CHAPTER – 2

REVIEW OF LITERATURE
2.1 Probiotics

Probiotics are defined as "live micro-organisms that confer a health benefit on the host when consumed in adequate amounts (WHO/FAO). Probiotics were first seriously studied at the Pasteur Institute in Paris at the turn of the 20\textsuperscript{th} century by several leading microbiologists, including Henry Tissier and Eli Metchnikoff. Metchnikoff, a Nobel Prize winner for Medicine in 1908, hypothesized that encouraging the colonies of non-harmful gut flora by adjusting the pH of the stomach could reduce many of the health problems associated with aging. Although this idea of using fermented dairy products to manage the pH proved incorrect, the concept of this theory gained attention and further research was conducted. In 1953, the name "probiotics" was officially given to the group of bacterial strains that had been found to positively enhance the functioning of the digestive tract.

Most probiotics are bacteria, which are small, single-celled organisms. Effects found from one species or strain of probiotics do not necessarily hold true for others, or even for different preparations of the same species or strain (Bengmark, 2003). Some of the commonly used probiotic bacteria include Lactobacillus, Bifidobacteria, and the yeast Saccharomyces boulardii (Table 2.1). They are most commonly used in the form of dairy products, fortified foods and as drugs. Today, evidence is emerging that probiotics offer innumerable benefits to the host by alleviating symptoms of lactose intolerance (Alvarez-Olmos and Oberhelman, 2001), known to prevent acute and traveler's diarrhoea (Bengmark, 2003; Blum et al., 2000), antibiotics associated diarrhoea (Blum et al., 2002), rotaviral diarrhoea (Dsouza et al., 2002), help to prevent the recurrence of cancers, especially bladder and colorectal cancer (Elliott et al., 2005).
Table 2.1 Different types of probiotics (Rastogi et al., 2011)

<table>
<thead>
<tr>
<th>Lactobacillus species</th>
<th>Bifidobacterium species</th>
<th>Streptococcus species</th>
<th>Saccharomyces species</th>
<th>Others</th>
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<tr>
<td>L. acidophilus</td>
<td>B. bifidum</td>
<td>S. thermophilus</td>
<td>S. boulardii</td>
<td>B. ooreus</td>
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<td>L. casei (hamnosus)</td>
<td>B. breve</td>
<td>S. salivarius</td>
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<td>E. coli</td>
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<td>L. fermentum</td>
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<td>Propionibacterium</td>
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<td>L. lactis</td>
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<td>L. salivarius</td>
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<td>L. reuteri</td>
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2.2 Characteristics of good probiotics

Fuller and Perdigon, (2001) listed the following as features of a good probiotic: It should be a strain, which is capable of exerting a beneficial effect on the host animal, e.g.

- It should be nonpathogenic and nontoxic
- It should be present as viable cells, preferably in large numbers
- It should be capable of surviving and metabolizing in the gut environment, e.g. resistant to low pH and organic acids
- It should be stable and capable of remaining viable for periods under storage and field conditions.

2.3 Health benefits of Probiotics

2.3.1 Probiotics and the immune system

Gill (2003); Gill and Guarner (2004) studies have shown that probiotics directly enhance the activity of human DC populations to promote Th1 differentiation. In animal studies, probiotic supplementation induces regulatory T-cell populations and in human studies probiotic ingestion increases production of regulatory cytokines (IL-10) in vitro.
Menard et al. (2005) analyzed the effect of oral administration of living LAB, or their conditioned media on the epithelial and immune functions of colitis-prone C57BL/6 IL-10 deficient mice. The results showed more reinforced intestinal barrier capacity and stimulation of Th 1 immune response, highlighting the involvement of LAB-derived components in host defence.

Johnson-Henry et al. (2005) determined that pretreatment of mice with a mixture of *L. rhamnosus* and *L. acidophilus* could attenuate *C. rodentium* induced colonic disease in mice. Results showed that probiotics (10⁹ cfu/ml) fed mice remained healthy and pretreatment with probiotics attenuates the effects of *C. rodentium* infection in mice.

Baken et al. (2006) demonstrated the stimulation or suppression of T helper (Th 1) mediated immune response by various strains. *L. casei Shirota* (LcS) mainly enhanced the innate immune response and promoted Th 1 mediated immune reactivity.

Mason et al. (2008) suggested some mechanisms for the effect of probiotics on intestinal microflora such as lowering the intestinal pH, release of gut protective metabolites, regulation of intestinal motility, and mucus production. Gastrointestinal mucosa is the primary interface between the external environment and the immune system. Whenever intestinal microflora reduces, antigen transport is increased indicating that the normal gut microflora maintains gut defenses. The nonpathogenic probiotic bacteria interact with the gut epithelial cells and the immune cells to start the immune signals. These bacteria interact with M cells in the Peyer's patches, with gut epithelial cells, and with associated immune cells.

Seth et al., 2008 and Ewaschuk et al., 2008 reported that the immune effects of probiotic bacteria are also mediated by soluble peptides which are secreted into the medium. The biochemical pathways mediating the probiotic effect on tight junction
function include protein kinase C and MAP kinase pathways which involves both redistribution and altered expression of the tight junction proteins occludin and claudins.

