

Role of a Probiotic Strain in the Modulation of Gut Microbiota and Cytokines in Inflammatory Bowel Disease

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Abstract

Human gut is one of the major niches for anaerobes which play diverse functional role in host physiology and development. Gut microbiota maintains a homeostasis and dysbiosis may contribute in pathogenesis of different diseases including inflammatory bowel disease (IBD). Probiotic intervention is an established approach to maintain microbial homeostasis for the prevention and treatment of different diseases. The efficacy of probiotic strain *Bacillus clausii* UBBC-07 has been demonstrated and established in various diseases but its efficacy in IBD is under reported. To assess the effect of *Bacillus clausii* UBBC-07 in IBD patients, a randomized controlled study was conducted. Patients were randomly allocated to either placebo or probiotic *B. clausii* UBBC-07 for four weeks along with the standard medical treatment (SMT). Enrolled patients were evaluated before and after intervention for GI survival of the given probiotic, change in GI microbiota, change in serum cytokines, serotonin and dopamine, symptoms of disease, physical, behavioral and psychological parameters.

B. clausii UBBC-07 showed good survival in IBD patients in the treatment group ($p < 0.01$) without any reported adverse event. Metagenomic analysis showed that the given probiotic strain was able to modulate the gut microbiota in treated group. Phylum *Firmicutes* was increased and phylum *Bacteroidetes* was decreased in the probiotic treated group. A significant increase was observed in the abundance of bacterial genera *Lactobacillus*, *Bifidobacterium* and *Faecalibacterium* in the probiotic treated group ($p < 0.01$) as compared to placebo group. These results indicated the given probiotic strain was able to modulate that gut microbiota in probiotic treated group. Significant increase was observed in IL-10 ($p < 0.05$) and variable decrease in the secretion of IL-1 β , TNF- α , IL-6, IL-17 and IL-23 in probiotic treated group. Change in the concentration of serum dopamine and serotonin in the treatment group was not significant as compared to placebo group. In the treatment group a significant decrease in the symptoms of IBD and improvement in the psychological parameter to various degrees was noted. These results indicated that probiotic strain *B. clausii* UBBC-07 affected the gut microbiota and cytokine secretion and shown efficacy in IBD patients.

Keywords: Probiotic, *Bacillus clausii* UBBC-07, Gut microbiome, IBD, Cytokines.

Introduction

Human gut is one of the most complex organization of microbes and human cells. The gut microbiome is predominantly comprised of bacteria which plays vital roles in human physiology and development. Human gastrointestinal tract (GIT) is an affluent and favorable niche for anaerobes therefore the major portion of gut microbiota are composed of strict anaerobes, which outnumber the facultative anaerobes and aerobes (Bull and Plummer, 2014; Claesson et al., 2009; Finegold, 1995; Loesche, 1969; Lozupone et al., 2012). Anaerobic bacteria are very sensitivity to oxygen often unable to survive in even in very low oxygen (Albenberg et al., 2014; Rolfe et al., 1978). Microbial interventions using probiotics to maintain the microbial homeostasis is an important preventive and therapeutic approach for different diseases including inflammatory bowel disease (IBD). Each probiotic has specific effects and few probiotics viz *Lactobacilli*, *Bifidobacteria* and *Bacillus* strains have already been proven their beneficial effects (Andrade et al., 2020). *B. clausii* is spore forming and resistant to low pH and able to survive in the intestinal environment. The spores of *B. clausii* and survive and transit through the human GI tract. Being a spore forming is the added advantage of *Bacillus clausii* as the probiotic of choice in the treatment of GI disorders.

