.

2.3.1 Experimental animal design: Animals were divided into nine major groups:

Group I*: Untreated Control group (not subjected to any treatment i.e. kept only on diet)*
Group II*: Positive control (25mgkg^{-1} Levamisole i.p for 17 consecutive days)*
Group III*: Immunized control (mice sensitized with SRBC and kept on normal diet)*
Group IV*: Lactobacillus delbrueckii 405* ***(LB 405)*** *for 17 consecutive days at the rate of 10^9cells day^{-1} mouse^{-1} as oral dose.*

***Group V**: DNA of Lactobacillus delbrueckii 405 **(DNA LB 405)** three injections in left tibialis anterior muscle [17] after 6 days at the rate of 75µg mL^{-1} $mouse^{-1}$.*
***Group VI**: Lactobacillus delbrueckii 403 **(LB 403)** for 17 consecutive days at the rate of 10^9cells day^{-1} $mouse^{-1}$ as oral dose.*
***Group VII**: DNA of Lactobacillus delbrueckii 403 **(DNA LB 403)** three injections in left tibialis anterior muscle [17] after 6 days at the rate of 75µg mL^{-1} $mouse^{-1}$.*
***Group VIII**: Bifidobacterium bifidium BD4 234 **(Bif 234)** for 17 consecutive days at the rate of 10^9cells day^{-1} $mouse^{-1}$ as oral dose.*
***Group IX**: DNA of Bifidobacterium bifidium BD4 234 **(DNA Bif 234)** three injections in left tibialis anterior muscle [17] after 6 days at the rate of 75µg mL^{-1} $mouse^{-1}$.*

2.3.2 Immunization
Sheep blood was collected in Alsever's solution in the ratio 1:2 and was centrifuged at 400 × g for 10 min at 4 ° C. The erythrocyte pellet obtained was washed and suspended in PBS (0.1 M, pH 7.2) for further use as per [18]. All mice were antigenically challenged intraperitoneally with a single dose (100µl ml^{-1} of 1×10^7 cells/ml) of sheep red blood cells (SRBC).

2.4. Humoral Immune Response
To assess the humoral immune response, blood was withdrawn from retro-orbital plexus of all SRBC antigenically challenged animals on day 0 (pre-immunized), 8th and 13^{th} (post immunization).The serum was separated and assayed by direct haemagglutination [19]. Titer was described as highest dilution capable of visible agglutination. The results were expressed as mean ± S.E.M. log titer of individual animals.

2.5. Cell mediated immune response
2.5.1. Delayed Type Hypersensitivity assay
Delayed Type Hypersensitivity response (DTH) was checked by foot pad swelling method [20]. All SRBC primed groups were challenged intradermally on day 15^{th} with SRBC suspension (1×10^7 100µl $saline^{-1}$) in the hind footpad. The control lateral paw was given equal volume of saline. Paw thickness was measured with micro-caliper at 24h interval up to 72h. The difference in paw thickness compared to control was taken as a measure of DTH and expressed in millimeter. Results are expressed as mean ± S.E.M. of footpad thickness up to 72h.

2.5.2. Total lymphocyte isolation from the spleen
Spleen was excised aseptically and lymphocytes were isolated by teasing the tissue. Cells were centrifuged (400 × g for 10 min at 4 ° C) and lysed by ACK lyses solution (0.5M NH_4Cl, 10mM $KHCO_3$ and 0.1 mM disodium EDTA, pH 7.2). Lymphocytes obtained were washed thrice in PBS, counted and adjusted to desired concentration in RPMI for further use.

2.5.3. Nitroblue Tetrazolium Reduction assay
NBT reduction test was evaluated by employing the method described by [21]. Briefly, the lymphocyte suspension was incubated with NBT and formazon formed was extracted in dioxan. The reduction in NBT was measured spectrophotometrically at 520 nm (Shimadzu, UV-1650 PC) against dioxan as blank. The results were expressed as mean ± S.E.M. of percentage dye reduced to formazon.

