CHAPTER 6

Immuno-modulatory study of the selected Probiotic organisms

6.1. INTRODUCTION:

Probiotics are the health benefiting bacteria that generally adhere to the human intestine for stimulating, regulating, and modulating various different functions which include metabolism, digestion, exclusion of the pathogenic bacteria, and epithelial immunity (innate and adaptive). [Hill *et al*., 2014] The gut environment is predominated by the beneficial microbiota and the non-digestible food which play a crucial role for balancing the gut microbial community [Bin *et al.*, 2018].

The immunity of the vertebrates is comprised of innate as well as adaptive immunity, where innate immunity has a non-specific shielding mechanism and adaptive is highly specific for the invading pathogens in the vertebrates [Tan *et al.*, 2015]. Both B cells and T cells exert an acquired immunity in response to the different upcoming antigens via some specific antigenic receptor recognition [Kau *et al.*, 2011].

The elite properties of the bacteria that are highlighted by the researchers include; anti-microbial, anti-inflammatory, anti-pathogenic, anti-allergic, anti-obesity etc. Large number of researchers supported the mechanism of the immuno-modulation which suggests that, antigenic fragments of the probiotic (such as, peptidoglygan layer) have the potential to overcome the barrier of intestinal cells and M cells in Payer's patches to initiate different modulation of the immunity. [Galdeano and Perdigón, 2004] The modulation of the host immunity is attributed by the probiotics by releasing cytokines including, IL, TNF, IFN, TGF and also certain chemokines from different host's immune cells (macrophage, mast cells, epithelial cells etc.). [Foligné *et al.*, 2010]

The probiotics (such as, *Bifidobacteria*) invading their cell wall components are able to regulate the immunity by stimulating the NO synthase that is represented by macrophages through TNF-α secretion. [Véronique, *et al.*, 2008] According several studies it was established that, the inflammatory process is the combination effect of pro- and anti-inflammatory immuno-modulators. IL-10 which is an anti-inflammatory cytokine, stimulate or inhibit various chemokines and cytokines which is responsible for intestinal inflammation. The probiotics also have the ability to act antagonistically with different infections and cancer by inhibiting the production of IL-12 which can activate NK cells by developing the Th1 cells [Chiba *et al.*, 2010].

In one of the *in vitro* study it was suggested that the cytokines like interleukin 1β, 8 and TNF α were induced by a probiotic strain *L. sakei* and can also enhance the production of TGF-β [Galdeano *et al.*,2007]. In addition to it some other cytokines also showed enhanced expression on the presence of such LAB group of bacteria in inducing adaptive immunity in the presence of some anti-inflammatory cytokines like interleukin 4, 5, 6 and 10 that is generated by Th 2 subsets, DCs, B cells and monocytes [Moore *et al.*, 2001]. Another cytokine IL 12 produced by antigen presenting cells and activates T- cells along with NK cells, which induces the stimulation of IFN-γ [Rogers *et al.*, 2005].

Thus to investigate the expressions of different pro- and anti- inflammatory cytokines, the current study was framed to evaluate the role of these cytokines while the disease induction period as well as during the treatment with the isolated strain of probiotics and also to find out the fluctuation in their expression in presence of different prebiotics as well treating them in combination to it (i.e., synbiotics).

6.2 MATERIALS AND METHODS:

6.2.1. FINE CHEMICALS

Tris-HCl (BioRad #161-0799), SDS (sodium dodecyl sulfate; Biorad #161-0301), DTT (dithiothreitol; BioRad # 161-0610) were obtained from BioRad, Berkeley, California. All the components of SDS PAGE along with PBST and p-nitrophenylphosphate solution and the antibodies for western blot were obtained from Hi-Media, India and Sigma (St. Louis, USA) and are of analytical grade.

6.2.2. INSTRUMENTATION:

The equipments used for the studies were: Cooling Centrifuge (REMI, India), Polyacrylamide Gel Apparatus (Genei, India), ECL (BioRad, Berkeley, California) and ELISA machine (Robonik, INDIA).

