The present work was conducted to find out the immunomodulatory potential and biotherapeutic efficacy of genomic DNA of a probiotic. The work was carried out in three phases; in Phase I six probiotic strains (*Lactobacillus casei subsp. casei* 17, *Lactobacillus brevis* 403, *Lactobacillus delbrueckii* 405, *Bifidobacterium bifidium* (BA3 233), *Bifidobacterium bifidium* (BD4 234), *Bifidobacterium bifidium* (BD1 235) procured from NDRI, Karnal were screened for immunological activity *in vitro* at two different concentrations i.e. $1 \times 10^6$ cells ml$^{-1}$ and $1 \times 10^9$ cells ml$^{-1}$. Three strains were selected based upon their variation in immune activity i.e. maximum active (LB 405), moderate active (LB 403) and minimum active (Bif 234). In Phase II, *in vivo* experiments were carried out. Isolated genomic DNA of each selected strain and its pure cell culture were tested for their immunopotential. In Phase III, experiments were carried to find out the mechanism of immunopotentiation by best selected strain or its DNA (LB405) in swiss albino mice. Further, the biotherapeutic efficacy of the isolated genomic DNA of probiotic was tested as the modulator of diabetes, hypercholesterememia and immunorestoration.

**Phase I**

First of all growth pattern of the procured six probiotic strains of *Lactobacillus spp.* and *Bifidobacterium spp.* was studied spectrophotometrically. Maximum growth of *Lactobacillus spp.* was observed after 18 hours whereas that of *Bifidobacterium spp.* maximum growth was after 20 hours. Similar results have been reported by many researchers earlier, who have studied the growth spectrophotometricaly and found the logarithmic phase of *Lactobacillus* between 6-16 hours (Gu *et al.*, 1998; Olson and Aryana, 2012) *Bifidobacterium* growth was observed to be maximum after 20 hours (Lin and Young, 2000; Su *et al.*, 2007; Elghali *et al.*, 2012).
Further, *in vitro* immunomodulatory potential of all the probiotic strains as tested by their iNOS, NBT and Phagocytic activity at two concentration of cells, revealed that although $1 \times 10^6$ cells ml$^{-1}$ showed the bioactivity but invariably the activity was more when $1 \times 10^9$ cells ml$^{-1}$ were employed. These results show direct correlation between the cell number and bioactivity. Similar to these studies other authors have also reported a direct correlation between bioactivity and cell number. Johnson-Henry *et al.* (2005) reported that a mixture of *L. rhamnosus* and *L. acidophilus* at $10^9$ cfu/ml was sufficient to attenuate *C. rodentium* induced colonic disease in mice. Mountzouris *et al.* (2010) showed that the therapeutic effect was evident at a minimum concentration of $1 \times 10^6$ CFU/ml but a total of some $10^8$ to $10^9$ probiotic microorganisms should be consumed daily for pronounced effect. Lin *et al.* (2011) also emphasized that dose selection is an important issue for probiotic studies and quoted that higher concentrations than $1 \times 10^6$ CFU/ml have beneficial effects on regulating T cell-mediated immune responses by attenuating mitogen-induced overactive immune responses and promoting Th1 immune responses. Evrard *et al.* (2011) studied that the probiotic *L. rhamnosus* induces a dose-dependent immunomodulation of human DCs. DNA microarray and qRT-PCR analysis showed that the probiotic induced a large-scale change in gene expression. It nearly modulated 1,700 genes, with 3-fold changes, but only with high doses.

Immune modulation by dietary bacteria has continued to be a subject of growing interest. It has been demonstrated that specific *Lactobacillus* strains can modulate host immunity, which positively correlates with enhanced resistance to various viral and
bacterial infections (Matsuzaki and Chin, 2000; Bhatia and Pawan, 2007; Kwon, 2010). Moreover, the beneficial effects of the probiotic strains vary not only at the species level, but at the strain level too (Kaur and Bhatia, 2012). In the present study, the six strains of probiotics could be placed in three categories based on the variability in bioactivity i.e. maximum, moderate and minimum. The following three strains one from each category was selected for further studies.

<table>
<thead>
<tr>
<th>Maximum activity</th>
<th>LB 405</th>
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<tr>
<td>Moderate activity</td>
<td>LB 403</td>
</tr>
<tr>
<td>Minimum activity</td>
<td>Bif 234</td>
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</table>

Our studies correlate the earlier findings that probiotic bacteria influence immune response and some of them have been employed as immunomodulators for complementary and alternative medicines (Bhatia and Pawan, 2007; Jennifer and Joshua, 2010; Megan and Kimberly, 2010; Bhatia and Kaur, 2012).