2.5.4. Inducible Nitric Oxide Synthase activity
Inducible nitric oxide synthase activity in lymphocyte suspension was evaluated by a previously described procedure by [21] using arginine. The color developed (indicating presence of citrulline) was measured spectrophotometrically at 540nm against RPMI and Griess reagent as blank and the results were expressed as mean ± S.E.M. of percentage enzyme produced.

2.5.5. Bactericidal activity
Bactericidal activity was determined by [21]. Briefly, the lymphocyte suspension was incubated with bacterial suspension (*Escherichia coli)* at 37° C for 60 min. The lymphocytes were lysed with sterile distilled water spread on agar plate and incubated at 37 ° C for 24 h. Bacterial suspension was spread in the control plate. Number of colony forming units (CFU) developed in control and test plates were counted and results were expressed as mean ± S.E.M. of bactericidal activity.

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.

RESULTS

3.1. Humoral Immune Response

In all groups i.e. Positive treated, Immunized Control, LB 405, DNA LB 405 ,LB 403, DNA LB 403, Bif 234 and DNA Bif 234 no anti SRBC antibody titer was observed on day 0. DNA LB 405 had a significantly higher antibody titer as compared to LB 405 (neat culture) as depicted in **(Figure 1).**The anti SRBC antibody titer of DNA LB 405 was found to be comparable to that of levamisole treated group which is an immune enhancer. Similarly, DNA of LB 403 and Bif 234 showed higher antibody titer as compared to neat cultures.

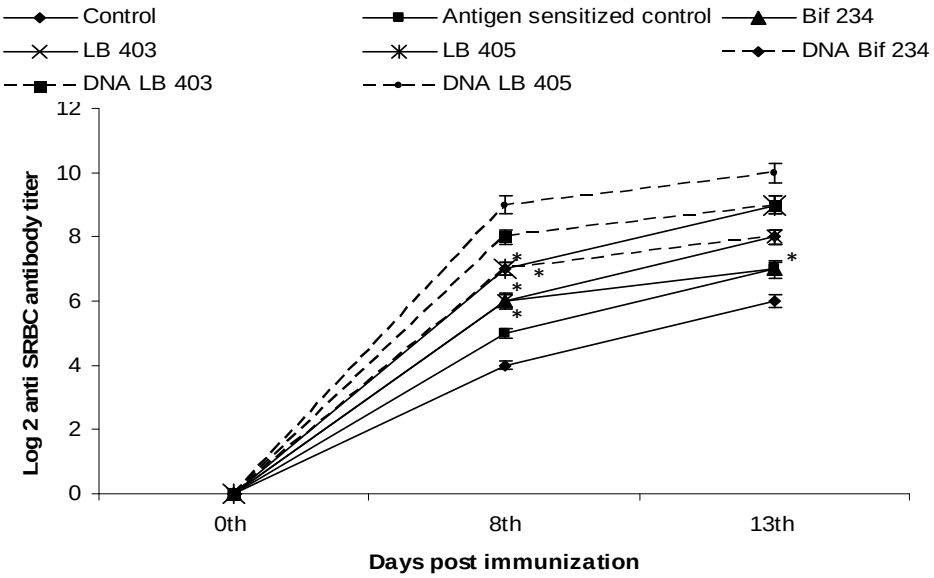

Figure 1. Effect of different groups on production of anti- SRBC antibody titer on pre-immunization(0^{th} day) and post-immunization (8^{th} and 13^{th} day).

