CHAPTER 4

Immunomodulatory Studies
CHAPTER 4.1

Immunomodulatory Effect of MBTU PBBM1 Spores in Balb/C Mice
4.1 IMMUNOMODULATORY EFFECT OF MBTU PBBM1 SPORES IN BALB/C MICE

4.1.1 Introduction

In normal healthy animals, the gastrointestinal tracts are colonized by a complex microflora containing many different species. A balance of these microorganisms in the gastrointestinal tract is important not only in promoting efficient digestion and maximum absorption of nutrients, but also in increasing the capacity of the host in excluding infectious microorganisms and hence preventing disease (Walter et al., 2003). Lactobacilli are common inhabitants of animal gastrointestinal tracts and many investigators have reported them to provide various positive health attributes. This group of “good” bacteria is generally called probiotics. Therefore, probiotics can be defined as living microorganisms that upon ingestion in certain numbers exert health effects beyond inherent basic nutrition (Gurner et al., 1998). Naidu et al., 1999 describes probiotics as microbial dietary adjuvants that beneficially affect the hosts’ physiology by modulating their mucosal and systemic immunity as well as improving the nutritional and microbial balance in their intestinal tracts. Probiotics are potentially useful in the management and treatment of various gastrointestinal diseases including diarrhea, inflammatory bowel disease, and colon cancer (Rolfe, 2000). Enhancement of non immunological gut defense barrier by probiotics may include maintenance of normal levels of intestinal permeability and microecology, which is commonly shifted in the event of intestinal infection by pathogenic bacteria. Vertebrate immune system can mount both innate and adaptive immune response in the event of infection by pathogenic microorganisms. Many types of immune cells are recruited to elicit an immune response and subsequently neutralize the pathogens. These cells
include epithelial cells, natural killer cells, macrophages, neutrophils, dendritic cells and lymphocytes. These cells are quickly activated in the event of infection leading to production of an array of humoral mediators. Some may change their physiology and become phagocytic, yet others get involved in antibody synthesis and secretion. These activated immune cells may provide immediate protection against pathogens or promote specific immune responses. Therefore, these cells are useful in the probiotic enhancement of immunologic barrier in the animal gastrointestinal tract.

Probiotics have profound effects on potentiating both arms of immune responses. For instance, oral administration of the probiotic, *Bifidobacterium breve* was shown in mice that had been previously challenged with cholera toxin to promote humoral immunity by enhancing the secretion of immunoglobulin A (IgA) (Yasui *et al.*, 1999). In an investigation conducted by De Simone *et al.*, (1993), bacterial cell wall products were not only enhances the proliferation of immune cells but also induce the expression of pro inflammatory cytokines, which are necessary for the maintenance of a stable Th1/Th2 balance. This delicate balance is important for the host immune function as it dictates whether a humoral (antibody production) or a cell mediated (cytotoxic T-cell) response should be mounted. Therefore, the influence of a probiotic strain of bacteria on the mammalian immune system can be easily evaluated through *in vitro* and *in vivo* measurement of cytokines, immunoglobulin production and lymphocyte proliferation. The use of antibiotics is associated with the emergence of antibiotic-resistant bacteria, which have become difficult to control and have exerted adverse effects on the consumers of animal products. With the above positive attributes of probiotic bacteria on the prevention and disease management, probiotics hold great potential as a better alternative to antibiotics in aqua
culture, poultry, animal husbandry. Bacterial species play important roles in maintenance of intestinal balance and enhancement of immunity. *Bacillus* species have the ability to colonize and survive through the gastrointestinal tract and interact with the intestinal epithelium. Intestinal microbial flora were intended to exert both harmful and beneficial effects on human health. Immunomodulative capacity is considered to be an important mechanism to support probiosis. A number of studies in humans and animal models have provided strong evidence that oral administration of *bacillus* spores stimulates the immune system. To evaluate probiotic effects on the immune system, it is very important to study the probiotic induced effects in animal models that mimic the physiological reality. *In vitro* probiotic studies identified MBTU PBBM1 isolated from milk, had strong probiotic properties such as acid, bile tolerance, non-haemolysis, lecithinase negative, resistance in artificial gastric and intestinal fluid and antagonism to enteric pathogens such as *Salmonella typhi*, *Salmonella paratyphi A*, and *Vibrio cholerae*. The present chapter is divided into two parts. First part of the study deals with the *in vivo* response of MBTU PBBM1 in Balb/c mice. This study investigated the *in vivo* effects of 30 days consumption of MBTU PBBM1 on humoral and cell mediated immune response as well as the potential infectivity and pathogenicity of these strain in Balb/c mice. For this study, spores of MBTU PBBM1 was given in two doses as a feed supplement in the diet of Balb/c mice and its immunomodulation were studied with focus on the animal’s humoral and cell mediated immune responses. Second part of the study deals with, preventive and curative effects of MBTU PBBM1 against cyclophosphamide induced immunosupression. Cyclophosphamide, a classical myelotoxic agent, has been used in this study to establish an immunosuppressed experimental model. The preventive and curative role of
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MBTU PBBM1 strain in immunosuppression were studied with focus on the animal’s haematology, immune responses and histological alterations in the small intestine.

4.1.2 Literature review

In modern medicine, study of the functioning of immune system plays an important role in understanding and diagnosis of the diseases. Disturbances in immune response are involved in the etiology as well as the pathophysiologic mechanism of many diseases. The modulation of immune response using various agents in order to reduce risks of infections is still an area of interest for research studies since many years (Ballow and Nelson, 1997). Chemotherapeutic agents available today are mainly immunosuppressive with added side effects. This leads the research to look forward into natural resources showing immunomodulatory activity (Patil et al., 2008) and the goal of therapeutic immunomodulation is to reduce host damage by shifting the damage-response curve to a point that is more beneficial to the host. Immune modulation via dietary supplementation may be a promising strategy for maintaining immune homeostasis in the healthy population. The concept of dietary components in enhancing immune function can also be used in the formulation of so-called immunonutrition, specific nutritional preparations for clinical use in critically ill patients with compromised immune functions. Substances such as glutamine, omega-3 fatty acids, arginine, glycine and/or ribonucleic acid have been added to standard nutritional support for formulation of “immunonutrition.” Pre-biotic and probiotic preparations are also used as immunonutritional strategies. European Commission concerted action program redefined probiotics as “a live microbial food ingredient that is beneficial to health” (Salminen et al., 1998). In a broadened definition, Naidu et al., (1999) describes probiotics as
microbial dietary adjuncts that beneficially affect the host physiology by modulating the mucosal and systemic immunity as well as improving nutritional and microbial balance in the intestinal tract. Microflora in animals is extremely important in protecting them against pathogenic infections. This fact is evident in the way germ-free animals become susceptible to infections in their intestines. Wells et al., 1996 found that, in order to kill the mouse with functional microflora, only $10^5$ spores of *Clostridium botulinum* are required. At the same time 50 spores alone are enough to kill germ-free mouse. *In vitro*, studies reveals that dozens of microorganisms have been shown to have desirable probiotic qualities. However, some ingested bacteria are normally killed in the host stomachs by gastric juices. According to Crabbe et al., 1968 a small number of strains have been shown to colonize the human gastrointestinal tract in clinical trial. Some scholars believe that this colonization is a prerequisite for any health benefits to be conferred. The effect of the probiotics on the intestinal ecosystem, may impact the consumer in some beneficial way.

For a given microorganism to function as an effective probiotic, it must have some important properties as in the case of some species of *lactobacilli* (Reid et al., 1990). The organism should be able to: adhere persistently to intestinal epithelial cells and mucus; reduce and exclude pathogenic adherence to healthy cells; reproduce in such a manner to allow rapid multiplication and colonization; produce reactive agents such as acids, hydrogen peroxides and bacteriocins that can hamper pathogen reproduction and multiplication; be safe, noncarcinogenic and nonpathogenic; resist various microbicides; and form a balanced flora.

A number of potential benefits arising from changes to the intestinal milieu through the consumption of probiotics have been proposed, including:
a) Increased resistance to intestinal infections, by pathogen interference, exclusion, and antagonism; b) reduction in blood pressure; c) reduction in serum cholesterol concentration; d) maintenance of mucosal integrity; e) alleviation of symptoms of lactose intolerance; f) reduction in allergy; g) stimulation of phagocytosis by peripheral blood leucocytes; h) immune system stimulation and modulation in human and animals; i) vaginal and urinary tract health; j) modulation of cytokine gene expression; k) adjuvant effect; l) regression of tumors; m) and reduction in carcinogens or co-carcinogen production. Some probiotics produce metabolic by-products that are antagonistic to establishment of pathogenic microorganisms. Strains of *Lactobacillus* may produce lactic acid and hydrogen peroxide that are not only toxic but might aid in inhibition and exclusion of potential pathogens (Gilliland and Speck, 1977).

### 4.1.2.1 The gut mucosal barrier

The small intestine is constantly exposed not only to food but also to a variety of antigens in the life of mammals. These exposures pose challenges to the host because some of the antigens may be pathogenic. The bacterial load in the small intestine is dynamic and keeps changing both in numbers and types. A well functioning mucosal barrier in the gut is important and may help in excluding establishment of pathogenic microorganisms (Janeway *et al*., 1999). Some antigens are absorbed across the mucosal epithelial-cell layer and may be processed in the lysosomes or may be eliminated by the mucosal immune system (Isolauri *et al*., 1993). It is well documented that the effect of commensal probiotic bacteria on the immune response of their host animals is through their close association with the gut lymphoid tissues found in the intestinal mucosa. The gut-associated lymphoid tissues are found in specific compartments in the host intestinal
tract. The Peyer’s Patches, which are areas rich in B-cell lymphoid follicles, are highly organized lymphoid tissues in the wall of the small intestine. The Peyer’s Patches are also resident to interfollicular populations of T cells. Large numbers of lymphoid and myeloid cells aggregate to form the lamina propria, which is found to be rich in immunoglobulin-A plasmablasts. The lamina propria is found scattered under the gut epithelium. Interspersed within the enterocyte monolayer are intraepithelial lymphocytes, which are made up of mostly CD8+ T-cell subsets (Janeway et al., 1999). It has been reported by various groups that the quantity and quality of immune cells in the Peyer’s Patches and lamina propria compartments depend on continuous stimulation provided by endogenous intestinal microflora (Cebra et al. 1999). In an investigation by Crabbe et al. (1968), they demonstrated that colonization of germ-free mice with different strains of lactic acid bacteria (LAB) induced the secretion of mucosal IgA from both the Peyer’s Patches and lamina propria. Since these LAB are also commensal organisms in many animals, they might be able to potentiate various indices of hosts’ humoral immune responses when used as dietary supplements.