Kim et al. (2009) proved that macrophage cultured with LAB strains resulted in increased production of NO and interleukin (IL)-1β, IL-6, IL-12 and TNF-α.

Nandakumar et al. (2009) concluded that consumption of beneficial bacteria such as *L. acidophilus* and *L. casei* reinforce intestinal mucous membrane immunity (mucosal immunity) as well as the body's global immunity (systemic immunity). *Lactobacillus* stimulates phagocytic activity and increases production of T and B lymphocytes and production of antibodies, particularly IgM, IgA and IgG.

Immunomodulatory activity of LABs on influenza virus (IFV) infection in relation to their efficacies was demonstrated by Shiro et al. (2011) in IFV-infected mice. It was found that *Lactobacillus plantarum* was effective in protecting the body weight loss of infected mice, reduced the virus yields in the lungs and prolonged the survival time without toxicity. The total numbers of infiltrated cells in the bronchoalveolar lavage fluid (BALF), especially macrophages and neutrophils, were significantly reduced. Further, it was observed that LAB enhances the interferon-α and Th1 cytokine production through intestinal immunity and the reduces TNF-α in the early stage of infection.

Ou et al. (2011) investigated that heat-killed lactic acid bacteria not only possess immunomodulatory functions but also provide the advantages of longer product shelf life, easier storage, and more convenient transportation. The results indicated that the adhesion of lactic acid bacteria decreases with increases in temperature. However, heat exposure did not influence immunomodulatory activity. Moreover, Th1 associated cytokines was increased and Th2 associated cytokines reduced. Similar to this, Bhatia and Pawan, (2007) observed that dead bacteria also enhance the immune response.
Cunningham-Rundles et al. (2011) studied that probiotic administration protects the gut surface and could delay progression of Human Immunodeficiency Virus type1 (HIV-1) infection to the Acquired Immunodeficiency Syndrome (AIDS). They proved that probiotic bacteria enhances growth in infants with congenital HIV-1 infection. Moreover, probiotic bacteria may stabilize CD4+ T cell numbers in HIV-1 infected children and are likely to have protective effects against inflammation and chronic immune activation of the gastrointestinal immune system.

Castillo et al. (2011) evaluated that the oral administration of a probiotic Lactobacillus modulates cytokine production and TLR expression improving the immune response against Salmonella enterica serovar typhimurium infection in mice. The oral administration of L. casei induces variations in the cytokine profile and provides an alternative way to reduce the severity of the infection.

Lin et al. (2011) indicated that probiotics have beneficial effects on regulating T cell mediated immune responses by attenuating mitogen-induced overactive immune responses and promoting Th1 immune response. They concluded that high concentrations ($\geq 1 \times 10^6$ CFU/ml) of probiotic inhibited mitogen-induced cell proliferation and arrested the cell cycle at the G0/G1 stage in both mitogen-stimulated spleen cells and in human peripheral blood mononuclear cells (PBMCs). In the results of low concentrations ($<$1 $\times 10^6$ CFU/ml), probiotic enhanced the production of IFN-$\gamma$ but inhibited the production of IL-4. Similar to this, Randhawa et al. (2011) proved that Lactobacillus delbruckii 405 and Lactobacillus casei subsp. casei 17 separately and in a 1:1 combination ($10^6$cells /ml) acted synergistically in vivo, as the effect was more profound in the lowering of cholesterol levels and in the augmentation of the immune system.
Evrard et al. (2011) studied that the response of the immune system to probiotics remains controversial. Some strains modulate the cytokine production of dendritic cells (DCs) *in vitro* and induce a regulatory response, while others induce conversely a pro-inflammatory response. These strain-dependent effects are thought to be linked to specific interactions between bacteria and pattern recognition receptors. Results revealed that the probiotic induced a dose-dependent immunomodulation of human DCs, at high doses.

Fanning et al. (2012) evaluated that *Bifidobacterial* surface-exopolysaccharide facilitates commensal-host interaction which was found through immune modulation and pathogen protection. The beneficial role for exopolysaccharide in modulating various aspects of *bifidobacterial*-host interaction includes the ability of commensal bacteria to remain immunologically silent and in turn provide pathogen protection.

Kaur and Bhatia, (2012) reported the immune modulatory potential of four strains of *Lactobacillus acidophilus* in swiss albino mice. The results showed that immunomodulation by *L. acidophilus* is strain specific and it is asserted that orally supplemented *L. acidophilus* could exert an indirect effect on T-lymphocyte activity through stimulation of other cell types, such as phagocytes.

Stoeker et al. (2013) observed that HIV infection is associated with intestinal mucosal dysfunction and probiotics offer the therapeutic potential to enhance the mucosal barrier in HIV+ patients. Response of immunocompromised hosts to probiotics was evaluated by orally administrating *Lactobacillus acidophilus* to cats with chronic feline immunodeficiency virus (FIV) infection. FIV infection significantly affected transcellular, but not paracellular, transport of small molecules across the intestinal epithelium. Additionally, probiotic treatment of FIV+ cats resulted in changes in cytokine release and mucosal leukocyte percentages that were not paralleled in FIV- cats. These results
suggested a novel role for FIV in upregulating transcellular transport across the gastrointestinal epithelial barrier and demonstrate the potential therapeutic use of probiotic bacteria to restore intestinal homeostasis.