6.2.3. SAMPLE PREPERATION:

The BALB/c mice were kept in starvation condition for a day before the dissection. Next day the mice were euthanized under isoflurane anesthesia and the blood was collected via heart puncture and left for clotting at 4°C for 2 hours which is followed by 15 mins centrifugation (3000 rpm) and the supernatant was collected and stored at -20°C for further use. The obtained serum was preserved at -80°C using liquid nitrogen. The protein content was analysed and the concentration was determined by the help of a popular assay by Bradford Reagents.

6.2.4. PROTEIN ESTIMATION USING BRADFORD'S METHOD:

Following the method of Bradford (1976), the total protein was estimated. The protein sample was prepared by the following method, in which the components added were, Coomassie brilliant blue G-250 (20 mg), methanol (10 mL) and orthophosphoric acid (20 mL) to prepare a final volume 200 mL by distilled water and then the extract was filtered for the further use which appears pale yellow in the test tubes and prior to the examinations the samples were stored in dark. BSA was added to the tubes with different concentrations (20 to 100 mg/mL) in 0.15 (M) NaCl and is considered as standard in this experiment. 5 µL of test samples and 95 µL of 0.15 (M) NaCl were added to each tube and to it 1 mL of Bradford's reagent was finally added. It was then kept at dark for 5 minutes incubation. The O.D. was measured at 595nm and the readings were plotted on a standard graph and to estimate the total protein concentration.

6.2.5. SDS - PAGE:

A 10% Polyacrylamide gel was used for separation and was run at 20 mA for approximately 120 min. The extracted and estimated protein was loaded in the well of the gel and was kept to run. After running of the protein its upto a specified target length they were transferred into a neutral buffer.

The stacking gel used for western blot is slightly acidic and has a lower acrylamide concentration for a better porosity that helps in better protein separation. The separating is basic in nature and has a higher polyacrylamide content making it less porous. Protein is thus separated by their size. The proteins while loading was slightly negative in charge, and thus will travel toward the (+) ve

electrode when a essential specific voltage is applied to it, as a high voltage can destroy the band due to over-heating.

6.2.6. WESTERN BLOT TECHNIQUE:

The proteins extracted from the intestinal tissue of the BALB/c mice were first homogenized and then were separated using SDS-PAGE and shifted to a nitrocellulose membrane. Subsequently, the blocking of membrane was done with 5% skimmed milk at 4ºC, and was washed with tween PBS. Post washing the membrane was incubated with specific primary antibody (i.e.; rabbit anti-IL1, anti –IL6, anti –IL10, anti –IL12, anti –TNF α, anti –IFN γ and anti –NF κβ) in 5% BSA and was incubated in 4ºC on a shaker overnight. After incubation, the membrane was washed using TBST for 5 minutes and the process was repeated for five times. It was then incubated with secondary antibody conjugated with HRP and TBST. Post incubation the membrane was again washed and developed using Horse Radish Peroxidase (HRP) substrate. It was then finally visualized in the dark room and photographed.

6.2.7. ELISA:

As per the instructions of the manufacturer (BD OptEIA set) to evaluate the serum antibodies by ELISA, each well of 96-well microplate was filled with buffer and incubated at 4°C for overnight and then treated the wells with PBST. Immediately after that 100 µl of blocking buffer was added. After repeated washing 100 µL mouse serum was added to it. Then all the plates were subjected to incubation for two hours which is followed by washing for three times, and 100 µL of conjugated ALP containing anti-mouse TNF α, IL-10 and IL-1β. Substrate used as *p*-nitro-phenylphosphate solution and the wells were incubated at dark. O.D. was taken at 405 nm.

6.3 RESULT AND DISCUSSION:

In this study, the expression of various cytokines was evaluated to establish a comparative study of the activity of pro- and anti- inflammatory immune-modulators in the response to the disease induction by the DSS (Dextran Sodium Sulfate) and also by the treatment with synbiotics. The pro-inflammatory cytokines that was used here for the evaluation of the successful disease induction by DSS on R2 are Interleukin 1β, 6, 12, TNF-α, IFN γ and NF-κβ. Subsequently, the effectiveness of the designed treatment of this study was re-confirmed by evaluating the expression of IL-10.

From the previous experiments, on the basis of DAI (Disease Activation Index) and the enzyme assay it was already an established fact that DSS is the most potent chemical inducer of colitis which successfully induced the disease on the animal model R2 and the most effective combination of treatment was also considered as the symbiotic that was applied on R9. Thus, the further study of immune-modulation was carried out taking R2 as the diseased sample and R9 as the treated sample.

