CHAPTER 1

INTRODUCTION
1.1 Probiotic bacteria

The growing awareness of the relationship between diet and health and the side effects of the allopathic medicines has led to an increase in demand for the food products that support health beyond providing nutrition and act as an alternative source of medicine. Microorganisms are extensively used in food processing industry and are present in the form of live, dead or as metabolites (Bhatia and Pawan, 2010).

The safeties of the microbes that have been used traditionally as probiotics have been confirmed through a long period of experience in benefitting the human health and have been granted the ‘Generally Recognized As Safe’: GRAS status. Probiotics are the best fortifying agents to make functional foods because due to their acid and bile tolerance characteristics, they can colonize the gut, and hence there is no need for repeated doses, if food habits which affect them are not changed (WHO/FAO).

The term probiotic is based on the Greek expression “probios” means ‘for life’. Since, the first discovery of the health effects of a probiotic by Metchnikoff (1908) and the introduction of the term “probiotics” by Kollath (1953), a lot of work has been done in this field. Probiotics have been defined as live microbial food supplements that beneficially affect the host by improving its intestinal microbial balance.

The predominant species used as probiotic agents belong to the group of Lactic Acid Bacteria (LAB). Among these, the most studied probiotics are particularly various species of Lactobacillus and Bifidobacterium. Lactobacillus species used as probiotic include L. acidophilus, L. casei, L. bulgaricus, L. fermentum, L. reuteri, L. rhamnosus and L. salivarius. However, many other organisms used as probiotics in humans include
*Escherichia coli*, *Streptococcus* sp., *Enterococcus* sp., *Bacteroides* sp., *Bacillus* sp. and *Propionibacterium* sp. Various strains of probiotics may act in synergism, hence some probiotic preparations contain mixtures of more than one bacterial strain (Rastogi *et al.*, 2011). Probiotics are normal inhabitants of human intestine and vagina and show may health benefits as given below.

### 1.2 Health enhancing effect of probiotic consumption

There are many health benefits of adding probiotic organisms to the diet, whether in a pure form such as *L.acidophilus* supplement, or in the form of traditional fermented and cultured foods. Some of the general and specific health benefits (Fig.1.1) of probiotics including impact on pathogenic strains have been definitively established such as:

- Improving intestinal tract health
- Lowering cholesterol
- Enhancing the immune system and preventing infections
- Synthesizing and enhancing the bioavailability of nutrients
- Reducing symptoms of lactose intolerance
- Decreasing the prevalence of allergy in susceptible individuals
- Reducing risk of certain cancers
- Reducing Inflammation

Due to their above mentioned properties, probiotic bacteria lead the researchers to place them as one of the food supplements as fortifying agents. Food sources of probiotic bacteria also include fermented dairy products like yoghurt, milk with live cultures and also sold as kefir, koumiss and yakult.
1.3 Proposed mechanism of action of probiotics

Probiotics are a part of normal flora. The mechanism behind their establishment and action in the body is depicted in Fig. 1.2. Some factors which contribute to their establishment in gut are:

1. Adhesion: Probiotics compete for attachment sites on the digestive tract wall to prevent colonization of pathogenic microorganisms. Detrimental bacteria like *Escherichia coli* attach to the gut wall and exert their harmful effects. Attachment is achieved by means of fimbriae made up of protein on the bacterial surface e.g. Lectins on bacterial surface recognize and selectively combine with specific oligosaccharide receptor sites in the
gut wall. *Lactobacilli* successfully disallow colonization of the disease provoking bacteria through competition for nutrients, immune system up-regulation, production of antitoxins (Marteau *et al.*, 2001; Dsouza *et al.*, 2002) and up regulation of intestinal mucin genes (Mack *et al.*, 1999).

**Fig. 1.2. Various mechanism of action of probiotic bacteria (Rastogi *et al.*, 2011)**

2. **Neutralization**: There is experimental evidence that live probiotic bacteria can neutralize endotoxins produced by pathogenic bacteria but their active substances have not been fully characterized. Probiotics reduce plasma levels of bacterial endotoxins (Vanderhoof and Young, 2001; Fioramonti *et al.*, 2003).