The development of probiotics for farm animals is based on the knowledge that gut microflora is involved in resistance to disease in mammals (Gill et al. 1998). In a clinical study with human volunteers, Alander et al. (1999) demonstrated that L. rhamnosus GG attached to human intestinal mucosa and the attachment persisted for about seven days following withdrawal of the probiotics. In a different clinical study, Miller et al., (1993) demonstrated that application of the same strain to premature infants did not decrease pathogen load even if it colonized the intestines. This investigation suggested that the probiotic was poorly antagonistic to pathogenic bacteria in vivo. However, the increased likelihood of gastrointestinal infection in infants
may be due to immaturity of their gut defense barrier. This barrier is important because it forms a protective phase between the infants’ internal environment and the potentially pathogenic factors in the external environment (Juntunen et al., 2001). Although the mechanisms behind the positive effects of probiotics on the host are not well known, one way in which probiotics may augment favourable health outcome on the host is by enhancing nonspecific (innate) and antigen-specific (adaptive) immune response (Miller et al., 1993; Juntunen et al., 2001).

4.1.2.2 Immune stimulation of probiotics

Stimulation of the immune system, or immunomodulation, is considered an important mechanism to support probiosis. A number of studies in humans and animal models have provided strong evidence that oral administration of spores stimulates the immune system. This tells us that spores are neither innocuous gut passengers nor treated as a food. A small proportion of spores have been shown to disseminate to the primary lymphoid tissues of the GALT (Peyer’s Patches and mesenteric lymph nodes) following oral inoculation (Duc et al. 2003) and in vitro studies have shown that phagocytosed spores can germinate and express vegetative genes but are unable to replicate (Duc et al. 2004a). Following oral dosing, anti-spore IgG responses could be detected at significant levels. Anti-spore IgG and secretory IgA (sIgA) could be produced by a normal process of antigen uptake by B cells. Detailed analysis of the subclasses showed IgG2a to be the initial subclass produced and this is often seen as being indicative of a type 1 (Th1) T-cell response (Robinson et al. 1997). Th1 responses are important for IgG synthesis but more importantly for CTL (cytotoxic T lymphocyte) recruitment and are important for the destruction of intracellular microorganisms (e.g., viruses, Salmonella spp.) and involve presentation of
antigens on the surface of the host cell by a class I MHC processing pathway. Support for Th1 responses has been provided by the analysis of cytokines in vivo that showed synthesis of IFN-γ and TNF-α in the GALT and secondary lymphoid organs when spores of B. subtilis or B. pumilus were administered to mice Duc et al. 2004. IFN-γ is an effector of cellular responses and could have been produced by an innate immune response probably including Natural Killer (NK) cells. A number of other studies have shown ex vivo synthesis of IFN-γ in rabbits or mice following dosing with B. clausii spores of the Enterogermina_ product (Muscettola et al. 1992). Bacillus firmus vegetative cells have been shown to stimulate the proliferation of human peripheral blood lymphocytes in vitro Prokesova et al., 1994. In this study B. firmus was shown to promote differentiation of B lymphocytes to Ig producing and secreting cells and was shown to be significantly more potent than other Bacilli tested (B. subtilis, B. coagulans, B. megaterium, B. pumilus, B. cereus and B. lentus). Another study involved a randomized trial of 30 elderly patients who were given B. clausii spores of Enterogermina. Lymphocyte subsets were determined from peripheral blood mononuclear cells and a significant increase in B lymphocytes bearing membrane IgA was observed but not unrestricted proliferation of all B lymphocytes (Fiorini et al., 1985) These results indicate that orally administered spores may be interacting with the GALT and priming B lymphocytes for IgA synthesis. An interesting study has shown that B. subtilis in combination with Bacteroides fragilis promoted development of the GALT in rabbits and led to the development of the pre immune antibody repertoire( Rhee, et al. 2004) Interestingly, neither species alone could induce GALT development, so this cannot be an antigen-specific immune response. Furthermore, at least one stress protein, Yqxm, secreted from B.
subtilis was shown to required for GALT development. On a more cautionary note, in vitro studies have shown that the proinflammatory cytokine IL-6 was produced in macrophages cultured with B. subtilis or B.pumilus spores (Duc et al 2004b). In commercially farmed shrimps, an undefined Bacillus species, Bacillus S11, has been shown to stimulate the primitive immune system of Penaeus monodon. Bacillus S11 cells were shown to increase phenoloxidase as well as antibacterial activity (against the shrimp pathogen V. harveyi) in shrimp hemolymph. Bacillus S11 was also shown to increase the levels of phagocytosis of hemocytes derived from hemolymph compared to control shrimps and levels were increased further after challenge of shrimps with V. harveyi. As with vertebrate studies that show cell wall peptidoglycan to be a potential immunogen, in shrimps, peptidoglycan has also been shown to stimulate granulocytes leading to higher levels of phagocytosis (Itami, et al. 1998).

4.1.2.3 Immune modulation by probiotics

Immune modulation by dietary bacteria has continued to be a subject of growing interest. Probiotics have been reported to facilitate stabilization of gut microflora and hence enhance gut defense against pathogenic microorganisms in a way that is non immunological (Salminen et al., 1998b). Although it is known that immune function tends to decline with age, supplementation twice daily with a probiotic Bifidobacterium lactis was found to significantly increase and improve various indices of immune function in a group of healthy elderly people in a double-blinded trial. The good attributes of the bacterial supplementation were observed in this group after about six week of trial time (Aruchalam et al., 2000). In other studies, probiotics have been shown to augment the humoral arm of adaptive immunity and subsequently enhance the immunologic barrier of the
intestine (Kaila et al., 1992). In the event of their action in animals as immune modulators, probiotics may either have up- or down-regulatory effects on various indices of immune response. Live probiotic microorganisms may stimulate production of cytokines and therefore enhance natural immune responses (Marin et al., 1997). Intestinal inflammation can be suppressed by viable probiotics after oral administration, which makes these microorganisms useful in controlling hypersensitivity reactions (Majamaa et al., 1996). In another independent investigation on the antiproliferative effects of five strains of probiotic bacteria, Pessi et al. (1999) studied the proliferation activity of mononuclear cells induced by phytohemagglutinin as a mitogen. Proliferation of these cells was carried out in the presence or absence of either unheated or heat-treated probiotic homogenates from L. rhamnosus GG, B. lactis, or L. acidophilus. They demonstrated that homogenates from these bacteria suppressed proliferation of mononuclear cells with mitogen treatment. The suppression was observed with both unheated and heat-treated homogenates. Their findings suggested that these homogenates might be used to generate non viable food products that are immunologically active. Low cost and convenience of administration makes immune modulation via the oral route a very desirable treatment. Due to large volumes of antigen required for oral immunization and that exposure to soluble protein antigen may induce oral tolerance, researchers are leaning towards the use of microorganisms to induce the desired immunity (Wells et al., 1996). The adherence to intestinal epithelium and mucus by probiotics may be an integral factor in the stimulation of the host immune system. Adhesion assists the bacteria in surviving host secretions and improves remarkably the chances of the probiotics colonizing the intestinal mucosa (Sami et al., 2001).
4.1.2.4 The role of probiotics in innate immunity

In mediating the innate or non-specific immunity in host animals, several types of immune cells are recruited and stimulated. These cells include neutrophils, eosinophils, macrophages, epithelial cells, natural killer cells and M cells. These cells monitor sites of pathogen entry and mount nonspecific immune responses including phagocytosis and natural killing (Janeway et al., 1999). It is reported that innate immune responses in animals can be modulated by consumption of specific lactic acid bacteria (Perdigon et al., 1995; Schiffrin et al., 1995). In a study conducted by Haller et al. (2000), human peripheral blood mononuclear cells (PBMCs) and purified lymphocyte subsets were capable of being stimulated by nonpathogenic gram-positive species of Lactobacillus. In vivo, interaction between nonpathogenic commensal bacteria and blood leukocytes may occur in definite compartments of the host’s mucosal immune system. They also reported that increase in the number and immune function of lymphoid effector cells at the mucosal level is heavily dependent on the presence of gut microflora. Secretion of interferon-α (INF-α) from purified natural killer (NK) cells was significantly increased in the presence of macrophages primed with bacteria, suggesting that probiotic bacteria are important in the activation of NK cells by macrophages (Haller et al., 2000). It is well documented that there is recirculation of the mucosal immune system. Cells of the mucosal-associated lymphoid tissue (MALT) recirculate within the mucosal system. Given that there is evidence of translocation of nonpathogenic bacteria through the epithelial barrier via M-cell pockets, there is every chance that these bacteria interact with several types of immune cells, which are resident in M-cell pockets. Probiotic bacteria might exploit this fact to potentiate host immune function (Neutra, et al. 1999).
Phagocytosis is an important arm of the innate immune system and is among the first line of defense that animals employ in the event of infection by pathogenic microorganisms. The nitro-blue tetrazolium (NBT) test is rapidly assuming importance as a method of assessing the phagocytic capability. Generation of active oxygen radicals represents an essential element of microbial killing. Thus, altered production of the oxygen radicals, superoxide anion and hydrogen peroxide after antibiotic exposure is a mechanism lowering antibacterial immune defense. Phagocytic cells are known to produce agents that are toxic to pathogens like reactive oxygen intermediates and lytic enzymes. They are involved in engulfing and destroying particulate antigens. Phagocytes may play an integral role in initiating inflammatory reactions. Salminen et al. (1998b) reported that intestinal inflammation might be as a result of imbalance in the intestinal microflora. Gill et al., (2001a) observed that the enhancement of the phagocytic capacity was in a dose-dependent manner. However, they also demonstrated that mucosal antibody responses were enhanced by live but not killed L. rhamnosus HN001. An in vitro investigation conducted by Pinchuk et al. (2001) demonstrated that a probiotic strain, Bacillus subtilis 3, was able to secret antibiotics that inhibited growth of Helicobacter pylori. They showed that the anti-H. pylori activity in cell-free supernatants was heat stable and protease insensitive. Their result suggested that other probiotic preparations might exhibit similar activities against pathogenic microorganisms.