**Phase II**

The experiments in this phase were carried out to find out, Firstly whether the genomic DNA can be used as an immunomodulator rather than pure cell, keeping in mind that in some weak subjects the probiotics cannot be given because they prove harmful rather than beneficial (Stone, 2010; Neel, 2012). Secondly, whether the immune activity of pure cell culture is parallel to their isolated DNA. Genomic DNA was isolated from three selected probiotic strains showing maximum (LB 405), moderate (LB 403) and minimum activity (Bif 234) and was subjected to *in vivo* tests for immunomodulatory potential by employing the NBT, iNOS, Bactericidal activity, Direct Haemagglutination test and DTH.
Parallel groups of animals fed with corresponding bacterial strain pure culture were also set up.

The Nitroblue Tetrazolium (NBT) reduction test is an indirect marker of the oxygen dependent bactericidal activity of phagocytes (Hellum, 1977). NBT dye with low reduction potential when phagocytozed is reduced to formazon, which is measured spectrophotometrically (Szczylik et al., 1979). The results showed that the genomic DNA of all the strains was better immune stimulator than the respective pure culture. Moreover, out of all the isolated DNA’s, the DNA of *Lactobacillus delbrueckii* 405 showed the maximum potential. It is clear from the results that variation in immunomodulatory activity by DNA’s of the selected strains was similar and parallel to that of their respective pure cultures. Hence, the DNA of moderate activity showing culture i.e LB 403 was also showing moderate activity. The functional ability of phagocytes 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 employed to measure the immunomodulatory potential (Park et al., 2001; Frankova and Zidek, 1998). The results of iNOS expression were parallel to that of NBT and bactericidal activity and in this test also the best results were obtained by DNA treatment. DNA of LB 405 showed higher anti-SRBC antibody titer and enhanced footpad reaction in SRBC immunized animals. The SRBC mediated immune response is a highly sensitive indicator of immunological integrity, which requires the coordinated interaction of various immune system cells and their products *i.e.* cytokines, antigen-presenting cells, T-lymphocytes and B-lymphocytes (Koganei et al., 2007). DTH is a good measure of cell mediated immune response and is assessed by foot pad swelling assay which is an expression of T- cell response (Mu and Sewell, 1994; Suvas et al., 2002). DNA of probiotics showed better
immune activity in the immunological tests i.e. NBT, iNOS and Phagocytotic activity, it shows that bacterial DNA is a good immunopotentiator. The proposed mechanism underlying the immune activity of DNA has been shown due to be presence of unmethylated CpG motifs. Similar to our results, Liu et al. (2003) reported that foreign plasmid DNA can induce humoral and cell mediated response immune in mice after administration via the gastrointestinal tract. Chuang et al. (2007) noted that the genomes of bacterial and viral DNA contain a much higher frequency of unmethylated CpG dinucleotides than those of vertebrates. This difference in genome structure allows the innate immune system of vertebrates to distinguish bacterial or viral DNA from self-DNA, and consequently to perceive a ‘danger signal’ when bacterial or viral DNA is encountered. Further, the authors reported that CpG motifs, including bacterial DNA and CpG ODNs (synthetic oligodeoxynucleotides containing unmethylated CpG), are capable of evoking a range of immunostimulatory effects in vertebrates and have a tremendous potential to be used as therapeutic agents and adjuvants. Similarly, Haiqi et al. (2007) reported that unmethylated CpG dinucleotides within specific flanking bases (referred to as CpG motif) are relatively abundant in bacterial DNA and are known to stimulate innate immune responses and stimulated expression of cytokine genes differentially. The observations indicated that Toll-like receptors (TLRs) recognize microbial components and initiate the innate immune responses that control microbial infections. Wells (2011) stated that the immunomodulatory mechanisms of Lactobacilli involve the innate pattern recognition receptors such as Toll-like receptors, nucleotide oligomerization domain like receptors and C-type lectin receptors. Binding with these receptors can activate antigen presenting cells and modulate their function through the expression of surface receptors, secreted cytokines and chemokines.
**Phase III**

The results of techniques employed in Phase II made it evident that the DNA of bacterial strain potentiates the phagocyte and T cells (Cell mediated immune response). In phase III, hence some more experiments were carried out to compare the impact of pure cell culture and its DNA on Th1 and Th2 response (IFN γ and IL-4 concentration respectively) and on B cell response to BSA.