** p<0.05 in comparison to sensitized control*

Table 1 Delayed type hypersensitivity response

Animal Groups	Foot pad thickness (mm)			
	Time periods (h) after SRBC challenge			
	0	24	48	72
Untreated Control	1.69 ± 0.01	1.69 ± 0.01	1.69 ± 0.01	1.69 ± 0.01
Positive Control	1.70 ± 0.02	1.86 ± 0.02**	2.01 ± 0.04	1.81 ± 0.03
Immunized Control	1.68 ± 0.01	1.72 ± 0.01*	1.70 ± 0.01*	1.67 ± 0.01
LB 405	1.69 ± 0.01	1.77 ± 0.03	1.85 ± 0.01	1.73 ± 0.03
DNA LB 405	1.70 ± 0.02	1.87 ± 0.02**	2.11 ± 0.01**	1.77 ± 0.01
LB 403	1.67 ± 0.01	1.74 ± 0.02	1.80 ± 0.01	1.70 ± 0.01
DNA LB 403	1.69 ± 0.01	1.83 ± 0.02**	2.06 ± 0.02**	1.73 ± 0.01
Bif 234	1.66 ± 0.02	1.70 ± 0.02	1.71 ± 0.01	1.52 ± 0.01
DNA Bif 234	1.67 ± 0.01	1.72 ± 0.01	1.74 ± 0.02	1.60 ± 0.03

The results are presents as mean ±S.E.M (n=6)

** p< 0.001 in comparison to untreated control*

*** p<0.05 in comparison to sensitized control*

3.2. Cell mediated immune response

3.2.1. Delayed type hypersensitivity

Effect of viable bacteria and DNA on T-cell response was studied by assessing the footpad swelling as a measure of Delayed type hypersensitivity. In untreated control group, no rise in footpad thickness was observed. However, DNA treated groups showed significant ($p< 0.05$) elicitation of the T-cells response as evident by an increase in foot pad thickness as compared to antigen sensitized control group and groups receiving neat cultures. It was found that DNA of LB 405 and LB 403 showed comparable rise in footpad thicknesss to that of levamisole treated group after 48 hours (**Table 1**).

3.2.2. iNOS activity

Cell mediated immune response is indicated in **Figure 2**. It was seen that DNA LB 405 treated group showed maximum activity which was significantly higher in comparison to control ($p< 0.001$) and orally treated group (LB 405).In, DNA treated group of LB 405 iNOS activity was 27.9 % higher than LB405 group (neat culture) . Similarly DNA treated groups of LB 403 and Bif 234 showed higher iNOS activity than neat culture groups of LB 403 and Bif 234 respectively.

3.2.3. NBT reduction

LB 405 neat culture and its genomic DNA treated group significantly increased ($p<0.05$) NBT reduction as compared to immunized control group. Similar to iNOS activity, NBT reduction activity of DNA LB 405 treated group was 24.05 % higher than LB 405 neat culture group **(Figure 2).**

3.2.4. Bactericidal activity

The effect of Probiotic DNA on bactericidal activity was studied in terms of number of colony forming units (CFU).The treatment of animals with DNA treated groups (DNA LB 405, DNA LB403, DNA Bif 234) reduced the number of colonies and thus enhanced the bactericidal activity as compared to neat culture groups (LB 405, LB 403, Bif 234) **(Figure 2).**