4.1.2.5 The role of probiotics in acquired immunity

Acquired or adaptive immunity is the response of antigen-specific lymphocytes to antigen and includes the development of immunological memory. Adaptive immune responses are generated by clonal selection of lymphocytes (Janeway et al., 1999). Acquired immunity involves
lymphocytes with receptors for specific antigens and presentation of the antigens in the context of two different major histocompatibility complexes (MHC) by antigen presenting cells (APCs). As a result, subsets of helper T cells (Th), the main effectors and regulators of cell-mediated immunity may be activated (Roitt et al., 1989). Upon activation by antigen or pathogen, T-cells are activated to synthesize and secrete a variety of cytokines that serve as growth, differentiation and activation factors for other immunocompetent cells. The types of cytokines produced during infection are key factors in determining whether a humoral (antibody production) or a cellular immune response is potentiated in the host animal. Among the first proinflammatory cytokines expressed by host immune cells after pathogenic challenge are tumor necrosis factor-alpha (TNF-α), interleukin 1α (IL-1α), IL-6 and interferons (IFNs) (Tracey and Cerami, 1993). It is well documented that cytokines produced later during microbial infection will either influence the development of cell-mediated immune response associated with T-helper type 1 cells (Th1) or a humoral immune response that is associated with T-helper type 2 cells (Th2). Gill et al., (2000 b) observed that feeding healthy mice with $10^9$ CFU of L. acidophilus, L. rhamnosus or B. lactis enhanced the proliferative responses of spleen cells to concanavalin A (Con A) and lipopolysacharide (LPS), which are T-and B-cell mitogens, respectively. The spleen cells from mice given these different bacteria expressed greater amounts of INF-α after stimulation with Con A than cells from control mice. When they assayed the levels of antibody responses after either oral or systemic administration of antigen, they found the levels to be higher in mice given the bacteria than in control mice. Their results suggested the use of LAB as feed supplements in mice was able to enhance several factors of both humoral and cellular immune
responses. Ibnou-Zekri et al., (2003) investigated the differential impacts of *L. johnsonii* and *L. paracasei* on the development of mucosal and systemic antibody responses in mice. Despite the fact that these two organisms had similar growth and adherence capacities to enterocytes *in vitro*, they showed marked differences in their patterns of colonization and translocation. They also promoted different immune responses at the mucosal and systemic levels *in vivo*. They demonstrated that of the strains tested, *L. johnsonii* colonized the intestines more efficiently than did *L. paracasei* in mice and that both strains activated mucosal B-cell responses evidenced by aggregation of cells of the Peyer’s Patches. They also showed that IgA secreting plasma cells were prevalent in lamina propria after association with either of the bacterial strains. Germ-free mice had either few or no IgA secreting cells even after association with either of the bacterial strains. They further showed that mice associated with *L. johnsonii* but not *L. paracasei* secreted increased amounts of *Lactobacillus*-specific IgA. Waard et al. (2001) investigated the effects of orally administered viable *L. casei* Shirota on immune response indices of Wister and Brown Norway rats. They used the *Trichinella spiralis* host resistant model. In their study, two weeks before and after *T. spiralis* infection, rats were fed with $10^9$ CFU of *L. casei* 5 days per week. They observed that *T. spiralis*-specific delayed-type hypersensitivity (DTH) responses were significantly augmented in mice fed *L. casei* than in control mice and significantly enhanced *T.spiralis*-specific antibody IgG2b in both types of rats. This type of DTH response is considered to be a manifestation of Th1 cell-mediated immunity. Yasui et al., (1999) reported that another probiotic bacterium, *B. breve* YIT 4064, was able to potentiate humoral immune response and that oral administration of this strain was able to augment production of antigen-specific antibodies.
4.1.2.6 Cyclophosphamide and immunosuppression

Conventional cancer treatments have many modalities, all directed at killing tumor cells or preventing their proliferation. Cyclophosphamide (CTX) is an alkylating agent, the most commonly used anticancer and chemotherapeutic drug. Cyclophosphamide (CTX) is one of the most commonly used anticancer agents and immunodepressant drugs for preventing graft rejection, treating some chronic autoimmune diseases and inducing experimental immunosuppression De Jonge et al., 2005. The main effect of cyclophosphamide is due to its metabolite phosphoramide mustard. This metabolite is only formed in cells that have low levels of ALDH. Phosphoramide mustard forms DNA crosslinks both between and within DNA strands at guanine N-7 positions (known as interstrand and intrastrand crosslinkages, respectively). This is irreversible and leads to cell death. Its cytotoxic effects are the result of chemically reactive metabolites that alkylate DNA and protein, by producing cross-links (Dollery 1999).

Immunosuppression and normal tissue injury are the major limitations of chemotherapy (Hersh and Freiriech 1968), which give rise to numerous side effects (Fraiser et al. 1991). CTX administration causes nausea, vomiting, mucosal ulceration, dizziness of short duration, transverse ridging of the nails, increased skin pigmentation, interstitial pulmonary fibrosis, alopecia, and hepatic toxicity (Hutter et al. 1969). It has been reported that oxidative stress mediated disruption of redox balance after CTX exposure generates biochemical and physiological disruptions. Several studies suggest that antioxidant supplementation can influence the response to chemotherapy as well as the development of adverse side effects that result from treatment with antineoplastic agents (Chakraborty et al. 2009).
Drugs that could reduce these side effects, as well as stimulate immunity, will be of great help in improving cancer treatment strategies. Recently there is an increase in interest in the search of probiotics that are capable of minimizing the toxicity induced by chemotherapy to normal cells without compromising its anti-neoplastic activity. However, LAB-containing functional foods are often included in the diet of immunocompromised patients, and it is known that agents with immunomodulatory activity may induce contrasting effects in normal and immunosuppressed individuals (Kundu et al., 1996; Labro, 2000). Therefore, it is very important to know whether probiotic strains are able to promote restoration of damaged immune functions or, on the contrary, to increase the severity of immunodeficiency.

4.1.3 Materials and Methods.

4.1.3.1 Animals

Male Balb/c mice (8 week old, (20±0.8) g were used in the study. Balb/c mice were obtained from Small Animal Breeding Station (SABS) under Kerala Veterinary and Animal Sciences University, Thrissur and the animals were kept in animal house of Bioscience department of Mahatma Gandhi University, Kottayam. Animals were housed in polypropylene cages and were given standard dry pellet (Sai Feeds, Bangalore, India) and drinking water ad libitum. The animals were maintained at a controlled condition of temperature of 26-28°C with a 12 h light: 12 h dark cycle. Bedding in cages was changed twice weekly. Care and use of animals under study were followed according to the institutional guidelines of M.G University.
4.1.3.2 Preparation of sample for *in vivo* study

4.1.3.2.1 Selection of Sample

*In vitro* studies in chapter I and II revealed that spores of MBTU PBBM1 possess effective desired probiotic characters than their vegetative cells. So in the present study spores of MBTU PBBM1 were selected to analyze the possible *in vivo* effects in Balb/c mice.

4.1.3.2.2 Preparation of spore sample

Spores of MBTU PBBM1 were cultured in Difco Sporulation medium for 48 hrs at 37°C. Culture was centrifuged at 1500 g for 10 min at 4°C and washed three times with PBS and resuspended in sterile PBS. For assessment of the number of viable spores, suitable dilutions of the bacterial suspensions were plated onto nutrient agar plates and CFU were counted after 24 hr at 37 °C. Two dilutions of bacterial suspension giving a colony forming unit of $10^8$ spores and $10^4$ spores was prepared separately in 0.1ml PBS.

4.1.3.3 Experimental design for persistence studies

Mice were assigned into 3 groups, which include two experimental groups and one control group. Each group comprised of six mice. Among the three groups, Group I received $10^8$ spores, Group II received $10^4$ spores of MBTU PBBM1 and Group III, the control group received 0.1 ml of sterile PBS alone.

4.1.3.3.1 Persistence Studies.

Determination of spore counts in faeces was studied after the oral administration of MBTU PBBM1 spores to the experimental groups. Two different oral doses of $1 \times 10^8$ spores and $1 \times 10^4$ spores were separately
prepared and were given three days consecutively to group I and group II mice by intragastric gavage. Group III was kept as control and they received 0.1ml of PBS alone. All the three groups were housed individually, and the cages were cleaned daily. One gram of freshly voided fecal pellet was collected separately for five consecutive days after three day continuous spore administration. Faecal pellets were homogenized separately, and their serial dilutions were made. For the isolation of spores, diluted suspensions of faeces were heat treated at 65°C for 20 min. Various dilutions were poured on nutrient agar plates and incubated for 48 h at, 37°C. Spore forming MBTU PBBM1 colonies per gm of faeces were counted, and they were recovered by re streaking on fresh nutrient agar plates. Colony morphologies were observed and presence of spores was confirmed by spore staining. Faecal spore counts of MBTU PBBM1 in mouse groups I and group II were compared with the control group III.

4.1.3.4 Experimental design for immunomodulatory studies

Mice were assigned into 3 groups, which included two experimental groups and one control group. Each group comprised of six mice. Among the three groups, group I received $10^8$ spores, group II received $10^4$ spores of MBTU PBBM1 and group III the control group received 0.1 ml of sterile PBS alone. All experimental treatments were via intragastric gavage. Spore treatments lasted for 30 days and the 31st day mice of all groups were sacrificed for further laboratory analysis.

4.1.3.5 Measurements of general health and growth

Throughout the experimental period, behavioural changes, activity, treatment-related illness or death, difference in hair luster between treated and control groups were monitored. Unhealthy symptoms were recorded
daily. Weight gain or loss of all spore treated groups was compared with the control group. Feed intake and water intake nature of animals were also observed.

4.1.3.6 Haematological parameters

4.1.3.6.1 Blood collection from experimental animals (Balb/c mice)

Blood samples from each individual mouse was collected from all groups by, a capillary pipette containing anticoagulant EDTA which was inserted in the lateral canthus and blood was collected from the retro orbital sinus. After collection, the pipette was removed and bleeding stopped when the eye returned to a normal position. Leukocytes were counted to determine differential percentage of white blood cells (lymphocytes, neutrophills, monocytes, and eosinophills). Total red blood cell (RBC), haemoglobin concentration (HB) and haematocrit were also determined.

4.1.3.6.2 Determination of total red blood cell (RBC) count (Chaudhari, 2000a)

Procedure

0.02 ml of blood was added to 3.98 ml of diluting fluid. The Neubauer chamber was charged with well-mixed dilute blood. The total number of red cells in the small square in the central ruled area of Neubauer counting chamber was counted using 40x objective of the microscope.

Total RBC count = Number of cells counted × 10,000 count/mm³
4.1.3.6.3 Determination of total white blood cell (WBC) count
(Chaudhari, 2000b).

Procedure

0.02 mL of blood was added to 3.98 ml of diluting fluid. The neubauer chamber was charged with well-mixed dilute blood. The total number of white blood cells in the four large corner squares of chamber was counted after 3-4 min.

Total number of WBC=Number of cells counted×50 count/mm$^3$

4.1.3.6.4 Determination of haemoglobin (Hb) in blood (Drakin and Austin, 1932).

Procedure

The study was conducted by using Agappe diagnostic kit. 0.02 mL of fresh whole blood was mixed with 5 mL of the cyanmeth reagent. The optical density was measured at 546 nm against reagent blank after incubation for 5 min. at room temperature. The optical density of standard solution corresponding to 60 mg/dl haemoglobin at 546 nm was read against reagent blank. Haemoglobin in the blood was calculated by using the following formula.

$$\text{Haemoglobin (g/dl)} = \frac{\text{O.D}_T \times 60 \times 0.251}{\text{O.D}_S}$$

4.1.3.6.5 Determination of haematocrit%

Procedure

Blood sample was taken in small sterile centrifuge tube and centrifuged at 1500 rpm for 10 min at 4°C. RBC layer was separated and cells were counted with an automated haemoanalyzer.
4.1.3.7 Humoral Immune Response

4.1.3.7.1 Serum collection

After the end of feeding period, and 8 hr of fasting, the animals of all groups were immediately exsanguinated by retro-orbital venous plexus puncture and the blood was collected into a 1.5 ml vial. The blood was allowed to stand for 2 hr at room temperature, then centrifuged at 5,000 rpm for 15 min at 4 °C to separate the serum. The sera were collected and stored at -20°C for later analysis.