Moreover, the study was performed on the basis of both the techniques, i.e., western blotting and ELISA to reconfirm the selection of the therapeutic combination designed here.

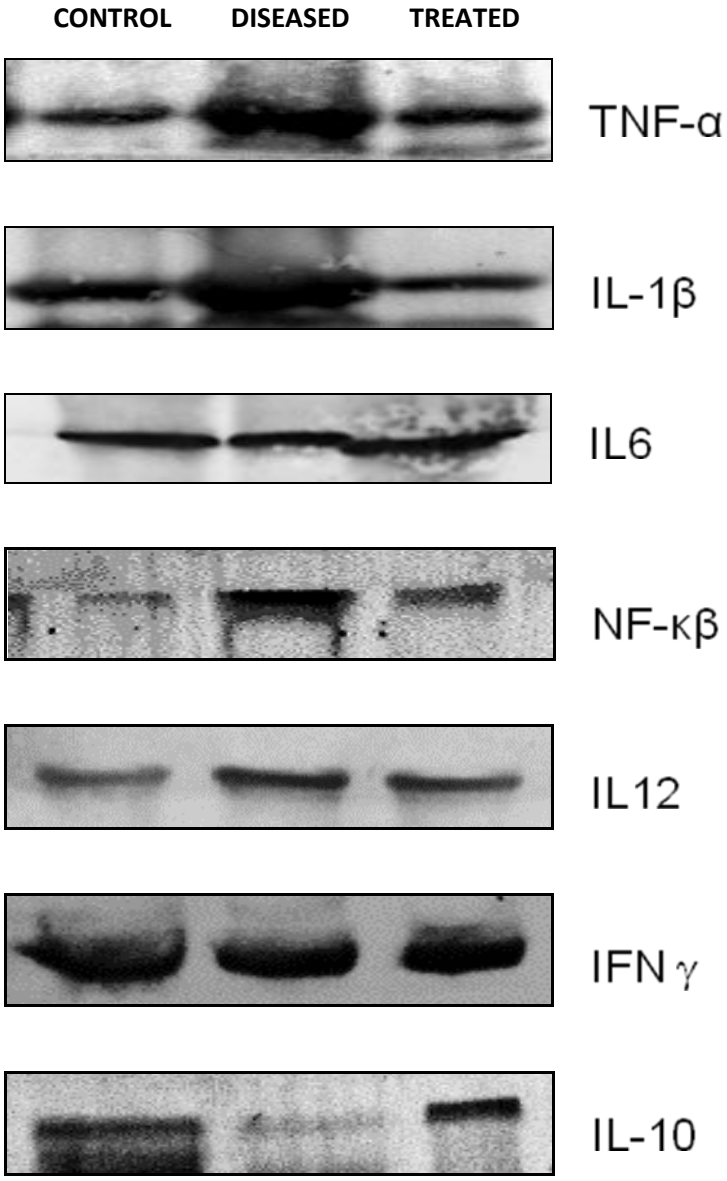

FIGURE 6.1: Western blot of different cytokine (TNF-α, IL-1β, IL6, NF-κβ, IL-12, IFNγ and IL-10).

The designated animal models referred here are, R1: Control; R2: Diseased with DSS; R9: Treated with synbiotics on colitis induced mice

The immune stimulatory property of the pro- and anti-inflammatory cytokines when symbiotic was used as a treatment on the BALB/c mice was evaluated by blotting technique. The combinations of probiotics and prebiotics were found to stimulate expression of IL-10 while down-regulating TNF-α, IL-1β, IL12 and NF-κβ. Along with that, the two pro- inflammatory cytokines that expressed equally in both the samples, i.e., in diseased and in treated.

Cross-talk with the mucosal epithelium determines the immune stimulatory effects of probiotics which directly impacts their ability to improve health benefit of the host [Duary *et al*. 2012]. Probiotic can induce different cytokines and chemokines and in this study the selected isolates PB1 to PB10 stimulates IL-10 expression.

IL-1β and IL-6 are cytokines that initiate an inflammatory response against antigen [Cammarota *et al.*, 2009]. However, the selected bacterial cultures displayed anti-inflammatory activity by higher expression of IL-10 and lesser expression of TNF-α, IL-1β, NF-κβ and IL-12 against DSS induced inflammation.