Bacillus species have been used to prevent or treat various GI disorders (Zhang et al., 2015). *Bacillus* probiotic Enterogermina, which includes *Bacillus clausii* has been reported to exert beneficial effects in the treatment of gastro-intestinal diseases (Urdaci et al., 2004). Inflammatory Bowel Disease (IBD) is a disorder of the GIT and Crohn's Disease (CD) and Ulcerative Colitis (UC) are its two main types (Sarlos et al., 2014). Etiology of IBD hypothesized that the commensal flora triggers an immune response that is further modified by specific environmental factors (Acharyya, 2018; Kedia and Ahuja, 2018, 2017). Probiotics is among the therapeutic approaches which has been tested to prevent and treat gastrointestinal inflammatory disease and have been found effective to cure this lifestyle disorders and improvement in the gut health. *Bacillus clausii* UBBC-07 (MTCC 5472) is a commercially available safe probiotic (Lakshmi et al., 2017; Upadrasta et al., 2016). The administration of *B. clausii* UBBC-07 was reported safe and effective to improve the symptoms of acute diarrhea in children and adults (Neelamraju and ratna sudha, 2015; Sudha et al., 2019, 2013). In this study, we assessed the effect of the probiotic strain *Bacillus clausii* UBBC-07 on adults IBD patients and the study was registered with Clinical Trials Registry (CTRI) -India (Ref-CTRI/2019/11/022087). Survival of the given probiotic *Bacillus clausii* UBBC-07 in the gut, change in gut bacteria, serum cytokines, IBD symptoms, and disease severity were evaluated before and after intervention.

Results

Survival of *Bacillus clausii* UBBC-07:

In both the study groups samples of the patients were evaluated for the presence of *B clausii* UBBC-07 before and after treatment. No *B clausii* UBBC-07 was detected in both the groups in the pre-treatment sample. In the probiotic treated group, in the UC and CD patient *B clausii* UBBC-07 was detected in 74.5 % and 79.6 % subjects respectively and the detection of given probiotic in UC and CD patients of treatment group was significant ($p < 0.001$) as compared to total absence or nil detection of *B clausii* in placebo group. These results indicated the survival of orally given probiotic strain *B clausii* UBBC-07 in GI tract of these subjects.

Metagenomic analysis:

Before and after intervention, Operational Taxonomic Units (OTUs) were estimated for various bacterial taxa, including phylum, class, orders, families, and genera in the UC patients of both the probiotic and placebo groups. Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Cyanobacteria, and Euryarchaeota were abundant in both study groups. In the treatment group, the average % OTUs of the phylum Firmicutes were 37.58 % and 48.0 % in the before and after intervention samples, respectively and in the treatment group's post-intervention sample, the abundance of the phylum Firmicutes increased significantly. In the treatment group, the average % OTUs of the phylum Bacteroidetes were 35.13 % and 33.14 % in before and after intervention samples, respectively. In post-intervention samples, the treatment group showed a drop in OTUs of the phylum Bacteroidetes. Results are summarized in Figure 1.

Among bacterial classes no significant change in OTUs of class Bacteroidia in before and after intervention samples was observed in treatment group. In the post-intervention sample, there was a drop in class Clostridia in the treatment group, and an increase in the placebo group. In the post-intervention samples of the treatment group, there was a drop in OTUs of the class Negativicutes and an increase in the placebo group. In the treatment group, OTUs of class Bacilli were 2.86 % and 11.92 % in pre and post intervention samples, respectively, while in the placebo group, the average percent OTUs of class Bacilli were 10.77 % and 3.85 % in pre and post intervention samples, respectively. There was a significant rise in OTUs of the class Bacilli in the treatment group's post-intervention samples and a drop in the placebo group. Results are summarized in Figure 2.