3. **Antibacterial activity**: *Lactobacilli* produce organic compound which inhibit the establishment of other bacteria. The lactic acid produced reduces the pH in the
intestine, which cannot be tolerated by harmful bacteria. Few probiotic bacteria produce H$_2$O$_2$ which inhibits growth of gram-negative bacteria. In addition Streptococcus and Lactobacillus spp. also produce small molecular size protein or antibiotics which inhibit the growth of pathogenic bacteria (Mason et al., 2008).

4. Prevention of amine synthesis: Coliform bacteria decarboxylate amino acid to produce amines, which are toxic irritate the gut and are concurrent with incidence of diarrhoea. Probiotic bacteria prevent proliferation of coliform bacteria by amine production.

5. Enhanced immune competence: Oral inclusion of young ones with Lactobacilli results in elevation of serum proteins and white blood cells (WBC) count. This aids in development of immune system by stimulation of the production of antibodies and increase in number of natural killer cells, monocytes, neutrophils and macrophages with enhanced phagocytic activity (Majamaa and Isolauri, 1997; Hooper and Gordon, 2001; Gill, 2003; Gill and Guarner, 2004). The immune response stimulation by live probiotics has been shown due to their establishment, continuous stimulus and adjuvant properties of cell wall or some proteins. Use of the Lactobacilli after rotavirus vaccination induces IgM-secreting cells and improves IgA seroconversion leading to enhanced immunoglobulin response. The positive effect of probiotics in innate and acquired immunity results most likely from an ability to bind to gut epithelium. After binding, antibody production is enhanced and the complement and reticuloendothelial system is activated. The interaction between probiotics and gut epithelial cells is termed as “bacterial-epithelial cross talk” (Walker, 2000) Probiotics exert their immunity enhancing effect by increasing both non-specific (e.g. phagocyte function, NK cell activity) and specific (e.g. antibody production, cytokine production,
lymphocyte proliferation, delayed-type hypersensitivity) host immune response (Tuo et al., 2011).

1.4 Cellular components of probiotic bacteria and their effect on host immunity

It has been reported that cellular components of LAB, as well as live bacteria and inactivated bacteria could exert immunoregulating effects (Miettinen et al., 1998; Cross et al., 2004). As the live bacteria and inactivated bacteria (Sashihara et al., 2006; Chuang et al., 2007), peptidoglycan, teichoic acid, cell surface protein, exopolysaccharide (Aattouri and Lemonnier, 1997; Amrouche et al., 2006a,b; Makino et al 2006) and unmethylated DNA (Kitazawa et al., 2003,2006;Iliev et al., 2008) from LAB could exert immunoregulatory effects through interaction with pattern recognition receptors (PRRs) of immunocompetent cells (Lebeer et al., 2008; Ng et al., 2009) because the cellular components are considered as ligands of PRRs (Pattern Recognition Receptors).

1.5 Prokaryotic DNA as immunenhancer

Current knowledge indicates that the prokaryotic DNA can activate immune response and has potential therapeutic application as shown in Table 1.1. The initial discovery of this phenomenon was reported by Tokunaga et al. (1984), who found out that in vivo DNA purified from mycobacteria (but not DNA from vertebrates) activated natural killer (NK) cells, fostered the release of IFN-γ by these cells and caused tumor regression. Later, it was confirmed (Shimada et al., 1986; Yamamoto et al., 1992) that bacterial DNA could enhance NK cell activity and induce B cell activation (Messina et al., 1991; Cong et al., 2003).