Kwon et al. (2013) studied the immunomodulatory effect of mixture of probiotics (IRT 5) that could suppress diverse experimental inflammatory disorders. The prophylactic and therapeutic effects of IRT5 probiotics was evaluated in experimental autoimmune encephalomyelitis (EAE), a T cell mediated inflammatory autoimmune disease of the central nervous system. Pretreatment of IRT5 probiotics before disease induction significantly suppressed EAE development. In addition, treatment with IRT5 probiotics to the ongoing EAE delayed the disease onset. Administration of IRT5 probiotics inhibited the pro-inflammatory Th1/Th17 polarization, while inducing IL10+ producing or/and Foxp3+ regulatory T cells, both in the peripheral immune system and at the site of inflammation. Collectively, data suggested that IRT5 probiotics could be applicable to modulate T cell mediated neuronal autoimmune diseases, including multiple sclerosis.

2.3.2. Reduction in serum cholesterol

Kiessling et al. (2002) evaluated that long-term consumption of fermented dairy products having probiotics over 6 months increases the HDL cholesterol and lead to the desired improvement of the LDL/HDL cholesterol ratio. A randomized, crossover, and placebo-controlled design trial involving 29 women to evaluate the hypocholesterolemic effect of yoghurt supplemented with L. acidophilus 145 and B. longum 913 was used. The crossover study, of 21 weeks’ duration, involved the administration of 300 g/day yoghurt, and the results obtained showed that HDL-cholesterol was increased significantly (P < 0.05) by 0.3 mmol/L and the ratio of LDL/HDL cholesterol decreased from 3.24 to 2.48.
Sindhu and Khetarpaul (2003) conducted another placebo-controlled study to evaluate the effects of a probiotic fermented food on serum cholesterol levels in 20 young Swiss mice. The experimental group was fed a food mixture containing probiotics and 1% cholesterol while the control group was fed food without probiotics, but containing 1% cholesterol for 42 days. The authors reported that the feeding of *L. casei* NCDC-19 (10⁹ CFU) and *Saccharomyces boulardii* (10⁹ CFU) caused a 19% reduction in the total serum cholesterol, while LDL cholesterol levels was reduced by 37% after the 42 day feeding trial.

Liong and Shah,(2005a) reported that cholesterol could be removed from media by *L. acidophilus* not only through assimilation during growth, but also through binding of cholesterol to the cellular surface. This mechanism was proposed when both non-growing cells and dead cells of probiotics were also found to remove cholesterol.

Liong and Shah,(2005b) postulated another hypocholesterolemic mechanism that involves the ability of certain probiotics to enzymatically deconjugate bile acids. Deconjugation of conjugated bile salts to deconjugated bile salts is catalyzed by bile salt hydrolase (BSH; cholyglycine hydrolase; EC 3.5.1.24), which is the enzyme that catalyzes the hydrolysis of glycine- and/or taurine-conjugated bile salts into amino acid residues and free bile acids. BSH activity has been detected in intestinal bacteria such as *Lactobacillus* and *Bifidobacterium* sp which reduced the cholesterol level.

Park et al.(2007) also evaluated the effects of probiotic on cholesterol metabolism in 36 male hypercholesterolemic rats. The authors found that the supplementation of *L. acidophilus* ATCC 43121 (2 × 10⁶ CFU/day) for 21 days not only reduced total serum cholesterol by 25%, but also significantly (P < 0.05) reduced very low density lipoprotein, intermediate density lipoprotein and LDL cholesterol, compared to the control.
Lye et al. (2010) provided experimental evidence to strengthen the hypothesis that probiotics could remove cholesterol via the incorporation of cholesterol into the cellular membrane and conversion of cholesterol to coprostanol. The strains studied may be potential health adjunct cultures in fermented dairy products with possible in vivo hypocholesterolemic effects.

Guo et al. (2011) conducted a meta-analysis of randomised controlled trials that evaluated the effects of probiotics consumption on blood lipids. These results indicated that a diet rich in probiotics decreases total cholesterol and LDL cholesterol concentration in plasma for participants with high, borderline high and normal cholesterol levels.

Wang et al. (2012) investigated the effect of adlay milk and adlay-soymilk fermented with Lactobacillus plantarum or Lactobacillus paracasei on lipid metabolism in hamsters fed with a cholesterol-enriched diet. Adlay milk and fermented adlay milk with or without soymilk administered to hamsters significantly decreased (p < 0.05) serum cholesterol levels and ratio of low-density lipoprotein cholesterol to high-density lipoprotein cholesterol, when compared to a high-cholesterol diet group; there was also a significant (p < 0.05) increase in the level of fecal cholesterol and triglycerides.

Similar to this, Starovoitova et al. (2012) selected potential probiotic lactobacilli for cholesterol-lowering properties and compare the effect of the Lactobacillus strains in vivo on lipid metabolism in rats fed with a high-lipid diet. Results indicated that Lactobacillus strains were able to lower cholesterol in vitro, and reduce cholesterol effectively in vivo.