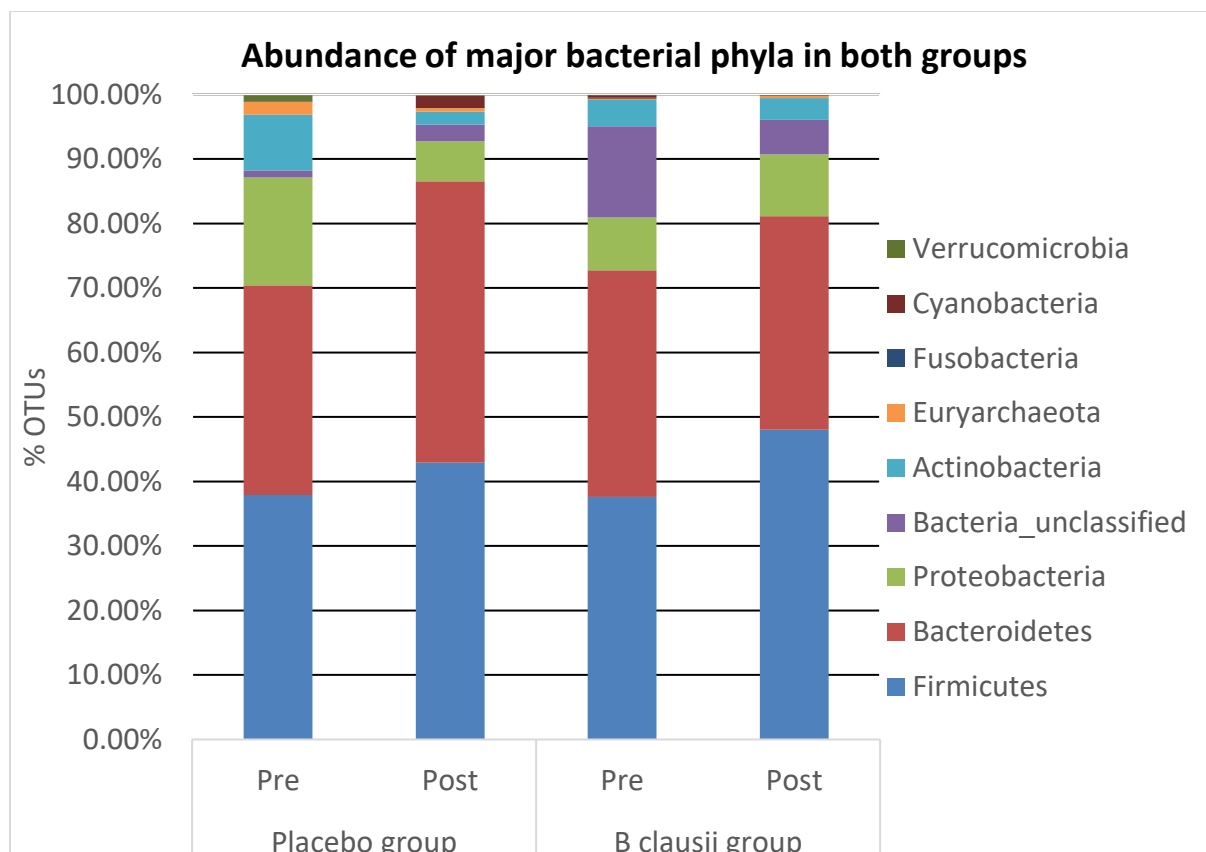


Figure 1: Abundance of major bacterial phyla (% OTUs) in both the study group

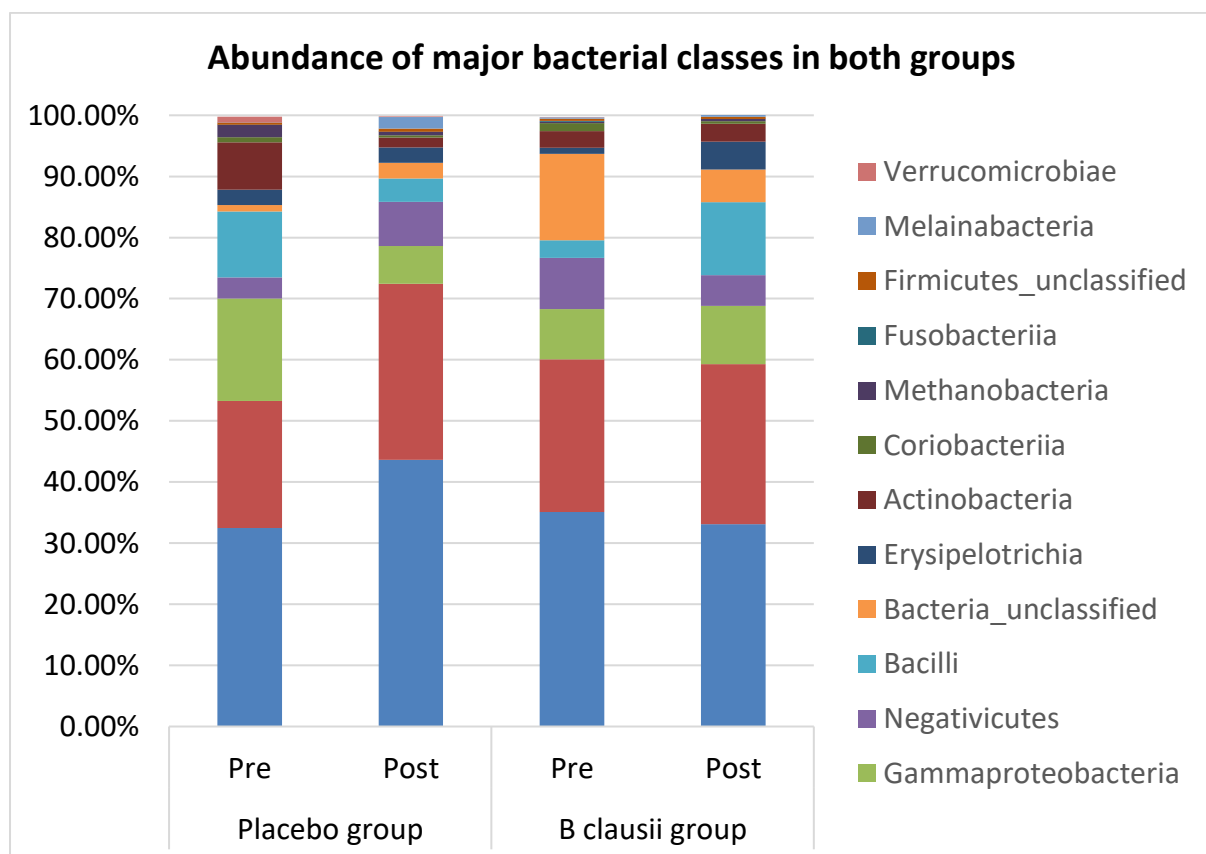


Figure 2: Abundance of major bacterial classes (% OTUs) in both the study group

Among bacterial orders in post-intervention samples in the treatment group, there was a drop in order Bacteroidales and an increase in the placebo group. In both groups, no significant changes in order Clostridiales were observed in post-intervention samples. After intervention there was a significant drop in order Selenomonadales in the treatment group and increase in the placebo group. In the treatment group, OTUs of order Lactobacillales was 2.79 % and 11.87 % before and after intervention, respectively, and in the placebo group, 10.66 % and 3.82 % before and after intervention, respectively. In post-intervention samples, there was a significant rise in the order Lactobacillales in the treatment group and a drop in the placebo group. In the treatment group, there was a significant rise in OTUs of the order Erysipelotrichales in post-intervention samples. In post-intervention samples, there was an increase in order Bifidobacteriales in the treatment group and a drop in the placebo group. Results are summarized in Figure 3.