Table 1.1. Immunostimulatory properties and potential therapeutic applications of CpG DNA (Olishevsky et al., 2003)
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<tr>
<th>Effects on immune system cells</th>
<th>Therapeutic applications</th>
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<td><strong>In vitro effects</strong></td>
<td><strong>In vivo effects</strong></td>
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<td>Rapid activation of B cells.</td>
<td>Induction of B cell</td>
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<td>proliferation and IgM,</td>
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<td>IgG2a production.</td>
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<td>Prevention of spontaneous and</td>
<td>Enhancement of</td>
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<td>induced apoptosis.</td>
<td>resistance to apoptosis.</td>
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<td>Direct and indirect</td>
<td>Increase in</td>
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<td>(via cytokines) activation on</td>
<td>development of innate</td>
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<td>NK cells.</td>
<td>and acquired immune</td>
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<td>Increase in expression of MHC</td>
<td>Properties of non-toxic</td>
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<td>and costimulatory molecules</td>
<td>immune adjuvants.</td>
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<td>by macrophages and dendritic</td>
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<td>cells.</td>
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<td>Concomitant production of Th-1</td>
<td>Induction of</td>
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<td>type cytokines; IL-6, IL-10,</td>
<td>extramedullary</td>
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<td>IL-12, IL-18, IFN-α, chemokines.</td>
<td>haematopoiesis.</td>
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In the mid-1990s, Krieg *et al.* (1995) gained additional insight into the motifs in bacterial DNA that are responsible for the immunostimulatory effects of bacterial DNA. To identify the bacterial DNA sequences responsible for NK activation, the research of immune stimulatory effects of synthetic single-stranded oligodeoxynucleotides (ODNs) was initiated by Carson and Raz (1997). The testing of hundreds of ODNs has revealed that the immunostimulatory effects of these ODNs were dependent on an cytosine-phosphodiester
bond-guanine or cytosine-phosphorothioate-guanine (CpG) dinucleotides that had direct NK stimulatory activity and were potent B-cell mitogens (Hennge et al., 2001; Krieg et al., 1995; Weiner, 2000). Vertebrate immune system has evolved innate immune defense pattern recognition receptors that detect unmethylated CpG motifs within bacterial DNA and these CpG motifs are suppressed and methylated (Weiner, 2000). Indeed, methylation of cytosine residues in the bacterial DNA or in the corresponding oligodeoxynucleotides destroyed their immunostimulatory activities (Krieg et al., 1995).

1.6 Effects of Unmethylated CpG DNA on immunocompetent cells

Unmethylated CpG DNA drive more than 95% of B cells into the cell cycle and also prevents B-cell apoptosis which may be associated with increased expression of several proto-oncogenes and oncoproteins directly and/or indirectly through a rapid and sustained activation of nuclear factor κB (NFκB) (Weiner, 2000; Iliev et al., 2008).

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 (Hennge et al., 2001; Olishewsky et al., 2003). As shown in Fig. 1.3. synthetic oligodeoxynucleotides containing CpG dinucleotides (CpG DNA) exhibit several immunological effects that has led to their use as therapeutic agents and adjuvants for various diseases. 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. These oligodeoxynucleotides containing CpG dinucleotides induce cytokine
production and have potent immunomodulatory activity (Agrawal and Kandimalla, 2002).

Fig. 1.3 Effects of oligodeoxynucleotides containing CpG dinucleotides on host immune cells (Agrawal and Kandimalla, 2002)

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\textsubscript{H}1 CD4 and CD8 T-cell responses (Krieg, 2006).
Over the past few years, there has been a dramatic increase in understanding the molecular and cellular effects of CpG DNA and its effects *in vivo* in animal models. Studies till date suggest that CpG DNA can be used in the treatment of a variety of diseases including infection, allergy and cancer. Moreover, many CpG motifs have been identified in probiotic bacteria (Takahashi *et al.*, 2006).

Keeping in view:

- The variation in bioactivity as shown by various probiotic isolate’s not only at generic but species and strain level also (Bhatia and Kaur, 2012),
- Health effects of probiotics and immunostimulation,
- CpG DNA having significant therapeutic potential,
- Hardly any literature is available where variations at the DNA level and controlled bioactivity have been studied *in vivo*.
AIMS AND OBJECTIVES

The present project was designed with an aim to compare the effect of probiotic bacteria and its DNA on immune response and evaluate its biotherapeutic efficacy with the following objectives:

1. Procurement of different probiotic strains (*Lactobacillus sp. / Bifidobacterium sp.*) and maintenance.

2. Screening of various probiotics for immunomodulatory activity under *in vitro* conditions and selection of probiotic strains having high, medium and low bioactivity.

3. Isolation of DNA from probiotic strains having high, medium and low bioactivity.

4. *In vivo* immuno-characterization of selected probiotic bacteria and with their DNAs.

5. Therapeutic studies of most potent DNA
   - Immunorestorer
   - Anti-cholesteremic
   - Anti-diabetic