Awaisheh et al. (2013) evaluated the effect of supplementation of probiotics and phytosterols alone or in combination on serum and hepatic lipid profiles and thyroid hormones of hypercholesterolemic rats. Mixed probiotics treatment consisted of 8 probiotic strains: 2 strains of each of Lactobacillus acidophilus, Lactobacillus...
casei, Lactobacillus gasseri, and Lactobacillus reuteri. The rats were fed for 8 wk with the given treatments in addition to a high-fat-high-cholesterol basal diet to induce hypercholesterolemia. Results showed that supplementation significantly reduced serum total cholesterol, low-density-lipoprotein cholesterol (LDL-C), high-density-lipoprotein cholesterol, and triglycerides compared with the controls. The symbiotic treatment was more effective in lowering LDL-C, whereas mixed probiotics treatment more effectively lowered serum total cholesterol and LDL-C than the phytosterol-containing treatment.

Kumar et al. (2013) observed that the probiotic Lactobacillus rhamnosus GG and Aloe vera gel improve lipid profiles in hypercholesterolemic rats. The combination of LGG and AV gel have a therapeutic potential to decrease cholesterol levels and the risk of cardiovascular diseases.

2.3.3. Roles of Probiotics on diabetes

Tabuchi et al. (2003) evaluated neonatally streptozotocin-induced diabetic mice which were given food containing Lactobacillus. The Lactobacillus cells improved glucose tolerance and the serum insulin level was significantly higher than in the control group at 30 min after glucose loading (p<0.05).

Yamano et al. (2006) studied that oral administration of Lactobacillus casei reportedly reduces blood glucose concentrations in a non-insulin-dependent diabetic mice. In order to determine if other lactobacillus strains affect glucose metabolism, they evaluated the effect of the probiotic strain Lactobacillus johnsonii La1 (LJLa1) strain on glucose metabolism in rats. Oral administration of LJLa1 via drinking water for 2 weeks inhibited the hyperglycemia induced by intracranial injection of 2-deoxy-D-glucose (2DG). Oral administration of LJLa1 for 2 weeks also reduced the elevation of blood glucose and
glucagon levels after an oral glucose load in streptozotocin-diabetic rats. They suggested that probiotics might improve glucose tolerance by reducing glucagon secretion via alteration of autonomic nerve activities.

Yadav et al. (2007) observed that probiotic fermented product dahi containing *L. acidophilus* and *L. casei* significantly delayed the onset of glucose intolerance, hyperglycemia, hyperinsulinemia, dyslipidemia and oxidative stress in high fructose induced diabetic male albino wistar rats. Values for blood glucose, glycosylated hemoglobin, glucose intolerance, plasma insulin, liver glycogen, plasma total cholesterol, triacylglycerol, low-density lipoprotein cholesterol, very low-density lipoprotein cholesterol and blood free fatty acids were increased significantly after 8 wk of high fructose feeding. However, the dahi-supplemented diet restricted the elevation of these parameters in comparison with the high fructose-fed control group.

Salami et al. (2008) reported the action of gliclazide, a sulphonylurea with beneficial extrapancreatic effects in diabetes, which may be enhanced by administering probiotics. They investigated the influence of probiotics on gliclazide pharmacokinetics and the effect of both probiotics and gliclazide on blood glucose levels in healthy and diabetic rats. They concluded that the probiotic treatment of diabetic rats increases gliclazide bioavailability and lowers blood glucose levels by insulin-independent mechanisms, suggesting that the administration of probiotics may be beneficial as adjunct therapy in the treatment of diabetes.

2.4 Prokaryotic DNA as immunenhancer

Among new approaches to immunization, the development of new adjuvants and immunomodulators is of special interest. Bacteria, their components and metabolic
products may serve as strong adjuvants with immunomodulating effects and may be used in immuno vaccinotherapy of different diseases.

2.4.1 History

The antitumor effects of microbial materials were firstly discovered in the 19th century. Coley (1893), a New York surgeon, performed a series of studies evaluating the antitumor activity of bacteria. In his initial studies, Dr. Coley injected vital *Streptococci* directly into the tumor masses of his patients. Such effort resulted in tumor regression in the patient that lasted for 7 years. The administration of such preparation known as “Coley’s toxin” that resulted in tumor regression was thought to be attributed to endotoxin.

Wiemann and Starnes (1994) reported that Coley’s original success was with *Streptococcus*, a gram-positive organism that does not produce endotoxin. In fact, bacterial extracts such as Coley’s toxins demonstrated remarkable effects in the immunotherapy of cancer and other diseases.

In the mid-1990s, Krieg *et al.* (1995) gained additional insight into the motifs in bacterial DNA that are responsible for immunostimulatory effects of bacterial DNA. It was observed that the vertebrate immune system has evolved innate immune defense pattern recognition receptors that detect unmethylated CpG motifs within bacterial DNA.

Scheule (2000) reported that bacterial DNA contains a much higher frequency of CpG dinucleotides than are present in mammalian DNA. Furthermore, bacterial CpG dinucleotides are often not methylated. It is thought that these two features in combination with specific flanking bases constitute a CpG motif that is recognized as a “danger” signal by the innate immune system of mammals and therefore an immune response is induced when these motifs are encountered. These immunostimulatory activities of bacterial CpG DNA can also be achieved with synthetic CpG...
oligodeoxynucleotides (ODN).