The results of present study revealed that IgM response started increasing by week 1 and then started decreasing after week 2 whereas IgG response started increasing from week 1 and on increase continued till week 3 as compared to the controls in which the response peaked at week 2 and then declined. IgM is the first immunoglobulin class produced in a primary and non specific response to an antigen. Whereas, IgG, the most abundant class in serum, is formed later on. Studies by Alberda *et al.* (2007) have also shown that probiotic therapy in critically ill patients with multiple organ dysfunction syndrome greatly enhance their immune activity with significant increase in IgA and IgG antibodies and reduced incidence of diarrhea in comparison to placebo-controls. Similarly, Tsai *et al.* (2008) and Ho *et al.* (2011) discovered that the total serum IgG level increased after the administration of *Lactobacilli* to Balb/c mice whereas Saiady (2010) found that with increase in IgG concentration, a significant increase in body weight was also observed pointing to the wellness effect of probiotics in newborn calves.

Bailon *et al.* (2004) observed a significant increase in serum IgG concentration in adult healthy dogs receiving *L. acidophilus* supplement indicating that probiotics might enhance the health and immune functions of animals
Similarly, administration of probiotic bacteria in chickens was shown to enhance specific, systemic antibody response and to stimulate the production of natural antibodies such as serum IgG and IgM (Haghighi et al., 2005). Similar to observations of present study, many authors observed that bacterial DNA containing unmethylated CpG motifs induces B cell proliferation, IgM and IgG2a production (Messina et al., 1991; Perez et al., 1994; Olishevsky et al., 2003). Takahashi et al. (2006) evaluated the efficiency of CpG DNA in preventing allergic responses by oral administration, which suppressed serum ovalbumin (OVA) specific immunoglobulin IgE levels and improved the OVA-specific IgG2a/IgG1 ratio in mice. Xu et al. (2008) revealed that CpG ODN inhibited class switching from IgM to IgE being potent inducer of Th1cytokine response to CD40 and IL-4 in B cells. These results imply a B cell-intrinsic, T cell-independent mechanism by which CpG ODN directly acts on B cells and inhibit IgE and IgG1 production leading to prevention of allergic symptoms.

In the present study, administration of probiotics as pure culture (LB 405) or DNA of LB 405 showed a significant up regulation of Th-1 cytokine (IFN-gamma production). Contrary to this, both LB 405 and DNA LB 405 downregulated Th-2 cytokine (IL-4 production). On the basis of cytokine profile, T-cell are further divided into Th-1 and Th-2 cells, each associated with a particular immune response to an antigen (Bretsch et al., 2001). T helper (Th) lymphocyte balance (Th-1/Th-2) is crucial in orchestrating the appropriate cytokine responses and hence remains as one of targets for immunomodulation and immune-based therapies. Th-1 type cytokines (IFN-gamma, IL-2) promote cell-mediated immunity
and Th-2 type cytokines (IL-4, IL-10) are responsible for humoral immunity leading to increased IgE responsible for inflammatory response. Over activation of either pattern can cause disease and either pathway can downregulate the other. Hence, the optimal immunotherapy should restore or maintain a well balanced Th1 and Th2 response, suited to the immune challenge as shown in Fig.6.1.

Fig.6.1. Cytokine-mediated differentiation of Th1/Th2 cells.

Undifferentiated naive T cells secrete specific cytokines after antigenic stimulus that polarizes, first into null T-helper cells (Th0), then into Th1 or Th2 cells (Bani et al., 2011)
Similar to observations in present study, many authors observed that probiotics as pure culture reinforced intestinal barrier capacity and stimulate Th1 mediated immune response (Menard et al., 2005; Baken et al., 2006; Kim et al., 2009; Cunningham-Rundles, 2011). It has been reported that changes in cytokine profile by CpG motifs present in bacterial DNA perform critical immunomodulatory function (Klinman et al., 1996; Millan et al., 1998). Olishevsky et al. (2003) reported that the activation of the immune system cells by CpG-DNA initiates a complex network of cell-cell interactions and cytokine production cascades that result in an overall enhancement of immune functions in an antigen-independent manner. Takahashi et al. (2006) found that the immunostimulatory oligodeoxynucleotide from the genomic DNA of the probiotic strain Bifidobacterium longum significantly inhibited immunoglobulin E (IgE) production and stimulated interferon-gamma (IFN-gamma) and IL-12 production, but did not affect IL-4 secretion in murine splenic cells of ovalbumin-primed BALB/c mice. Ghadimi et al. (2008) studied the effect of different probiotics, Lactobacillus rhamnosus GG, Lactobacillus gasseri (PA16/8), Bifidobacterium bifidum (MP20/5), and Bifidobacterium longum (SP07/3), on the Th-1 and Th-2 responses of peripheral blood mononuclear cells (PBMCs) from healthy subjects and from patients with allergy against house dust mite to Staphylococcus enterotoxin A (SEA) and Dermatophagoides pteronyssinus (Dpt). To elucidate the molecular basis of these effects, the effects of bacterial genomic DNA were compared with the effects of viable bacteria. It was found that bacterial DNA inhibited IL-4 and IL-5 secretion
whereas IFN-gamma stimulation by their DNA was more pronounced in allergic subjects.