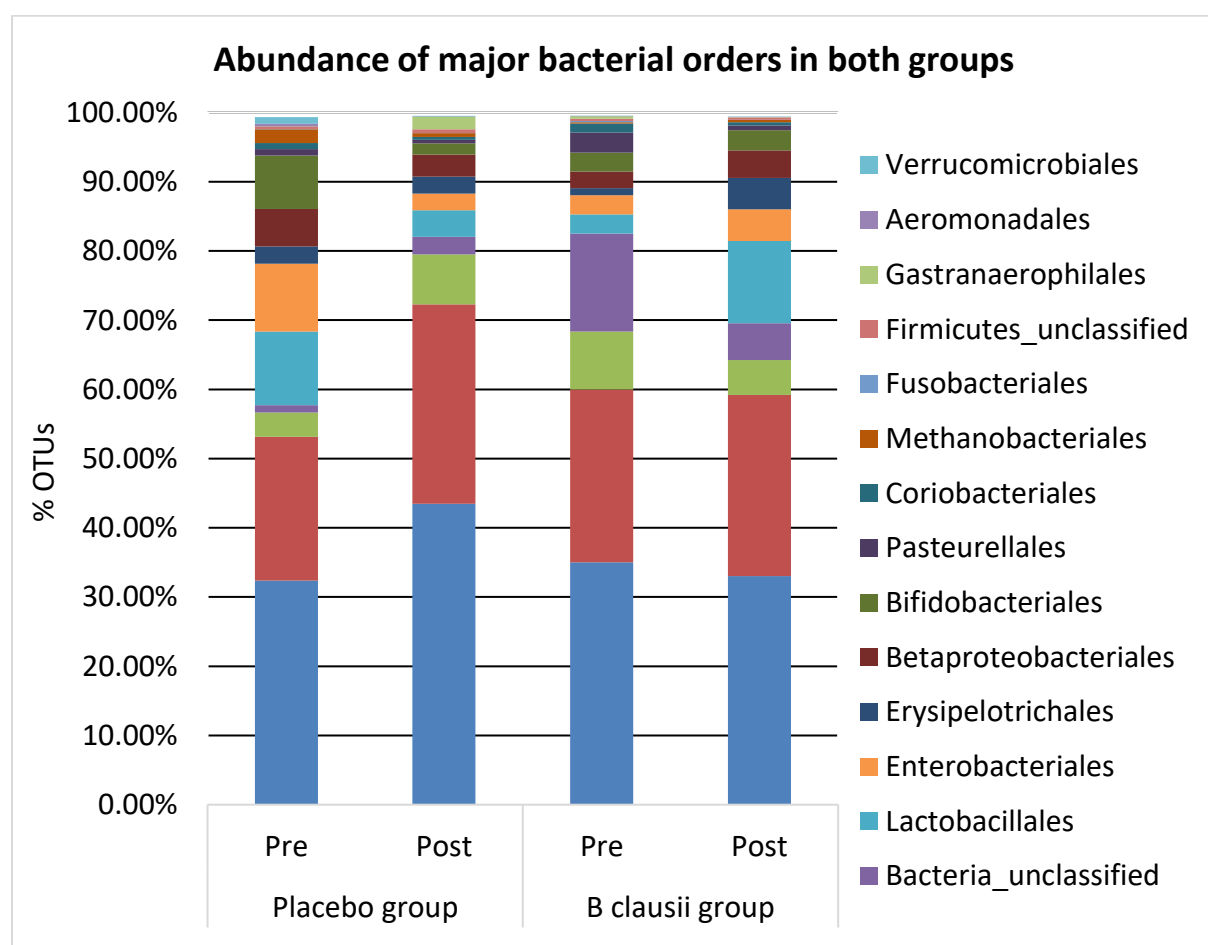


Figure 3: Abundance of major bacterial orders (% OTUs) in both the study group

Among bacterial families in both groups, there was an increase in OTUs of the Prevotellaceae in post-intervention samples. In post-intervention samples in the treatment group, there was an increase in the family Ruminococcaceae, while in the placebo group, there was a decrease. In post-intervention samples, there was a decrease in the family Lachnospiraceae in the treatment group and an increase in the placebo group. There was a decrease in the family Veillonellaceae in the treatment group and an increase in the placebo group, in post-intervention samples. In the treatment group, OTUs of the family Lactobacillaceae were 2.12 % and 6.03 %, respectively, and 6.36 % and 3.22 % in before and after intervention, respectively. In post-intervention samples, there was a significant rise in the OTUs of Lactobacillaceae in the treatment group and a drop in the placebo group. In post-intervention samples from the treatment group, there was an increase in the family Bifidobacteriaceae, while in the placebo group, there was a drop. Results are summarized in Figure 4.