Mutwiri et al.(2003) proved that recognition of CpG motifs by the innate immune system required engagement of Toll-like receptor 9 (TLR-9), which induced cell signaling and subsequently triggered a pro-inflammatory cytokine response. Cytokines released by these cells in response to CpG motifs in turn activate other immune cells, such as Natural Killer cells and T cells, and can drive the development of adaptive immune responses.

2.4.2 Cellular immunology of CpG DNA

Yi et al.(1999) investigated that unmethylated CpG dinucleotides in bacterial DNA or synthetic CpG-S DNA rapidly activate murine B-cells to secrete IL-6, IL-10 and IgM, as well as their proliferation.

Cong et al.(2003); Iliev et al.(2005); Xu et al.(2008); Klinman et al.(2009) studied that the self-stabilized CpG DNAs activated human B cells and induced plasmacytoid dendritic cells to secrete high levels of IFN-α.

Olishevsky et al.(2003) investigated that CpG DNA promotes lytic activity of NK cells and the secretion of IFN-γ. However, the stimulatory effect is not sufficient since CpG DNA does not stimulate highly purified NK cells which are potently stimulated by type-1 IFNs, IL-12 and TNF-α as shown in Fig.2.1.

Huang et al.(2005); Talati et al.(2008); Yabuki et al.(2010) reported that monocytes and macrophages are induced to produce proinflammatory cytokines including IL-6, IL-12, IFN-α, IFN-β, TNF-α, IL-1β, and IL-18 and mediate antibody dependent cellular cytotoxicity (ADCC). Phenotypic changes of APCs are similar to those observed in B cells and included the upregulation of MHC class-I and MHC class-II.
Krieg (2006) proved that unmethylated CpG DNA activated 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<sub>H</sub>1 CD4 and CD8 T-cell responses.

![Diagram of immune response](image)

Fig.2.1 The effects of CpG DNA on cells of innate and adaptive immunity (Olishevsky et al., 2003).

### 2.4.3 Immunomodulatory properties of CpG DNA

Synthetic oligodeoxynucleotides (ODN) containing immunostimulatory “CpG motifs” interact with Toll-like receptor 9 to initiate an immunostimulatory cascade that culminates in the maturation, differentiation and/or proliferation of multiple cell types, including lymphocytes, dendritic cells, NK cells, monocytes and macrophages (Klinman et al., 1996; Stacey et al., 1996; Hemmi et al., 2000; Takeshita et al., 2001; Gursel et al., 2002; Hornung et al., 2002). Together, these secrete cytokines and chemokines that create
a pro-inflammatory (IL-1, IL-6, IL-18 and TNF) and Th1-biased (IFN gamma and IL-12) immune milieu (Krieg et al., 1995; Klinman et al., 1996; Ballas et al., 1996; Halpern et al., 1996; Hemmi et al., 2000; Takeshita et al., 2001; Ishii et al., 2002). In humans, TLR9 is primarily present within human B cells and plasmacytoid DC, while in mice multiple cells of the myeloid lineage (including monocytes, macrophages and DC) express TLR 9 and directly respond to CpG stimulation (Bauer et al., 2001; Kadowaki et al., 2001; Krug et al., 2001).

Cong et al. (2003) studied the ability of self-stabilized CpG DNAs to stimulate human B-cell proliferation and interferon-α (IFN-α) secretion in plasmacytoid dendritic cell (pDC) culture assays. Self-stabilized CpG DNAs activated human B cells and induced plasmacytoid dendritic cells to secrete high levels of IFN-α. While both stimulatory and secondary structures in CpG DNAs were required for pDC activation, CpG motifs are sufficient to activate B cells.

Hacker et al. (2004) investigated the presence of bifidobacteria and lactobacilli and their DNA in the human placenta. DNA from intestinal bacteria was found in most placenta samples. The results suggested that horizontal transfer of bacterial DNA from mother to foetus may occur via placenta. Although the newborn infant was considered immunologically immature, when exposed to bacterial DNA, they may programme the infant’s immune development and can improve immune response.

Klinmann (2004) reported that CpG oligodeoxynucleotides (ODNs) directly activate human B cells and plasmacytoid dendritic cells, creating an immune milieu that is rich in pro-inflammatory and T helper 1 (T_{H1})-type cytokines. This innate immune response forms a foundation on which antigen-specific adaptive immunity is based. CpG ODNs facilitate the generation of humoral and cellular vaccine-specific immunity by
improving the function of professional antigen-presenting cells (APCs) as shown in Fig.2.2.

Chuang et al. (2007) suggested 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. CpG motifs with different sequences have been shown to induce various types or levels of immunostimulatory responses whereas the immunostimulatory effects of CpG motifs are species-specific.