4.1.3.7.2 Enzyme linked immunoabsorbent assay for immunoglobulins.

4.1.3.7.2.1 Quantification of serum Immunoglobulins

Procedure

Serum antibody levels of IgA, IgE, IgG, and IgM were analyzed using the mouse IgA, IgE, IgG, and IgM ELISA quantitation kit. Serum antibody levels of all groups under study were assayed according to the procedure on Quantitation Kit (Mouse Ig G ELISA kit catalog number: E-90G, Mouse Ig M ELISA kit catalog number: E-90M, Mouse Ig A ELISA kit catalog number: E-90A, Mouse Ig E ELISA kit catalog number: E-90E CRL Laboratories). Diluted serum samples for enzyme assay were prepared according to the specific kit instructions. Briefly, IgA, IgE, IgG, and IgM levels in test sera were measured by sandwich enzyme-linked immununoabsorbent assay (Sandwich ELISA). The microtiter plate provided in kit has been pre-coated with antibody specific to immunoglobulins. All reagents were brought to room temperature before use. 100 μL of standards in various concentrations and test samples were pipetted in duplicate into pre designated wells. The microtiter plate was incubated at room temperature for thirty minutes. The plates were kept covered during incubation. After
incubation, the contents of the wells were aspirated. Each well was completely filled with appropriately diluted wash solution and aspirated. Washing step was repeated for three times. Wells were completely filled with wash buffer and the contents were aspirated. Residual buffer was removed by sharply striking on absorbent paper. After a total of four washes 100 µL of appropriately diluted Enzyme-Antibody Conjugate was added to each well and incubated at room temperature for thirty minutes. The Plate was covered and kept in dark during incubation. Wells were washed and blotted. 100 µL of TMB substrate solution was added into each well and incubated in the dark at room temperature for precisely ten minutes. After ten minutes, added 100 µL of Stop Solution to each well. The absorbance was determined at 450 nm in an ELISA reader. The concentration of immunoglobulins in the samples was then determined by comparing the O.D. of the samples to the standard curve. Equivalent levels of test immunoglobulins were calculated by comparing the levels with a reference curve generated using immunoglobulin standards. Results were expressed as the concentrations of each immunoglobulin in serum.

4.1.3.7.3 Haemagglutinin antibody titer

Principle

The non-agglutinated SRBC will settle to the bottom of the well as a clear button while agglutinated cells settle as a diffuse mat. The maximum dilution of antisera at which a clear agglutination is observed gives the titre of the antibody.
4.1.3.7.3.1 Preparation of SRBC

Fresh sheep blood was collected from the local slaughter house in vials under sterile conditions in sterile freshly prepared Alsevere’s solution in a 1:1 proportion. Blood was kept in refrigerator and processed for the preparation of SRBCs batch, by centrifugation at 2000 rpm for 10 minutes and washing with PBS (10 mM sodium phosphate buffer containing 150 mM NaCl, pH – 7.4) 4-5 times and then suspending into buffered saline for further use and finally adjusting to a concentration of $1 \times 10^8$ cells/ml for immunization and challenge.

4.1.3.7.3.2 Assessment of antibody titer (Puri et al., 1994)

To determine the serum antibody response to SRBC, a direct hemagglutination technique was used. Followed by the spore treatment, animals in all groups including control were immunized by intra peritoneal administration of 0.1 ml of $1 \times 10^8$ SRBC/mouse. The day of immunization was considered as Day 0. On Day 1, blood samples were collected from all test animals by puncturing the retro-orbital plexus in ependroff tube. The serum was collected as previously described in section 4.3.7.1, from each mouse of all groups and were used to estimate the haemagglutinating antibody (HA) titre against the antigen SRBC. Two fold serial dilutions of individual serum samples of all groups were made with PBS. 25 µl of this diluted mice serum samples from all groups were added in the wells from columns two to twelve. The first column of wells was considered as blank and it has only phosphate buffered saline. 25 µl of 1% SRBC in PBS were added to all wells for a final volume of 50 µl. The plates were then shook for 1 min’ and incubated at 37°C for 1 hr to determine agglutination titers. The
reciprocal of the highest dilution of the test serum giving agglutination was taken as the antibody titer.

4.1.3.8 Cell mediated immune responses.

4.1.3.8.1 Nitroblue tetrazolium (NBT) Assay (Freeman and King, 1971).

Principle

In this test, we measured the respiratory burst- the intracellular killing by oxidative mechanism by macrophages. Upon activation, macrophages are highly effective at generating reactive oxygen species (ROS) by a process known as the respiratory burst. NADPH oxidase of macrophages facilitates the transfer of one electron from cytosolic NADPH to molecular oxygen captured from the medium. The product of this reaction, the superoxide anion (O\textsuperscript{−}), can then be converted to other reactive oxygen metabolites, including hydrogen peroxide and hypochlorous acid. Nitro blue tetrazolium (NBT) is an electron acceptor used to detect indirectly the production of superoxide by stimulated macrophages. Superoxide reduce the yellow soluble NBT to blue black formazan, an insoluble material that get precipitated and can be seen microscopically within the cell and hence used as a qualitative assessment of superoxide production.

4.1.3.8.1.1 Isolation of peritoneal macrophages (Ray et al., 2010)

After the end of feeding period, each mouse of all groups were euthanized and sprayed with 70% ethanol and mounted on the styrofoam block on its back. By using a scissors and forceps a cut was made on the outer skin of the peritoneum and gently pulled it back to expose the inner skin lining of the peritoneal cavity. 0.5 ml of ice cold PBS (with 3% FCS) was injected into the peritoneal cavity using a 27g needle. The needle was slowly pushed into the peritoneum being careful not to puncture any organs.
After injection, the peritoneum was gently massaged to dislodge any attached cells into the PBS solution. A 25 g needle, was, attached to a 5 ml syringe and inserted into the peritoneum and the fluid was collected while moving the tip of the needle gently to avoid clogging by the fat tissue or other organs. Cell suspension was collected and kept on ice after removing the needle from the syringe. An incision was made in the inner skin of the peritoneum and while holding up the skin with a forceps, the remaining fluid were collected from the cavity by the use of plastic Pasteur pipette which was transferred in collection tube kept in ice. The collected cell suspension was centrifuged at 1500 rpm for 8 minutes, at 4°C and the supernatant was discarded and the cells were resuspended in PBS for counting. Trypan blue exclusion was used to check the viability of the immune cells. Equal part of 1% trypan blue solution was added to equal part of cell suspension and mixed well. Cells were loaded into hemocytometer and the number of stained and unstained cells were counted in the hemocytometer.

4.1.3.8.1.2 Preparation of Nitro-blue tetrazolium (NBT)dye

Commercially available, Stock solution was prepared as 0.1% NBT in saline with gentle heating. 'Working' NBT solution was made by mixing 1 ml of the stock solution with 1ml phosphate-buffered saline. This solution must be made fresh for each batch of tests.

Procedure

For the assay, 0.1 ml peritoneal cells from all the study groups were incubated with 0.1ml NBT for 30 min at 37°C with 5% CO₂. After incubation, a drop from each sample was carefully transferred on to a grease free glass slide. The smear was allowed to dry and fixed with absolute ethanol for 3 minutes. Then, the slides were stained with saffranin for 3
minutes; air dried and observed under 40X objective for 100 macrophages for calculating percentage of macrophages with formazan granules.

4.1.3.8.2 Delayed type hypersensitivity (DTH) response
(Karthikumar et al., 2011)

Principle

T cells are immune response mediators and play an important role in establishing and maximizing the capabilities of the adaptive immune response. By using SRBC the stimulation of the T-lymphocyte activity was checked by measuring the foot pad swelling.

Procedure

Followed by the spore treatment for 30 days, animals in all groups including control were immunized by intraperitoneal administration of 0.1 ml of 1x10^8 SRBC/mouse. The day of immunization was considered as Day 0. On the day 1 (24 hr after immunization), animals in all the groups were challenged by subcutaneous administration of 0.2 ml of SRBC 1% into the right hind foot pad, while a 0.2 ml of PBS was administered in the left hind foot pad to serve as control. DTH response was measured at the 48 hr after the immunization and expressed as percent increase in foot pad swelling (FPS). The inflammation percentage was calculated as per Manosroi et al. 2005.

\[
\text{Inflammation (\%)} = \left( \frac{\text{FPS after treatment} - \text{FPS before treatment}}{\text{FPS before treatment}} \right) \times 100
\]
4.1.3.8.3 Proliferation of splenic lymphocytes (Bujalance et al., 2009)

Principle

The MTT Cell Proliferation Assay measures the cell proliferation rate. Assay is based on the ability of a mitochondrial dehydrogenase enzyme (by generating reducing equivalents such as NADH and NADPH) from viable cells to cleave the tetrazolium rings of the pale yellow MTT and form a dark blue formazan crystals, which is largely impermeable to cell membranes, thus resulting in its accumulation within healthy cells. The resulting intracellular purple formazan can be solubilized and quantified by spectrophotometric means.

4.1.3.8.3.1 Isolation of splenocytes (Shalini et al., 2009)

Animals of all groups under study were sacrificed by cervical dislocation and the skin underlying the peritoneal region was removed and spleen was collected aseptically. The spleen were teased apart in cold Hank’s balanced salt solution with a sterile needle and forceps. The teased cells were transferred to a sterile centrifugation tubes. Clumps were allowed to settle in a centrifugation tube kept in ice for 20'. Supernatants was collected in separate tubes and resuspended in sterile RPMI 1640 media (Himedia) with HEPES buffer (20 mM), sodium bicarbonate (24 mM) and gentamicin (100 ug/ml). 1 ml upper portion of the medium containing the splenocytes were aseptically transferred using micropipette to a 15 ml sterile centrifuge tube. Tube was kept to stand in ice for two minutes. After that cell suspensions were transferred to fresh centrifuge tube and centrifuged at 1000 g for 5 min, at 4°C. Cells were washed once again in RPMI-1640 medium and then 1ml of erythrocyte lysis buffer were added to the pellet. After 45 sec, 5 mL of culture medium was added and centrifuged
at 1000 g for 5 min. Lymphocytes were washed twice with RPM1 culture medium to remove the traces of lysis buffer and finally suspended in 1 mL of RPMI 1640 containing 10% FCS. Trypan blue exclusion was used to check the viability of the immune cells.