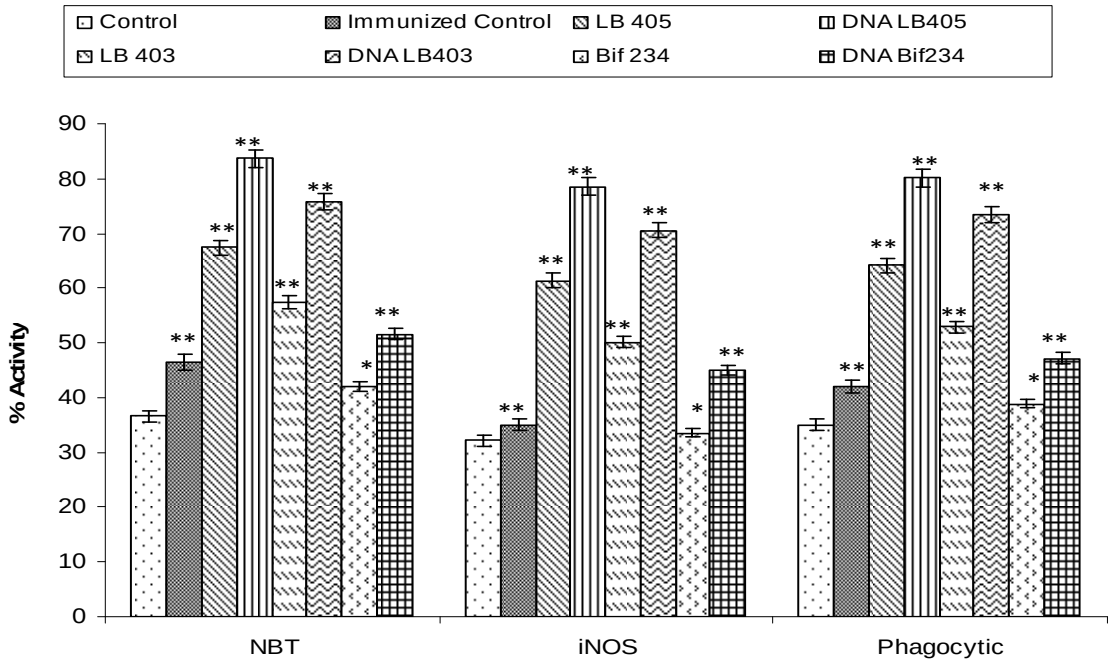

Figure 2. Influence of different groups on iNOS activity, NBT reduction and Phagocytic activity. The results are expressed as mean ± S.E.M (n=6)

** p< 0.001 in comparison to untreated control*
*** p<0.05 in comparison to sensitized control*

DISCUSSION

In the present study, the immunomodulatory efficacy of *Lactobacillus delbrueckii 405, Lactobacillus brevis 403 ,Bifidobacterium bifidium BD 234* and their isolated DNA(DNA LB 405, DNA 403, DNA Bif 234) were compared on the basis of evoking the immune response in Swiss albino mice. Results revealed that isolated DNA of the probiotic showed higher potency than the whole cell i.e. live probiotics.

In our study anti SRBC antibody titer development, Delayed Type Hypersensitivity, Nitroblue Tetrazolium Reduction test, Inducible Nitric Oxide Synthase as well as Bactericidal activity were higher in DNA treated animals than only probiotic treated groups. These results show that both the Humoral as well as Cell mediated immune response are elicited more by genomic DNA than probiotic alone. The possible mechanism for this could be the activation of T mediated B cells by the CpG sequences present in the nucleotides which results in the secretion of cytokines.

Earlier [22] reported that Unmethylated CpG dinucleotides in bacterial DNA or synthetic CpG sequence (CpG S) DNA rapidly activate murine B cells to secrete IL-6, IL-10 and Ig M as well as their proliferation. The authors observed that within 30 minutes after CpG DNA stimulation *in vivo*, IL-6 mRNA levels increased in liver, spleen and thymus cells. Serum IL-6 protein was markedly increased within 1 hour of stimulation. Immunostimulatory CpG and non CpG Oligodeoxynucleotides (ODNs) have already been identified from the genomic DNA of probiotics [23].

Levamisole is a well-known stimulant of B cell, T cell, monocytes and macrophages. Hence, Levamisole was used as a positive control while studying the effect of probiotics on immune response. In our experiments, animals were sensitized with SRBC. SRBC mediated immune response is a highly sensitive indicator of immunological integrity and requires coordinated interaction of various immune system cells [24] and our study is a mirror of this coordination between the various immune system cells.

NBT reduction test is an indirect marker of the oxygen dependent bactericidal activity of the phagocytes and metabolic activity of granulocytes or monocytes [25,26]. Present results indicate that probiotic DNA is capable of stimulating the immune function of macrophages as evidenced by an increase in NBT reduction and bactericidal activity in all the treated groups. The functional ability of macrophages was evident from increased expression of iNOS that oxidizes L-arginine to citrulline and nitric oxide. The iNOS activity is correlated to bactericidal activity of macrophages and has been documented as a measure of immunomodulatory potential [27].

CONCLUSION

It is concluded that genomic DNA of probiotics is a better immune enhancer than whole bacterial cell suspension and should be exploited for therapeutic potential in treatment of variety of diseases inc d.luding infection, allergy and cancer.

Acknowledgement
The financial aid to the department in the form of FIST grant for the purchase of instruments by DST, Govt. of India is fully acknowledge

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