Medina et al. (2007) investigated that the immunostimulatory effects of genomic DNA from different B. longum strains led to the production of the proinflammatory cytokines IFN-gamma and TNF-α. DNA extracted from pure cultures of the probiotic mixture and from human faeces collected after probiotic ingestion influence cytokine production by PBMC decreasing IL-1β and increasing IL-10. The ability of the probiotic mixture to attenuate experimental colitis was mediated by DNA via TLR9 signalling. It has been suggested that the high guanine cytosine (GC) content (58–61%) of the Bifidobacterium genus could favour IL-10 production. The differential effects exerted by the B. longum strains could be due to differences in the presence or redundancy of CpG motifs in their DNAs, as it is demonstrated that some of these motifs exert a more pronounced immunomodulatory effect than others.

Iliev et al. (2008) showed that pure cells, cell wall components and some soluble factors from genomic DNA of Lactobacillus rhamnosus GG (LGG) are a potent inducer of splenic B cell proliferation, CD86/CD69 expression, interleukin (IL)-6, IL-12, IL-18, interferon gamma (IFN-gamma), tumour necrosis factor alpha (TNF-alpha) mRNA expression and IFN-gamma/IL-12p70 protein production assays.

Talati et al. (2008) demonstrated the role of bacterial DNA in macrophage activation by group B streptococci (GBS) which is an important cause of neonatal sepsis and meningitis. Data suggested that more than one-third of the macrophage TNF response to whole, antibiotic-treated GBS is triggered by bacterial DNA via TLR9. Similarly, more than half of macrophage IL-6 and IL-12 production under these conditions was stimulated via the CpG DNA/TLR9
pathway. TNF is an early response gene in activated macrophages, while NO production is
delayed and largely due to autocrine and paracrine signals that lead to increased iNOS protein
expression.

Mutwiri et al. (2009) reported that CpG oligodeoxynucleotides (ODN) activate the
immune system and are promising immunotherapeutic agents against infectious diseases
and cancer. CpG ODN are potent immune activators in mice, their immune stimulatory
effects are often less dramatic in humans and large animals. This disparity between rodents
and mammals has been attributed due to the differences in TLR9 expression in different
species.

Yabuki (2010) investigated CpG oligonucleotides activate the immune response in
burned mice as immunosuppression after burn injury increases the risk of sepsis and
multiple organ failure. CpG treatment partially reversed the reduction of class II antigen
expression and synthesis of cytokines (interleukin-12, tumor necrosis factor-α, interleukin-
6, and interleukin-1) by splenic macrophages after burn injury.

2.5 Applications of CpG DNA

2.5.1. Protection against infectious diseases

Huang et al. (2005) demonstrates that bacterial DNA plays an important role in the
macrophage response to a heat-killed intracellular pathogen Brucella abortus.

Talati et al. (2008) recently reported that the bacterial DNA/TLR9 pathway is
implicating in early host defense against Streptococcus pneumoniae.

Zhu et al. (2008) proved protective effect of CpG-DNA against mastitis induced by
Escherichia coli infection in a rat model. An intramuscular injection of CpG-DNA
(200 μg) induced more rapid migration of polymorphonuclear leukocytes (PMNs) from the
blood to mammary tissue at the initial stage of infection, stimulated the secretion of IL-6
and TNF-α at different time points, reduced viable *E. coli* in mammary tissues and decreased the activity of *N*-acetyl-β-d-glucosaminidase (NAGase). CpG-DNA also promoted the expression of its specific receptor TLR-9 mRNA in mammary tissue.

Patel *et al.* (2008) demonstrated that intramuscular administration of oligodeoxynucleotides containing CpG motifs (CpG-ODN) induces protection in neonatal chicks against a lethal challenge of *Escherichia coli* by increasing the expression of IL-1β, IL-6, IL-8, IL-10, IL-18, IFN-γ and MIP-3α mRNAs in the spleen and IL-10 and IFN-α in bursa of Fabricious of chicks that had received CpG.

Han *et al.* (2009) contributed to a better understanding on the therapeutic effects of CpG-DNA against infectious diseases. Defensins have a broad range of antimicrobial activity against bacteria, fungi, and viruses. The expression of human β-defensin-2 (hBD-2) is prevalently observed in epithelial cells and is induced by bacterial infection. It was proved that expression of the hBD-2 gene and release of hBD-2 protein into the medium is up-regulated in response to CpG-DNA in human B cell line RPMI 8226.


Judy *et al.* (2012) investigated that the prophylactic application of CpG oligonucleotides augments the early host response and confers protection in acute melioidosis. Melioidosis is an infectious disease caused by a Gram-negative bacterium,
*Burkholderia pseudomallei*, found in soil and water. It exists in acute and chronic forms. Symptoms may include pain in chest, bones or joints, cough, skin infections, lung nodules and pneumonia. Protection of CpG treated animals was associated with recruitment of inflammatory monocytes and neutrophils into the lungs prior to infection. These responses correspond with early control of bacterial growth, a dampened inflammatory cytokine/chemokine response, reduced lung pathology and greatly increased survival.

### 2.5.2 Development of effective vaccine/Vaccine adjuvant

Krieg *et al.* (1995) proposed the mechanisms contributing to the strong adjuvant activity of the CpG motifs for inducing humoral immunity may include:

i) synergy between TLR9 and the B cell receptor (BCR) preferentially stimulating antigen specific B cells ; ii) inhibition of B cell apoptosis improving B cell survival (Yi *et al.*,1999) iii) enhanced IgG class switch DNA recombination, which may enhance the maturation of the immune response (Liu *et al.*,2003;He *et al.*,2004).