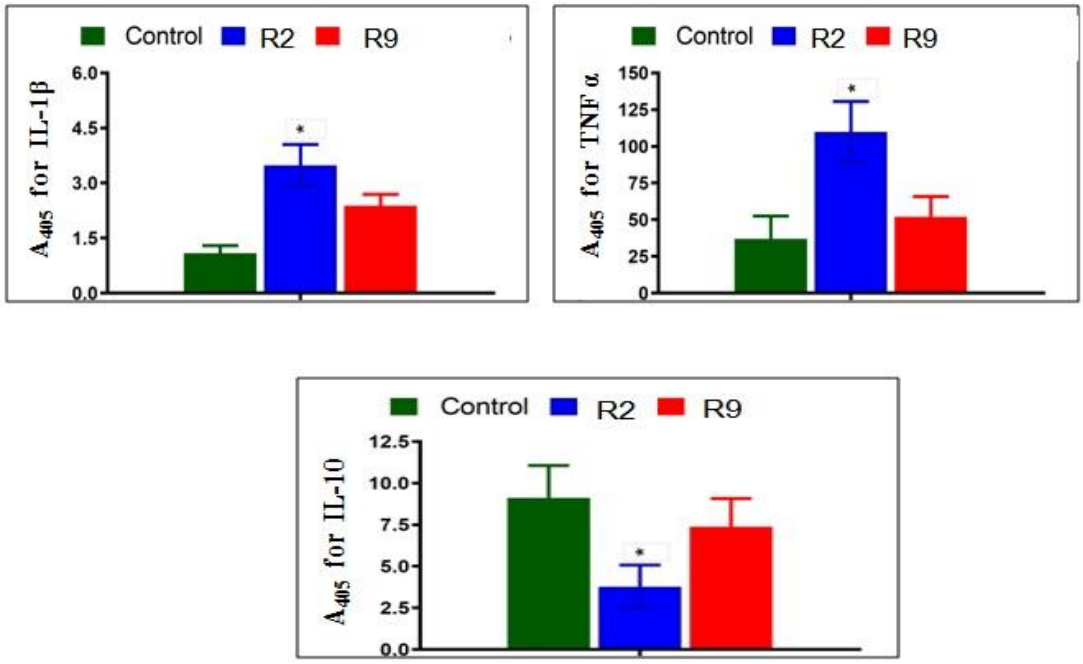

FIGURE 6.2: The comparative analysis of the different cytokines by ELISA.

It was observed from the present study, that the two potent cytokines IL-1β and TNF-α have eminently increased in diseased serum in which the colitis was induced with DSS. At the same time, it also showed decreased level in the serum collected from the animal that was treated with synbiotics which contains the combination of all the selected probiotics (PB1- PB10) and prebiotics (inulin & FOS).

In the same study it was also observed in **FIGURE 6.2**, that the expression of IL-10 being has significantly increased at the treated serum in compare to the diseased serum of the BALB/c mice.

6.4. CONCLUSION:

Immunomodulatory properties of 10 selected probiotic bacteria were analyzed on BALB/c mice. The study revealed prominent induction of interleukin 10. Seven isolated strains of *Streptococcus thermophilius*, and one strain of *Lactobacillus fermentum* showed a therapeutic effect against colitis induced mice, referring the probiotics as a potential agent to predict the immune-based selection criteria.

Selected probiotic bacteria have the potentiality to counteract the inflammation of the intestine which was initiated by multiple pro-inflammatory cytokine. Thus, it can be considered as an alternative treatment towards IBD, which is a rapid health aspect in most of the countries [Peran *et al.*, 2005].

The present study concluded that the selected probiotic strains along with the prebiotics have the potential to regulate the immune-stimulatory role of the different cytokines (both pro- and anti-inflammatory cytokines). The fact was re-confirmed by evaluating the same serum by western blotting and ELISA. And, in both the cases, it was evident that, the concentration of pro-inflammatory cytokines increases in the diseased serum whereas, the anti-inflammatory cytokines elevates in the treated sample. Thereby, it can be concluded that the effectiveness of the colitis inducer as well as the formulated synbiotics is very is very evident and successful in this study.