**Procedure**

Proliferation of spleen cells was measured by methyl thiazolyl tetrazolium (MTT) incorporation as described by Buja-lance with minor modifications. Cell suspension containing $1 \times 10^7$ viable cells/mL were made in RPMI-1640 (Himedia) containing 10% FCS. 100 µL volumes of cell suspension were dispensed into 96-well flat bottomed tissue culture plates. Cells were allowed to grow with and without 10 µL mitogen (1.5 µg/mL concanavalin A) to determine the difference between cell proliferation. Plates were incubated in a humidified CO$_2$ incubator under 5% carbon dioxide at 37⁰C for 72 hours. Methyl thiazolyl tetrazolium(SRL Laboratories) was dissolved in RPMI-1640(Himedia) at 5 mg/mL and filter sterilized through 0.22 µm filters. 10 µL of MTT were added to each well, and the plates were incubated at 37⁰C for 4 hours. During this period, formazan crystals will be formed at the bottom of each well .70 µL of supernatant was removed from each well and discarded; Cell dissociation solution (20% wt/vol sodium dodecyl sulfate, 50% vol/vol dimethyl sulfoxide, pH 4.5) were added to each well and incubated overnight at 37⁰C. Spleen cells proliferation were measured by determining the absorbance at 570 nm using an automated microplate reader (Lisa plus), Proliferation ratio, were calculated according to the following equation: proliferation ratio = (test OD$_{570}$ − control OD$_{570}$)/control OD$_{570}$ × 100%, where OD$_{570}$ is the optical density measured at 570 nm.
4.1.3.9 Bacterial translocation test and persistence in ileal section

Bacterial translocation and persistence of MBTU PBBM1 in all treated and untreated groups were analyzed in the small intestine, blood, liver, and spleen. 50 µL samples of blood were serially diluted and pour plated. Plates were incubated at 37°C for 72 hrs. Tissue samples were homogenized in buffered peptone water (1g/L) and 100 µL of the resulting homogenates were serially diluted and pour plated in nutrient agar. After 48 hr of incubation, presence of MBTU PBBM1 were checked and confirmed.

4.1.3.10 Histopathological examination of intestine

After 30 days of spore feeding mice from all groups were sacrificed and dissected. A small portion of small intestine was taken and fixed in 10% formaldehyde. Immediately after sacrifice, tissue specimens were fixed in 10 per cent buffered formalin. Specimens were dehydrated by passing through ascending grades of alcohol, cleared in xylene, impregnated and embedded in paraffin. Thin sections were cut (3-5 µm) by using microtome and were stained using hematoxylin and eosin.

4.1.3.11 Statistical Analysis

Results are expressed as means ± SDs. Data were analyzed using 1 way Anova analysis of variance.

4.1.4 Results

4.1.4.1 Persistence Studies

Persistence of MBTU PBBM1 in gastrointestinal tract was determined by the study of faecal microflora of treated groups. MBTU PBBM1 counts were found to be higher in faeces, immediate after the third day of spore feeding and later the spore counts were found to be decreased
with the increase in time. Experimental group I (which received $10^8$ spores) has stable spore counts even after the 5th day of the last spore feeding. Experimental group II (which received $10^4$ spores) failed to persist in the faeces even after third day of spore administration. This proves that a particular level dose of spores were required for persistence in the gastrointestinal tract. In the untreated suspensions of control group there was no visible spore counts of MBTU PBBM1 strain. The count of spores is given in Table 4.1.1 reflects the actual, number of viable units of spores.

### Table 4.1.1: Persistence of MBTU PBBM1 spores

<table>
<thead>
<tr>
<th>Name of groups</th>
<th>Viable counts (log CFU/gm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
</tr>
<tr>
<td>Group I (received $10^8$ spores)</td>
<td>7.14±.08</td>
</tr>
<tr>
<td>Group II (received $10^4$ spores)</td>
<td>7.03±.07</td>
</tr>
<tr>
<td>Group III (Normal control)</td>
<td>0</td>
</tr>
</tbody>
</table>

### 4.1.4.2 Measurement of general health and growth

Spore treated groups were analyzed for any marked changes in general health. No adverse clinical signs were observed in any of the mice during the period of experimentation. No diarrhea or other treatment related sickness or death was recorded. General health appearance did not differ significantly between mice fed with probiotics strain and non fed control. Different doses of probiotics strains did not significantly affect the feed and water intakes. Data on weekly live body weight gain is shown in Figure 4.1.1. There was no significant difference in specific growth rate between the
treatment group and control group (P>0.05). Mice of all treated and untreated control mice exhibited almost similar growth and weight.

![Figure 4.1.1 Mice live body weight](image)

**Figure 4.1.1 Mice live body weight**

### 4.1.4.3 Hematological studies

A change in haematological parameters from the normal status is considered as a disease indicator. Safety assessment of probiotic treatment can be done by studying and comparing haematological parameters of probiotic fed mice and non fed control. The effects of feeding of MBTU PBBM1 spores on haematological parameters is shown in Table 4.1.2. No significant differences in differential counts were detected among the dose dependent treatment groups and control group. There was no significant difference in RBC, HB, HT, and WBC among the mice in the different groups.
Table 4.1.2: Haematological parameters of mice orally administrated with MBTU PBBM1 spores for 30 days *

<table>
<thead>
<tr>
<th>Haematological parameters</th>
<th>Group I (received $10^8$ spores)</th>
<th>Group II (received $10^4$ spores)</th>
<th>Group III (Normal control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemoglobin (g/l)</td>
<td>14.3±.6</td>
<td>14.2±.4</td>
<td>14.6±1.2</td>
</tr>
<tr>
<td>RBC ($10^3$/mm$^3$)</td>
<td>5.9±0.10</td>
<td>5.8±0.12</td>
<td>5.7±0.18</td>
</tr>
<tr>
<td>WBC ($10^3$/mm$^3$)</td>
<td>4.7 ± 0.37</td>
<td>4.4 ± 0.25</td>
<td>4.9 ± 0.29</td>
</tr>
<tr>
<td>HT (%)</td>
<td>42±0.004</td>
<td>42±0.00</td>
<td>42±0.07</td>
</tr>
<tr>
<td>Neutrophil (%)</td>
<td>19.4±2.37</td>
<td>19.2±3.46</td>
<td>19±2.72</td>
</tr>
<tr>
<td>Lymphocytes (%)</td>
<td>74.1±2.21</td>
<td>73.9±3.71</td>
<td>74±2.65</td>
</tr>
<tr>
<td>Monocytes (%)</td>
<td>2.96±0.42</td>
<td>2.84±0.58</td>
<td>2.92±1.6</td>
</tr>
<tr>
<td>Eosinophils (%)</td>
<td>0.81±0.04</td>
<td>0.83±0.02</td>
<td>0.84±0.08</td>
</tr>
</tbody>
</table>

*All values $P >0.05$ (control vs. probiotic-fed animals). WBC: white blood Cells, RBC: red blood Cell; HT: Haematocrit

4.1.4.4 Serum Immunoglobulins levels

*In vivo* humoral immune response of MBTU PBBM1 spores in mice was measured by checking the level of serum immunoglobulins Ig A, IgE, IgG, IgM. Serum IgA and IgG levels of spore fed mice were found to be significantly higher in comparison with the control group($P<0.05^*$). Increase in serum immunoglobulin Ig A and Ig G in spore treated groups was in a dose dependent manner ($P< 0.05^*$). See Table:4.1.3. Comparison of serum immunoglobulins revealed that there is no marked variations in IgE and Ig M between spore treated groups and control ($P> 0.05^*$).
Table 4.1.3: Humoral immune response

<table>
<thead>
<tr>
<th>Name of Group</th>
<th>Serum Immunoglobulins µg/ml</th>
<th>Haemagglutinin Titer Mean ±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ig G</td>
<td>Ig A</td>
</tr>
<tr>
<td>Group I (received 10^8 spores)</td>
<td>820±120</td>
<td>680±114</td>
</tr>
<tr>
<td>Group II (received 10^4 spores)</td>
<td>640±164</td>
<td>595±108</td>
</tr>
<tr>
<td>Group III (Normal control)</td>
<td>620±88</td>
<td>525±74</td>
</tr>
</tbody>
</table>

P< 0.05* when compared to control group.

4.1.4.5 Haemagglutin antibody titer

Development of anti SRBC antibody titer in mouse serum of treated groups untreated controls was evaluated by haemagglutination method. The antibody titers was determined by examining the plates for mat formation. Spore treated groups showed an increased response in antibodies against SRBC compared to the control group. Group I (which received spores of 1 x 10^8) animals had an enhanced antibodies against SRBC in the serum than Group II (which received spores of 1 x 10^4) animals. This reveals that spores of MBTU PBBM1 can enhance humoral immune response in a dose dependent manner. Haemagglutination of antibody titers against SRBC is given in Table 4.1.3.

4.1.4.6 Nitroblue tetrazolium (NBT) Assay

NBT is an electron acceptor used to detect indirectly the production of superoxide by peritoneal macrophages. Respiratory burst of cell can be seen microscopically and hence used as a qualitative assessment of superoxide production. See figure: 4.1.3. Group I animals (which receives 1 x 10^8 spores) and Group II animals (which received 1 x 10^4) spores possessed significantly enhanced percentage of cells reducing NBT (p< 0.05) than untreated controls.
group. Among the studied groups, group I animals showed significant increased percentage of cells reducing NBT than the group II. See figure 4.1.2.

<table>
<thead>
<tr>
<th></th>
<th>Percentage of cells reducing NBT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Group</td>
<td>7</td>
</tr>
<tr>
<td>Group receiving 10^8 spores</td>
<td>12</td>
</tr>
<tr>
<td>Group receiving 10^4 spores</td>
<td>9</td>
</tr>
</tbody>
</table>

**Figure 4.1.2: Nitroblue tetrazolium (NBT) Assay**

**Figure 4.1.3: Photomicrograph showing cells undergoing respiratory burst (200 X magnification)**
4.1.4.7 Delayed type Hypersensitivity

Delayed-type hypersensitivity was measured by the extent of local inflammation determined by the increase in sole thickness after a second dose of SRBCs. Mice which received different dosages of MBTU PBBM1 spores showed DTH responses in a dose dependent manner. The DTH responses of mice of various treated and untreated controls are shown in Figure 4.1.4 and 4.1.5& 4.1.6. Group I animals (which received $1 \times 10^8$ spores) and Group II animals (which receives $1 \times 10^4$ spores showed significantly enhanced DTH ($p<0.05$) than untreated controls group. Among the studied groups, group I animals showed significant increased DTH than the group II animals.

![Figure 4.1.4: Delayed type Hypersensitivity (DTH)](image-url)
Figure 4.1.5: Mouse foot pad control

Figure 4.1.6: Delayed type Hypersensitivity of group I animals
4.1.4.8 Splenic lymphocyte Proliferation.

Spleen immune function was evaluated through the analysis of lymphocyte proliferative response. Administration of spores caused significant increase in the proliferative activity of splenic immune cells (stimulated by 1.5 μg/mL concanavalin A). Mice treated with MBTU PBBM1 spores showed significantly higher splenic lymphocyte proliferations than the untreated control group ($P < 0.05$). The responses in group I (which received $1 \times 10^8$ spores) and group II (which receives $1 \times 10^4$ spores) animals were found to be dose dependent. (See figure 4.1.7).

![Image of Splenic lymphocyte Proliferation](image.png)

**Figure 4.1.7: Splenic lymphocyte Proliferation**

4.1.4.9 Bacterial translocation test and persistence in ileac section

Translocation of administered spores to various internal organs of treated group after probiotic treatment will reveal the pathogenic nature of treated spores. There was no bacterial growth in the cultures, which indicates that the visceral surface was not contaminated. Ileac sections showed
presence of MBTU PBBM1 which again confirms persistence of MBTU PBBM1 in gastrointestinal tract. No bacteraemia was detected in any of the groups of animals.