Vabulas *et al.* (2000) reported that CpG induce stronger Th1 immune responses than any other single vaccine adjuvant compared in mouse models. This Th1 biased response is maintained even in the presence of Th2 promoting vaccine adjuvants such as alum (Sugai *et al.*,2005) and with vaccination in both young and elderly mice (Weeratna *et al.*,2001;Zhou *et al.*,2003;Alignani *et al.*,2005).

McCluskie and Davis,(2000) quoted that most vaccine studies with CpG DNA have been performed using subcutaneous or intramuscular injection, but they are also effective as mucosal vaccine adjuvants for the respiratory tract , vaginal mucosa (Kwant and Rosenthal,2004), oral or intrarectal vaccination (Dong *et al.*,2005) conjunctival vaccination (Nesburn *et al.*,2005), and even for transcutaneous immunization (Berry *et al.*,2004). Vaccination through mucosal routes has succeeded in inducing both local and
systemic humoral and cellular immune responses including enhanced protection against infectious challenge (Gallichan et al., 2001; Dumais et al., 2002).

Manning et al. (2001) findings suggest that the incorporation of CpG ODNs into vaccine formulations provided to the aged could prove useful in the development of more effective vaccines for the elderly. The immunization of aged mice with Diphtheria toxoid in formulations containing unmethylated immunostimulatory CpG motifs, promotes the successful development of immune response. Aged mice given vaccines containing CpG oligodeoxynucleotides (ODNs) expressed primary and secondary systemic humoral immune responses having isotype profiles consistent with an enhancement in Th1 type immunity. Dendritic cells (DCs) were determined to represent one of the cellular targets of CpG ODN activities in aged mice.

Weerantna et al. (2001) investigated that in mice, vaccine doses can be reduced by approximately 99% if a CpG ODN vaccine adjuvant is incorporated. In theory, a strong vaccine adjuvant might reduce the dose of vaccine antigen needed to induce protection, with the potential for reduced costs and improved safety. Such an effect could be extremely important in the setting of influenza vaccination, where production of adequate quantities of a new vaccine to a pandemic strain may be limiting.

Kojima et al. (2002) observed adjuvant effect of multi-CpG motifs on an HIV-1 DNA vaccine. Based on evidence that the immunogenicity of DNA vaccines can be augmented by the addition of CpG motifs, 5–20 additional CpG motifs were cloned into a pUC-derived plasmid. These results suggest that plasmids containing multiple CpG motifs may improve the immunogenicity of DNA vaccines.

Sugai et al. (2005) proved that a CpG-containing oligodeoxynucleotide acts as an efficient adjuvant, counterbalancing the Th1/Th2 immune response in diphtheria-tetanus-
pertussis vaccine. Diphtheria-tetanus-pertussis (DPT) vaccine contains not only aluminum hydrate (alum) to enhance the immune response to the vaccine ingredients, but also has a principal ingredient for pertussis toxin (PT). However, both adjuvants strongly promote T helper Th2 type immune responses. Th1 and Th2 type immune responses are counterbalanced in vivo, and a Th2 prone immune response is not effective against intracellular infections but promotes IgE production, which is related to allergic disease. Administration of DPT vaccine with CpG-ODN (DPT-alum/ODN) to mice significantly reduced the total IgE levels and increased the anti-PT specific IgG2a titer in serum, in comparison with ordinary DPT vaccine (DPT-alum).

Cooper et al. (2005) evaluated CpG DNA in human clinical trials as adjuvants for hepatitis B surface antigen either in combination with alum or alone.

McCluskie and Krieg (2006) investigated that with the increasingly widespread recognition of their strong immune effects, CpG DNA have become the vaccine adjuvant of choice in many experimental models, including antigens such as peptide or protein antigens, live or killed viruses, dendritic cell vaccines, autologous cellular vaccines, and polysaccharide conjugates.

Strandskog et al. (2008) first time reported that Double-stranded RNA and CpG DNA induced immune responses in Atlantic salmon. dsRNA and CpG DNA in fish mimic viral recognition, resulting in an enhanced innate immune response that could be used for vaccination.

Maeyama et al. (2009) examined the effects of oligo B, which is a synthetic CpG-DNA, in mucosal administration of Bacillus Calmette Guérin (BCG) and diphtheria toxoid (DT). Co-administration with oligo B enhanced BCG induced delayed type hypersensitivity to purified protein derivative (PPD) in guinea pigs. The analysis of
antibody subclasses showed that intranasal administration of oligo B induced not only IgG1 but also IgG2a, IgG2c and IgA anti-DT antibodies. In contrast, there was no or little production of the anti-DT serum IgE. Therefore CpG DNA can be exploited as a powerful adjuvant in mucosal immunization.

Maurer et al. (2011) examined that cytosine-phosphorothioate-guanine (CpG DNA) oligonucleotides (ODN) boost cytokine and costimulatory molecule expression on murine bone marrow-derived dendritic cells (mBMDC). It was concluded that CpG-DNA acts as potent adjuvant for vaccination therapies. CpG-ODN mediated immunotherapy represents a potentially inexpensive, safe, easy-to-produce, and easy-to-handle treatment alternative.