In the present study, biotherapeutic potential of selected LB 405 strain and its DNA of LB 405 was compared for Anti-diabetic activity, Anti-Cholesteremic activity and Immuno Restorative activity.

The combination of standard drug (glyburide) and DNA of 405 (Group VI) exerted an antihyperglycemic effect at 2 h and showed better reduction in glucose levels (37.68 %) than glyburide alone (25.13 %). However, LB 405 showed only 2.71 % decrease in glucose level in comparison to DNA LB 405 which showed 16.7 % decrease. According to Salami et al. (2008) gliclazide an oral antihyperglycemic agent, used for treatment of Non-insulin dependent diabetes mellitus, increases both basal insulin secretion and meal stimulated insulin release. They reported the action of a sulphonylurea with beneficial extrapancreatic effects on glucose level, which may be enhanced by administration of probiotics. Moreover, many studies have proven that probiotics reduces the blood glucose concentrations (Tabuchi et al., 2003; Yamano et al., 2006; Yadav et al., 2007; Bhatia et al., 2012). However, no study is available which shows the effect of DNA of probiotic as hypoglycaemic agent.

It is very well accepted fact that diabetes is a risk factor for cardiovascular disease. Diabetes leads to the development of state of hypercholesterolemia and hypertriglyceridemia (Pyorala et al., 1987). The abnormalities in lipid metablosim in diabetes generally lead to elevation in the levels of serum lipids and lipoproteins that play role in antherosclerosis (Mitra et al., 1996). Insulin deficiency in diabetes induces
synthesis of lipases that increases the generation of free fatty acid in plasma and liver and thus, increases oxidative stress. Excess of fatty acids promote their conversion into cholesterol and triglycerides with concomitant increase in low density lipoprotein cholesterol (Ghebremeskel et al., 2002). The role of probiotics in reducing the cholesterol level is well known (Lin and Chen, 2000; Pereira and Gibson, 2002; Liong and Shah, 2005; Zhang et al., 2008; Sirilun et al., 2010; Lye et al., 2010; Ooi et al., 2010). Moreover, the relationship between immune response and diabetes, cholesteremia and diabetes lead us to study the effect of DNA of probiotics on hypercholesteremic mice. But no study is available showing the effect of bacterial DNA or probiotic DNA on cholesterol level. The present study revealed that the DNA of probiotic strain alone or in the combination with standard drug reduced the total cholesterol levels 1.3 and 1.9 times more than LB 405 alone or in combination with standard drug. Lee et al. (2010) investigated factors involved in serum cholesterol reduction by L. acidophilus using a mutant that had decreased cholesterol reduction ability. Similar to present study, Ramasamy et al. (2010) investigated removal of cholesterol by 12 lactobacillus strains in vitro. The lactobacillus strains also showed differences in their ability to remove cholesterol from the growth medium (26.74-85.41%). Significant (P<0.05) correlations were observed between cholesterol removal and deconjugation of sodium taurocholate. In vivo studies, Bhatia and Pawan (2007); Bhatia and Pawan (2010) and Randhawa et al. (2011) proved the cholesterol lowering activity of probiotics using single and multiple strains revealed that combination of probiotics reduced the total cholesterol levels 25-29% as compared to the 11% only by the standard drug treatment. Raghavan et al.(2011) performed in vitro study to reduce the cholesterol with Lactobacillus as probiotics to a significant level. From this
study, they concluded that the high level of cholesterol could be removed by growing cell from media, which can be used as adjuvant to lower serum cholesterol *in vitro*.

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%). Corticosteroids are powerful immunosuppressors 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 (Sternberg, 2001). 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 (Masera *et al.*, 1999). Dennis and Monad (1986) also studied the effect of corticosteroid in murine B cells and found that endogenous/exogenous corticosteroid are able to influence the immune system. Similar to this, Kwon *et al.* (2010) 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. 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. Lollo *et al.* (2012) reported that probiotic cheese attenuates exercise induced immune suppression in Wistar rats. 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, Maloney et al., 1997; Weiner et al., 1997; Wooldridge et al., 1997; Pegram et al., 1998; Smith and Wickstrom, 1998; Krieg et al., 2000; Stacey et al., 2000; Warren et al., 2000; Jahrdsorfer et al., 2001; Hennge et al., 2001; Kawarada et al., 2001 proved that bacterial DNA containing unmethylated CpG DNA acts as immune enhancer 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. 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 (anti-diabetic, anti-cholesteremic, immunorestorer) even in immunocompromised host.