4.1.4.10 Histopathological Examination

Intestinal segments stained with haematoxylin-eosin examined by light microscopy demonstrated normal morphology in all animals. No apparent differences were found between, the probiotic fed group and non treated control. Histological examination showed that MBTU PBBM1 fed mice has no signs of inflammation, degeneration or necrosis of the intestinal mucosa. See Figure 4.1.8 & Figure 4.1.9

Figure 4.1.8 Histology of control group III animals

Figure. 4.1.9 Histology of MBTU PBBM1 spores treated animals.
4.1.5 Discussion

Probiotics are being developed commercially for both human use, primarily as novel foods or dietary supplements, and in animal feeds for the prevention of gastrointestinal infections, with extensive use in the poultry and aquaculture industries. Among the large number of probiotic products in use today are bacterial spore formers, mostly of the genus *Bacillus*. (Hong et al., 2004). *In vitro* studies revealed that the strain ‘MBTU PBBM1’ has the capability for exerting probiotic effects. Present study was aimed to evaluate the *in vivo* colonization, safety assessment and immunomodulatory effects of MBTU PBBM1 spores after 30 days continuous oral administration in experimental model. In order to exert probiotic effects the probiotic strain should persist in gastrointestinal tract (GIT). Stable colonization in gastrointestinal tract was noticed in group I animals which received $10^8$ spores of MBTU PBBM1. The group II animals which received $10^4$ spores had no stable colonization in GIT. This indicates that a particular dose is necessary for the establishment of administered spores in the gastrointestinal tract of mouse. Stable persistence of spore counts in faeces after the fifth day of last spore administration again indicates the colonization capability of strain MBTU PBBM1. This persistence study results are supported by the study of Duc *et al* 2004a that some strain of *bacillus* could persist in gastrointestinal tract of mouse. Safety is the most important criterion for selection of new probiotic strains, and there has been considerable debate on appropriate safety testing for new probiotic strains proposed for human consumption (Saarela *et al*. 2000). Of the safety criteria currently proposed for probiotics, the absence of pathogenicity and infectivity are regarded as the most important factor for consideration (Conway, 1996; Huang *et al*. 2004). Present study focused on the infectivity and pathogenicity of
MBTUPBBM1 strain in Balb/c. Animals of all treated groups were found to be healthy when compared to control group. No abnormal behaviour, and no significant weight reduction were observed between spore treated groups and control group. These results indicated that the MBTU PBBM1 spores in two different doses had no adverse effect on the general health status of mice after the 30 days continuous ingestion. Probiotic feeding will not interfered with the normal health status of host. So these above safety assessment results agrees in accordance with findings of Donohue et al. (1996) and Momose et al (1987). Changes in haematological parameters from the normal status are considered as a disease indicator. Increase in peripheral blood neutrophils or eosinophils are useful indicators of bacterial infection. Treated groups and untreated control have no significant change in haematological parameters. This reveals that MBTU PBBM1 spores had no adverse effects on red blood cell count, hemoglobin, and other haematological parameters. These observations again support that mice experienced no infection resulting from the treatment with this strain.

Stimulation of the immune system, or immunomodulation, is considered an important mechanism to support probiosis. A number of studies in humans and animal models have provided strong evidence that oral administration of Bacillus spores stimulates the immune system. (Hong et al 2009). Serum immunoglobulin levels represent a non-specific, relatively insensitive measure of immune function, however total serum immunoglobulin level might moderately reflect the actual humoral immunity in vivo. Stimulation of antibody production appears to be an important mechanism by which bacteria may interact with the host immune system in numerous ways and act to influence the host immune response and increase resistance to infection (Huang et al., 2004; Jian-Kui et al., 2009). Serum Ig A
and Serum Ig G antibodies are implicated in host defense against bacterial infections. MBTU PBBM1 spores induced the stimulation of serum Ig A and serum Ig G in treated groups in a dose dependent manner. This means that spores can modulate humoral immune response. In the blood, IgA interacts with an Fc receptor called FcαRI (or CD89), which is expressed on immune effectors cells to initiate inflammatory reactions. IgG antibodies are predominantly involved in the secondary antibody response; it can bind to many kinds of pathogens and protects the body against them. The entry of the antigens by the oral route is essential to induce the immune response. This fact has been demonstrated by Wostmann and Pleasants, 1991 in germ free mice, receiving an antigen-free diet, where serum IgA and IgG levels were diminished. They concluded that IgA levels depended primarily on the presence of microflora, whereas in the case of IgG levels, diet was the more important factor. Ig M(natural antibodies) and Ig E (allergic antibodies) in serum usually increase early in the course of infection and after that they will remain to a lesser extent. Present study revealed that MBTU PBBM1 has not induced significant increase in these two serum antibodies.

Humoral modulation capacity of MBTU PBBM1 spores was studied by haemagglutination studies. SRBC mediated immune response is a highly sensitive indicator of immunological integrity and requires a coordinated interaction of various cells of immune system. After 30 days continuous ingestion of MBTU PBBM1 spores, a significant response in antibody production against SRBC was found in group I compared to group II. The enhancement of antibody responsiveness to SRBC in mice, may be due to the enhanced responsiveness of macrophages and B. lymphocyte subsets involved in the antibody synthesis (Benacerraf, 1978). Therefore, the
augmentation of the antibody production response to SRBC confirmed the enhancement of humoral immune response.

Macrophage activation by MBTU PBBM1 spores was studied by NBT assay. NBT reduction test is an indirect marker of the oxygen dependent bactericidal activity of the phagocytes and the metabolic activity of granulocytes and monocytes. (Hellum et al. 1977, Dubaniewicz et al. 2004). Kosak et al. (1998) found that orally administered B. subtilis leads to a rapid induction of interferon production by mononuclear cells, which stimulated the activity of both macrophages and NK cells. Present study revealed that MBTU PBBM1 spores treated groups were found to show increased NBT reduction in a dose dependent manner than the control group. This reveals that spore treatment will help in the oxygen dependent bactericidal activity.

Association between Delayed type hypersensitivity and acquired cellular immunity remains controversial, both are manifestations of T-cell–mediated immunologic memory (Baldridge et al. 1990, Tsukada et al. 1991). Cui et al. 2008 reported that DTH responses increased with increasing dosage of two commercial strains. T cells are immune response mediators and play an important role in establishing and maximizing the capabilities of the adaptive immune response. The interaction of sensitized T-cells, with antigen presenting cell, results to the release of cellular mediators, such as histamine, initiation of arachidonic acid metabolism (Grisworld et al., 1982) and eventually to interferon-γ synthesis that will lead to DTH reaction. DTH reactions in treated groups was found to be dose dependent and the group I animals showed increased DTH than the other groups. As expected, in the positive result, the change of thickness in treated groups may be due to the proliferation of the T lymphocytes and the enhanced cell-mediated immunologic function. Lymphocyte proliferation in response to mitogens is
commonly examined when analyzing the efficacy of immunomodulatory agents, and thereby, evaluate the effects of probiotic bacteria on the immune function (Kirjavainen et al., 1999; Campbell et al., 2000; Gill 2001b). It was found that MBTU PBBM1 spores when given to mice, slightly stimulated the spleen cell proliferation in response to Con A, in a dose dependent manner. Exact component involved in enhancing function of MBTU PBBM1 spores on lymphocytes proliferation has not yet understood. Previous study reports shown that specific components or secretions of human *Lactobacillus* or *Bifidobacterium* strains, including chromosomal DNA, cytoplasm, cell wall structures, and exopolysaccharides, can act as mitogens and induce lymphocyte proliferation (Kitazawa et al 2001, Olaya et al., 1998, Amrouche et al 2006,). Indeed, increased T-cell proliferation may act as a wash-out mechanism for pathogenic microbial agents. Through this lymphoproliferation study it was supposed that these MBTU PBBM1 spores strains may have the potential to improve the cellular immunity.

Translocation is recommended as an indicator of probiotic toxicity, because it is the first step in the pathogenesis process for many opportunistic strains indigenous to the lumen. (Steffe and Berg, 1983). No bacteremia and no translocation of bacteria was associated with MBTUPBBM1 treatment. Spore counts in the ileac section revealed the evidence of persistence of spores in gastrointestinal tract. This again supports the faecal persistence of spores which was previously described in section 4.1.4.1. Histological alterations in small intestine colonized by probiotic are indirect indicators of infection, but in this study any abnormal macroscopic changes in the small intestine of animals treated with MBTU PBBM1 spores was observed. Dose dependent studies revealed that group which receiving 1x10⁸ spores showed an effective humoral and cell mediated immune response in balb/c mice. So this dose can
be decided as an appreciable measure which can evoke an effective humoral and cell mediated immune response. Studies revealed that this strain could colonize gastrointestinal tract which would further act as a key to the initiation of immunomodulation. The previous result of *in vitro* adhesion studies also supports the colonization capability of MBTU PBBM1 spores. Present results demonstrates that 30 days consumption of MBTU PBBM1 spores had no adverse effects on animals’ general health status, haematology, gut mucosal histology parameters. The bacterial translocation was not also observed. The entire study results suggests that the ingestion of MBTU PBBM1 would enhance the immunity indicating immunomodulatory effect.
CHAPTER 4.2

Preventive and curative role of MBTU PBBM1 Spores on cyclophosphamide induced Immunosuppression in Balb/C Mice
4.2 PREVENTIVE AND CURATIVE ROLE OF MBTU PBBM1 SPORES ON CYCLOPHOSPHAMIDE INDUCED IMMUNOSUPPRESSION IN BALB/C MICE

4.2.1 Materials and Methods

4.2.1.1 Animals

Male Balb/c mice 8 week old, weighing (20-21) g used in present study were obtained from small animal breeding station under Kerala Veterinary and Animal Sciences University, Mannuthy, Thrissur. The animals were cared in same way which was discussed in section 4.1.3.1.

4.2.1.2 Spore Preparation

In vivo immunomodulatory studies in chapter 4.1 revealed that spores of MBTU PBBM1 can induce immune responses in a dose dependent manner. Hence the present study was aimed to analyze the possible in vivo effects of MBTU PBBM1 spores in a dose of $1 \times 10^8$ spores in immunosuppressed Balb/c mice. Aliquots of $10^8$ spores, were prepared in 0.1 ml of sterile PBS in the previous manner which was described in section 4.1.3.2.

4.2.1.3 Preparation of Immunosuppressant

In the present study cyclophosphamide (CTX) (Khandelwal Laboratories Ltd. Mumbai) was used as immunosuppressant in Balb/c mice. Drug was dissolved in 0.9% NaCl and administered orally for ten consecutive days to mice at the dose of 30 mg/kg body weight to induce immunosuppression.
4.2.1.4 Experimental design

Experimental set consisted of five groups and each group consists of six animals. Among the five groups, Group I (Preventive) received MBTU PBBM1 spores for 28 days followed by the ten days treatment of immunosuppressant cyclophosphamide. This group explains the protective effects contributed by MBTU PBBM1 spores against cyclophosphamide induced immunosuppression. Group II (Preventive control) received 0.1 ml of PBS for 28 days followed by the ten days treatment of immunosuppressant cyclophosphamide. This group remained to be immunosuppressed positive control and also revealed the toxic effects of cyclophosphamide. Group III (Curative) received ten days treatment of immunosuppressant cyclophosphamide followed by the treatment of MBTU PBBM1 spores for 28 days. This group explains the curative role contributed by MBTU PBBM1 from immunosuppression induced by cyclophosphamide. Group IV (Curative control) received ten days treatment of immunosuppressant cyclophosphamide followed by the treatment 0.1ml of PBS for 28 days. This group explains the normal curing after immunosuppression induced by cyclophosphamide Group V (Normal control) received 0.1 ml of PBS control for all the days up to the end of experiment.