**2.5.3 Prevents development of allergic diseases**

Liu et al. (2003) showed that the Th1-biased immune response induced by CpG DNA may facilitate the development of improved allergy vaccines, to reprogram the pathogenic Th2 allergic response. CpG DNA redirect the allergic Th2 response in allergic mice, preventing inflammatory disease manifestations. A conjugate of a CpG DNA to a portion of the ragweed allergen has been evaluated in human clinical trials as an allergy vaccine, with encouraging evidence for a selective and specific redirection of the allergic Th2 response towards a non-allergic and non-inflammatory Th1 response, and showing apparent clinical benefit with reduced allergic symptoms (Simons et al., 2004).

Kitagaki et al. (2005) quoted that oral administration of CpG DNA can protect against eosinophilic airway inflammation in murine model of asthma in association with reduction of antigen-specific IgE in a dose dependent manner. CpG-ODNs may be useful as a component of oral immunotherapy to promote tolerance in established asthma.

Takahashi et al. (2006) reported the antiallergic activities of the immunostimulatory CpG, identified from genomic DNA of *Bifidobacterium longum* BB536 from *in vitro* and
in vivo studies. The authors evaluated the efficiency of CpG DNA in preventing allergic responses by oral administration. Oral administration of BL07S suppressed serum ovalbumin (OVA) specific immunoglobulin (Ig) E levels and improved the OVA-specific IgG2a/IgG1 ratio. ODN BL07S increased Th1 cytokine and decreased Th2 cytokine production in splenocytes.

Cong et al. (2007) reported that intranasal immunization with chlamydial protease-like activity factor (CPAF) and CpG deoxynucleotides enhances protective immunity against genital Chlamydia muridarum infection. I.n. or intraperitoneal (i.p.) vaccination with CPAF plus CpG deoxynucleotides (CpG), an alternative T helper 1 (Th1) adjuvant, induced robust CPAF-specific IFN-γ responses and elevated levels of serum antibody and vaginal IgA production. CPAF + CpG vaccinated animals displayed accelerated genital chlamydial clearance and inflammatory cellular infiltration compared to mock-immunized (PBS) challenged animals. Together, CpG deoxynucleotides are an efficacious alternative Th1 adjuvant with CPAF to induce protective anti-chlamydial immunity.

Suzuki et al. (2007) demonstrated, for the first time, that immunotherapy with CpG DNA conjugated with a T-cell peptide is useful in preventing and treating allergic conditions.

Xu et al. (2008) proved the ability of immunomodulatory unmethylated CpG oligodeoxynucleotides (ODN), which are potent inducers of Th1 cytokines, to prevent allergic symptoms in mice immunized and sensitized with allergen. In vitro analysis revealed that CpG ODN inhibited class switching from IgM to IgE and IgG1 in response to CD40 and IL-4 in B cells, and this effect did not correlate with up-regulation of IFN-α production. These results imply a B cell-intrinsic, T cell-independent mechanism by which
CpG ODN directly acts on B cells and inhibits IgE and IgG1 production leading to prevention of allergic symptoms.

Klinman *et al.* (2009) suggested that CpG oligonucleotides can act as adjuvants for vaccines targeting infectious diseases. These effects are optimized by maintaining close physical contact between the CpG ODN and the immunogen. Co-administering CpG ODN with a variety of vaccines has improved the humoral and/or cellular immune responses, culminating in enhanced protective immunity in rodent and primate challenge models.

Kaburaki *et al.* (2011) suggested that Japanese cedar pollen allergen (Cry j 1) conjugated with CpG oligodeoxynucleotide (Cry j 1–CpG) immunization can induce Cry j 1-specific Th1 immune responses, thereby inhibiting IgE response to the pollen allergen.

### 2.5.4 Antitumor immunization

The antitumor effects of microbial materials were firstly discovered in the 19th century. Coley (1893), a New York surgeon, performed a series of studies evaluating the antitumor activity of bacteria. Bacterial extracts such as Coley’s toxins demonstrated remarkable effects in the immunotherapy of cancer and other diseases.

Cornet *et al.* (2006) proved that CpG DNA activate dendritic cells *in vivo* and induce a functional and protective vaccine immunity. Study of the CD8 response obtained after antigenic challenge suggested that a functional memory response is induced upon vaccination with ODN-CpG. Thus, MHC class I-restricted epitope in combination CpG DNA is a promising and rather simple cancer vaccine formulation.

Weerantna *et al.* (2009) reported that CpG DNA or Synthetic oligodeoxynucleotides containing immunostimulatory CpG motifs (CpG ODN) act as ligands for TLR9. CpG DNA can be used successfully in tumor immunotherapy in animal models as well as in human clinical trials.
Zhou et al. (2010) reported that synthetic oligodeoxynucleotides containing CpG motifs (CpG DNA) can activate immunocompetent cells which offer the potential advantage of antitumor activity. In this study, cationic liposomes were complexed with CpG DNA (CpG DNA lipoplex) to prevent pulmonary metastasis following intranasal administration in mice.