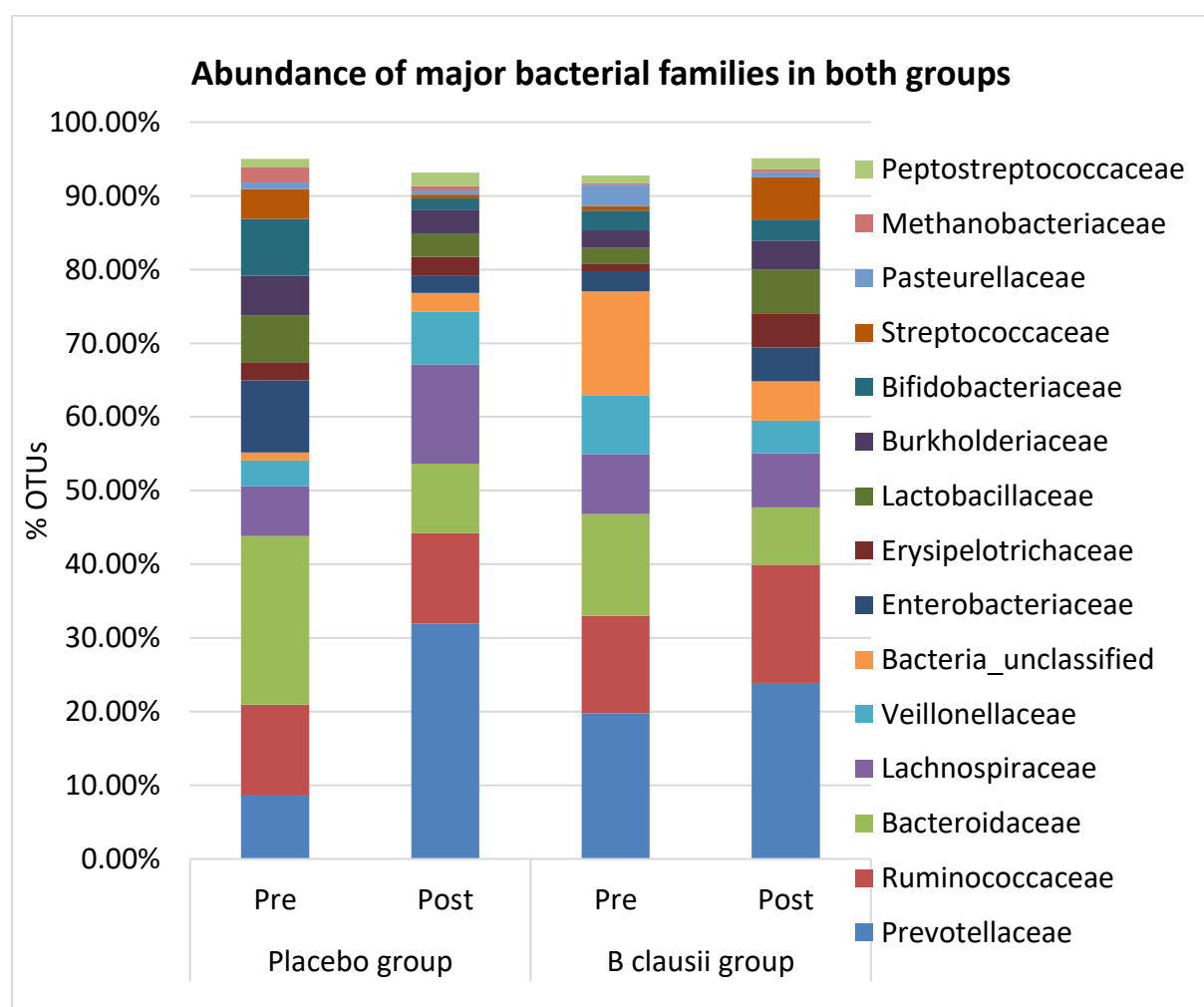


Figure 4: Abundance of major bacterial families (% OTUs) in both the study group

Among bacterial genera, the abundance of *Lactobacillus*, *Bifidobacterium*, *Faecalibacterium*, *Lachnospira*, *Blautia*, and *Alistipes* were increased and abundance of bacterial genera *Bacteroides*, *Clostridia*, *Dialister*, *Megasphaera*, *Roseburia* and *Olsenella* and *Megamonas* were decrease in the post intervention samples in the treatment group. In the post intervention samples an increase in genus *Prevotella* and decrease in genus *Bacteroides* were observed in both treatment and placebo group. Before and after intervention in the treatment group, the *Faecalibacterium* were 7.76 % and 14.28 % respectively, and in the placebo group, 9.23 % and 8.01 % respectively. There was a significant increase in OTUs of the genus *Faecalibacterium* in post intervention samples in the treatment group, while in the placebo group, there was a decrease in OTUs of the genus *Faecalibacterium* in post intervention samples. The average % OTUs of the *Lactobacillus* were 1.69 % and 5.05 % in the pre and post intervention samples in the treatment group, respectively, and 7.33 % and 3.49 % in the placebo group, respectively. In post-intervention samples, there was a significant rise in the genus *Lactobacillus* in the treatment group and a drop in the placebo group. Before and after intervention the OTUs of the *Bifidobacterium* were 2.05 % and 2.41 %, respectively in the treatment group and 6.03 % and 1.42 % before and post intervention, respectively in the treatment group. In post-intervention samples, there was an increase in the genus *Bifidobacterium* in the treatment group and a drop in the placebo group. Results are summarized in Figure 5.