4.2.1.5 Measurements of general health and growth

Throughout the experimental period, behavioural changes, activity, treatment-related illness or death, difference in hair luster between treated and control groups were monitored. Unhealthy symptoms, weight gain or lose of all groups were recorded. Feed and water intake nature of animals were observed.
4.2.1.6 Hematological parameters

Blood samples were collected from each individual mouse of all groups. Haematological parameters of all groups were analyzed according to the procedures described in section 4.1.3.1. Blood from all the groups were collected immediately on the first day after the study period. Haematological parameters such as total red blood cell (RBC), haemoglobin concentration (HB), haematocrit, total counts of white blood cells and differential count of lymphocytes, neutrophil were determined in the same manner which was previously discussed in 4.1.3.6

4.2.1.7 Humoral Immune Response

Humoral antibody responses of MBTU PBBM1 spores and CTX treated groups were studied and compared with normal control.

4.2.1.7.1 Hemagglutinin antibody titer

Haemagglutination antibody titer of all study groups were analyzed according to the procedure described previously in 4.1.3.7.3

4.2.1.8 Cell mediated immune responses

Nitro blue tetrazolium Assay (NBT), Delayed type hypersensitivity (DTH) response, Proliferation of splenic lymphocytes were studied in all groups according to the procedures described previously in section 4.1.3.8

4.2.1.9 Histopathological examination

After the end of the experimental period, mice from all groups were sacrificed and dissected. Immediately after sacrifice a small portion of small intestine were taken and washed carefully in sterile PBS tissue and was fixed in 10% buffered formalin. Specimens were dehydrated by passing through ascending grades of alcohol, cleared in xylene, impregnated and embedded
in paraffin. Thin sections were cut (3-5 um) and were stained using hematoxylin and eosin.

4.2.2 Results

4.2.2.1 Measurements of general health and growth

Cyclophosphamide treatment affected the general health and growth of the mice. CTX positive control (group II) mice showed lethargy, lack luster pelage, reluctance to environmental activity, fur piloerection, and reduced food and increased water intake. No death occurred during the time of CTX administration. Significant weight lose was observed in this group. Gastrointestinal problems observed because faeces were found to be loose in nature. Group I (Preventive) animals had no signs of gastrointestinal upsets. Intake of food and water remained to be normal. Group I (Preventive) animals showed an increased body weight than CTX induced positive control (group II). Compared to CTX positive control (group II), group I animals showed a better behavioural pattern. Group I and group II results revealed that MBTU PBBM1 spores treatment prior to CTX administration can enhance growth and prevent CTX induced unhealthy symptoms.

During the time of CTX administration, group III (Curative) animals showed unhealthy symptoms, but after the treatment of MBTU PBBM1 spores most of the symptoms were alleviated. Animals had no signs of gastrointestinal upsets and the animals were found to be active. Intake of food and water remained to be normal. Group III animals possessed a significantly increased body weight (P<0.05) than CTX treated group IV (Curative control). Compared to group IV animals, group III animals showed a better behavioural pattern. Reduced food intake of group IV animals resulted in the weight loss. Group III and group IV results revealed that
MBTU PBBM1 had an added capability in curing CTX induced unhealthy symptoms. Group V normal control which received PBS alone had no adverse clinical signs in any of the mice during the period of experimentation. No sicknesses were recorded. Feed and water intakes were not affected the MBTU PBBM1 treated, group I and group III, showed significantly (P<0.05) better health and growth pattern than CTX alone treated groups. (See Figure 4.2.1)

4.2.2.2 Hematological studies

Changes in haematological parameters from the normal status are considered as a disease indicator. The effects of various treatments on haematological parameters are shown in Table 4.2.1. CTX treatment in animals, reduced the total WBC and neutrophil, lymphocyte counts. Total RBC and haemoglobin levels of all treated groups and normal were not different significantly (P>0.05). Haematological parameters of group I (Preventive) mice showed significant difference (P<0.05) from group II (CTX positive control)
mice. Results from group I and group II animals revealed that prior treatment of MBTU PBBM1 spores could prevent CTX induced reduction of the total WBC, neutrophil and lymphocyte counts. Group III (curative) animals showed significantly increased (P<0.05) total WBC and neutrophil, lymphocytes count than the group IV (curative control) animals. This revealed that MBTU PBBM1 spores, had an added curative capability than natural curing, of group IV animals. Group IV (curative control) animals showed improvements in haematological parameters which explains the natural curing after CTX administration. Group V (untreated normal control) possessed normal level of haematological parameters. Compared to normal control, all treated group showed a significant reduction in WBC levels.

Table 4.2.1 Haematological Parameters

<table>
<thead>
<tr>
<th>Haematological parameters</th>
<th>Group I (Preventive)</th>
<th>Group II (CTX positive control)</th>
<th>Group III (Curative)</th>
<th>Group IV (Curative control)</th>
<th>Normal Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemoglobin (g/l)</td>
<td>12.2±.6</td>
<td>12.1±.4</td>
<td>12.4±.2</td>
<td>12.3±0.2</td>
<td>12.7±.4</td>
</tr>
<tr>
<td>RBC (10^3 /mm^3)</td>
<td>5.7±0.10</td>
<td>5.6±0.18</td>
<td>5.5±0.14</td>
<td>5.3±0.20</td>
<td>5.9±0.2</td>
</tr>
<tr>
<td>WBC (10^3 /mm^3)</td>
<td>3.12±.31</td>
<td>1.98±.45</td>
<td>4.01±.02</td>
<td>3.02±.14</td>
<td>4.59±.22</td>
</tr>
<tr>
<td>Neutrophil (%)</td>
<td>6±2.37</td>
<td>2.71±3.46</td>
<td>8.82±2.16</td>
<td>3.94±3.46</td>
<td>16.8±2.72</td>
</tr>
<tr>
<td>Lymphocytes (%)</td>
<td>39±2.2</td>
<td>21.21±3.6</td>
<td>42±1.7</td>
<td>32±2.4</td>
<td>71.4±2.8</td>
</tr>
</tbody>
</table>

4.2.2.3 Humoral Immune response

4.2.2.3.1 Haemagglutinin antibody titer

Development of SRBC antibody titer in mouse serum of different treated groups and untreated control were evaluated by haemagglutination method. Antibody titer was determined by visualizing plate for mat formation in microtiter plate. Antibody titers of various treated groups and
normal control are shown in Table 4.2.2 Group I (Preventive) animals showed a significantly (P<0.05) higher antibodies against SRBC compared to Group II (CTX positive control) animals. CTX alone treated group II animals showed suppressed antibody titer against SRBC than normal control group V. Results of group I and II revealed that MBTU PBBM1 treatment enhanced antibody titer against SRBC.

Group III animals (Curative) showed significantly increased (P<0.05) amount of antibody titer against SRBC than group IV animals (Curative control). This result clearly showed the enhanced role of MBTU PBBM1 spores, in curing CTX induced suppression on antibody production against SRBC. Group IV animals showed a higher antibody titer against SRBC than normal control group. Among groups I &III, group I possessed an enhanced antibody titer against SRBC than group III animals.

Table 4.2.2 Haemagglutination studies.

<table>
<thead>
<tr>
<th>Name of Groups</th>
<th>Haemagglutinin Titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I (Preventive)</td>
<td>128±8.7</td>
</tr>
<tr>
<td>GroupII (CTX positive control)</td>
<td>8±4.5</td>
</tr>
<tr>
<td>GroupIII (Curative)</td>
<td>64±8.7</td>
</tr>
<tr>
<td>GroupIV (Curative control)</td>
<td>32±4.3</td>
</tr>
<tr>
<td>GroupV Normal Control</td>
<td>16±2.3</td>
</tr>
</tbody>
</table>

4.2.2.4 Cell mediated immune responses

4.2.2.4.1 Nitroblue tetrazolium (NBT) reduction Assay

NBT is an electron acceptor used to detect indirectly the production of superoxide by peritoneal macrophages. Percentage of NBT reducing cells of different treatment groups are given in Figure 4.2.2. Group I animals (Preventive) showed significantly increased number of cells reducing NBT (P<0.05) than group II animals (CTX positive control). Group II animals
showed a very less amount of cells reducing NBT. Results of group I and II revealed that MBTU PBBM1 spores treated group I animals showed a preventive effect against CTX induced suppression on the percentage of NBT reducing cells.

Group III (Curative) animals showed significantly (P<0.05) increased NBT reducing cells than group IV (Curative control) animals. This result clearly showed the, enhanced role of MBTU PBBM1 spores, in curing CTX induced suppression on the percentage of NBT reducing cells. This curing capability was found to be higher than group IV animals which reflected the natural curing after CTX treatment. Group V untreated normal control possessed normal percentage of NBT reduction.

![NBT Assay](image)

**Figure 4.2.2: Nitroblue tetrazolium (NBT) reduction Assay**

### 4.2.2.4.2 Delayed type Hypersensitivity(DTH)

Delayed-type hypersensitivity was determined by the extent of local inflammation and was measured by the increase in sole thickness after a second dose of SRBC. DTH reactions of all groups are shown in Figure 4.2.3.
Group I (Preventive) animals showed an increased sole thickness and it was significantly different (P<0.05) from group II (CTX positive control) animals. Group II animals showed no increased sole thickness because of the CTX induced suppression on cell mediated immune responses. Results of group I and II revealed that MBTU PBBM1 spores treated group I animals showed, a protective effect against CTX induced suppression on cell mediated immune responses.

Group III (Curative) animals showed significantly (P<0.05) increased delayed type hypersensitivity than group IV (Curative control) animals. This result clearly showed the enhanced role of MBTU PBBM1 spores, in curing CTX induced suppression on cell mediated immune responses. This curing capability was found to be higher than group IV animals which showed natural curing after CTX treatment. Untreated normal control group V possessed normal level of delayed type hypersensitivity.

![Figure4.2. 3 Delayed type hypersensitivity(DTH)](image)
4.2.2.4.3 Splenic lymphocyte Proliferation.

Spleen immune function was evaluated through the analysis of lymphocyte proliferative response. Lymphocyte proliferative percentage in animals receiving different treatments showed different proliferation rate. Results of proliferation studies were given in figure 4.2.4, Group I (Preventive) animals showed a significantly (P<0.05) increased proliferation than group II (CTX positive control). Group III (Curative) animals showed significantly increased proliferation (P<0.05) than group IV (Curative control) animals. This result clearly showed the, enhanced role of MBTU PBBM1 spores, in curing CTX induced suppression on splenic lymphocyte proliferation. This curing capability was found to be higher than group IV animals which explain natural curing after CTX treatment. Untreated normal control group V possessed normal level of splenic lymphocyte proliferation.

![Figure 4.2.4: Splenic lymphocyte proliferation](image-url)
4.2.2.5 Histopathological Examination

Intestinal segments stained with haematoxylin and eosin was examined by light microscopy demonstrated histology of treated groups and normal control and positive control. The positive control exhibited all histological anomalies induced by CTX. (See figure 4.2.5). It was found that after CTX treatment, intestinal villi was shrunked and height was reduced. The proximal part of the villus was covered by large misshapen cells and the villus was considerably shrunken in size and finally collapsed into small mounds. Crypt architecture was completely lost and brush border discontinuity was observed in intestinal villi. Group I (Preventive) animals showed a markedly better histology than group II (CTX positive control) animals. Crypt architecture were slightly lost but not as much of group II. Shrinkage of villi was found to be less when compared to group II CTX positive control. Figure 4.2.6 revealed that prior MBTU PBBM1 spores treatments had a protective role in CTX induced anomalies.

Histological studies in the group III (Curative) animals revealed the curative role played by MBTU PBBM1 spores in curing the anomalies induced by CTX treatment. Shrinkage of villus and crypt architecture were observed. Histological studies revealed that group III animals possessed a better healing of anomalies than group IV (Curative control) animals. (See figure 4.2.7.) Histological studies in group IV animals revealed the natural curing capabilities after CTX treatment. The villus remained to be shrinked and not much improvement was observed in crypt architecture. See figure 4.2.8. Untreated normal control group V possessed normal histology of small intestine. See figure 4.2.9.
Figure 4.2.5: CTX induced histological anomalies of Group II

Figure 4.2.6: Preventive effect of MBTU PBBM1 spores (group I) against CTX induced histological anomalies

Figure 4.2.7: Curative effect of MBTU PBBM1 spores (group III) in CTX induced histological anomalies
4.2.3 Discussion

Many probiotic effects are mediated through modification of the immune functions and this has been demonstrated by investigations on immunologically intact animals or humans, with the exception of some studies on elderly subjects (Gill et al., 2001), and in nude mice (Wagner et al., 1997) and malnourished mice (Cano and Perdigon, 2003; Hidemura et al., 2003). Immunomodulatory studies on MBTU PBBM1 spores in section 4.1 revealed
that this strain can enhance both humoral and cell mediated immunity. Present study was aimed to investigate preventive and curative effects of probiotic strain MBTU PBBM1 against cyclophosphamide induced immunosuppression. Cyclophosphamide, a classical myelotoxic agent, has been used in this study to establish an immunosuppressed experimental model. CTX is a non specific cytotoxic agent that can inhibit both humoral and cell mediated immunity. The preventive and curative role of MBTU PBBM1 spores in immunosuppression was studied with focus on the animal’s haematology, immune responses and histological alterations in small intestine. Group I (preventive) results explained preventive capability of MBTU PBBM1 spores in CTX induced immunosuppression. Group II (CTX positive control) results explained the possible toxic effects of CTX administration. Group III (curative) results explained curative capability of MBTU PBBM1 spores in CTX induced immunosuppression .Group IV (curative control) explained natural curative effects in CTX induced immunosuppression. Group V (normal control) remained as untreated normal control.

Measurement of general health and growth revealed that MBTU PBBM1 spore treated groups can tolerate the CTX induced immunosuppression both in preventive and curative manner. These results suggest that MBTU PBBM1 spores had a protective role against CTX induced immunosuppression which helps in maintaining normal health status. This may be attributed by the production of health promoting substances such as vitamins and aminoacids. Sanders et al., 2003 supports the health promoting aspects of sporeformers as probiotics. MBTU PBBM1 spores treatment in animals before and after immunosuppression revealed that these groups of animals had reduced CTX induced ill effects. Haematological parameters of treated groups were significantly different from the normal control. CTX treatment
significantly induces leucopenia, neutropenia, and lymphopenia. This was in good agreement with previous study, that the cyclophosphamide treatment lead to the decrease of WBC and lymphocyte counts in Balb/c mice, (Xiao et al., 2011). Group I and group III animals possessed an increased count of neutrophil, lymphocytes and WBC than group II and group IV animals after CTX treatment. This revealed that MBTU PBBM1 spores had both preventive and curative role in CTX induced immunosuppression. This preventive and curative capability in hematopoietic recovery may be due to the influence of certain growth factors from MBTU PBBM1 spores which may further stimulated colony stimulate factors of both granulocytes and agranulocytes.

Okuyama et al., 1989 Smith et al.,1984 Zhu et al., 1987 found that cyclophosphamide affects lymphocyte function, delayed-type hypersensitivity and is selectively toxic to B cells. Haemagglutination studies revealed that CTX treatment in mice suppresses its humoral immune responses. Selective toxicity of CTX to B cells affects the humoral immune system of mice. So anti antibody titer against SRBC after CTX treatment were found to be lower in group IV and group II. Group IV animals shown an increased antibody titer than normal control against SRBC revealed the slight immune enhancive effect induced by CTX. Jonathan et al.,2000 found that CTX treatment in low doses will make stress in immunesystem followed by the enhancement of humoral immune system. Antibody titers of group I and group III were found to be significantly higher than group II and group IV animals. This preventive and curative capability of MBTU PBBM1 may be due to the enhancement of humoral immunity of mice which positively increase the antibody titer against SRBC. The enhancement of antibody responsiveness to SRBC in mice may be due to the enhanced responsiveness of macrophages and B. lymphocyte
subsets involved in the antibody synthesis (Benacerraf, 1978). Previous results in chapter 4 (section 4.1.4.5) also supports the present haemagglutination study results.

Stimulation of normal phagocytic cells elicits a non-mitochondrial burst of respiration in which there is a huge increase in oxygen consumption and the production of remarkable amounts of superoxide anion in polymorphonuclear cells (Borregaard 1985). The respiratory burst is a distinguishing feature of phagocytes that serves as an important defense mechanism against invading pathogens necessary for effective microbiocidal action (Jiz et al., 2007). The production of reactive oxygen species reflected stimulation of cellular activation and phagocytosis in relation to extracellular killing. (Jiz et al., 2007). However, cyclophosphamide treatment, suppressed viable macrophages which reduced NBT. Percentages of cells reducing NBT after CTX treatment were found to be lower in CTX alone treated, group IV and group II. Group I and group III results explained the preventive and curative role of MBTU PBBM1 in improving the percentage of cells reducing NBT via macrophage activation. Previous immunomodulatory studies in chapter 4 (section 4.1.4.6) again supports the present results of NBT assay.

Delayed type hypersensitivity and acquired cellular immunity remains controversial, both are manifestations of T cell mediated immunologic memory (Baldridge et al., 1990, Tsukada et al., 1991). In the present study mice foot-pad thickness (delayed type hypersensitivity DTH) was studied using SRBC antigen. When this cellular antigen, was administered in group II, the foot pad thickness was brought down and was much reduced. This results was supported by the reports of Laemmli et al., 1970 that CTX treatment results in reduced DTH reaction. Results revealed that DTH after CTX treatment was found to be lower in group IV and group
II. This may be due to the suppression in cell mediated immunity. Previous studies have reported that, in animals treated with CTX at the time of antigenic challenge, DTH responses were depressed, as a result of the loss of DTH effectors functions (Gill & Liew, 1978; Mitsuoka et al., 1976; Diamantstein et al., 1981; Rondinone et al., 1983). Group I and group III results explained the preventive and curative role of MBTU PBBM1 against CTX induced suppression in delayed type hypersensitivity. Results clearly indicated that certain mediators involved in DTH, was elevated during the treatment of MBTU PBBM1 and these groups showed a comparatively increased level of foot pad thickness. Previous studies revealed that interaction of sensitized T-cells, with antigen presenting cell, resulted in the release of cellular mediators, such as histamine, initiation of arachidonic acid metabolism (Grisworld et al., 1982) and eventually to interferon-γ that will lead to DTH reaction.

Lymphocyte proliferation in response to mitogen is commonly used to evaluate the effects of probiotic bacteria on the immune function. Previous immunomodulatory studies revealed that MBTU PBBM1 treatment in normal mouse stimulated splenic lymphocyte proliferation. (Ref section 4.1.4.8). However, here the suppressive effect of cyclophosphamide on the Concanavalin A driven lymphoproliferation was enhanced by MBTU PBBM1. Splenic lymphocyte proliferation after CTX treatment was found to be lower in group II and group IV animals. Group I and group III results explained the preventive and curative role of MBTU PBBM1 against CTX induced suppression in lymphocyte proliferation. This preventive and curative capability may be due to the enhancement of lymphoproliferation in mice by MBTU PBBM1. A probiotic strain of *L. plantarum* was a potent inductor of cytokine release by mononuclear peripheral blood leukocytes from healthy human donors (Miettinen et al., 1996). Based on its capacity to
increase the production of interleukin-10 (IL-10) by macrophages and T cells from the intestinal mucosa, possible use of *L. plantarum* in the treatment of inflammatory bowel diseases has been considered (Pathmakanthan *et al*., 2004). This IL-10 was able to promote proliferation of B cells and CD8+ T cells, and to antagonize the immunosuppressive effect of cyclosporine (Moore *et al*., 2001). Therefore, the induction of IL-10 might be involved in the stimulatory effects of MBTU PBBM1 on the proliferative capacity of splenocytes from immunologically intact or compromised mice.

The intestinal mucosa plays an important role in gut barrier function as it prevents potential pathogens and toxigenic substances from invading systemic tissues (Ma *et al*., 1990). Previous studies on oral administration of MBTU PBBM1 (Ref section 4.1.4.10) had no adverse effects on the integrity of the gut mucosa, thus further suggesting that these strains were non-toxic. Histological studies revealed that group II animals’ exhibited all anomalies induced by CTX. Group I and group III results explains the preventive and curative role of MBTU PBBM1 against CTX induced adverse effects on small intestine. This preventive and curative capability may be due to the colonization and further interaction of the MBTU PBBM1 strain on intestinal mucosa. From the results it was evident that probiotic strain MBTU PBBM1 was capable for producing some beneficial substance which have interacted directly with cyclophosphamide to retard the drug’s immunosuppressive properties. Potent colonization in gastrointestinal tract can convert the toxic effects of CTX. *Bacillus* species (*B. subtilis, B. firmus, B. megaterium* and *B. pumilus*) have recently been shown to convert genotoxic compounds to unreactive products *in vitro* and this has been proposed as a probiotic mechanism, if this could occur in the intestine (Caldini *et al*., 2002).
Histopathological studies on group IV results revealed the natural curing of anomalies which was induced by CTX.

Immunomodulatory studies in normal mice have proved that the strain MBTU PBBM1 has a significant role in improving both humoral and cell mediated immunity. CTX induced immunosuppressive studies show that MBTU PBBM1 spores could potentially prevent and reverse immunosuppressive effects of cyclophosphamide, possibly by immunomodulating mechanisms. The above potentials of MBTU PBBM1 spores provide strong information sufficient enough to indicate its possible use as a probiotic agent after further studies.