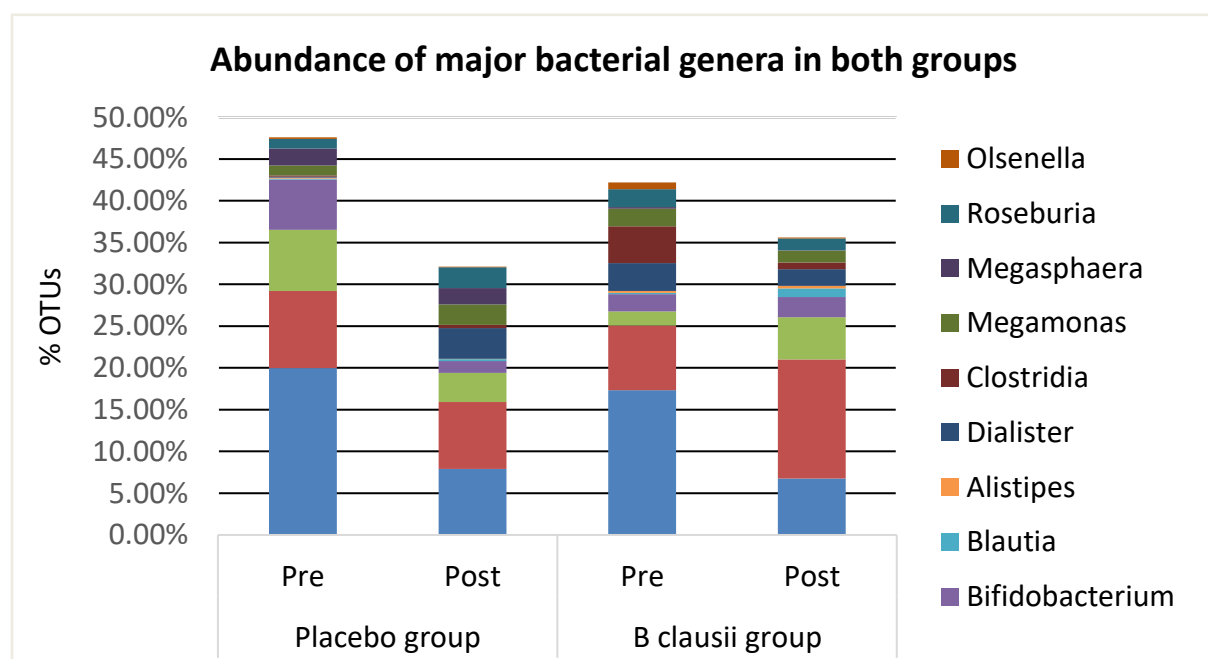


Figure 5: Abundance of major bacterial genera (% OTUs) in both the study group

Cytokine

Serum concentration of different cytokines were measured before and after intervention in the enrolled patients. Results indicated that the probiotic strain *Bacillus clausii* UBBC-07 was able to modulate the serum levels of cytokines in IBD patients. In the probiotic group serum IL10 level were 21.6 ± 4.8 pg/ml and 31.5 ± 5.2 pg/ml pre and post intervention in UC patients ($p < 0.05$) while the difference was not significant ($p = ns$) in the placebo group. In the CD patient in the probiotic treated group serum IL10 level were 18.4 ± 4.86 pg/ml and 28.4 ± 4.9 pg/ml pre and post intervention ($p < 0.05$) while in the placebo group the difference was not significant ($p = ns$). In the treatment group serum IL6 were 44.5 ± 5.6 pg/ml and 31.5 ± 4.4 pg/ml pre and post intervention in UC patient ($p < 0.05$) while in the placebo group the difference was not significant ($p = ns$). In the UC patients, serum IL 17 level were 39 ± 6.3 pg/ml and 24.5 ± 4.5 pg/ml before and after intervention patient and the difference was significant ($p < 0.05$) in the treatment group while in the placebo group the difference was not significant ($p = ns$). In the CD patient serum IL17 level were 45.4 ± 4.8 pg/ml and 28.6 ± 7.4 pg/ml before and after intervention and the difference was significant ($p < 0.05$) in the treatment group while the difference was not significant ($p = ns$) in the placebo group. Serum IL23 were 898.5 ± 54.6 ng/ml and 705.6 ± 46.7 ng/ml pre and post intervention in UC patient in the treatment group ($p < 0.05$) while the difference in the placebo group was not significant (p, ns). In the CD patient serum IL23 level were 902.6 ± 48.5 ng/ml and 734.6 ± 40.8 ng/ml pre and post intervention in the treatment group ($p < 0.05$) while in the placebo group the difference was not significant (p, ns). In the UC patient serum IL-1 β level were 384 ± 45.5 pg/ml and 246 ± 38.4 pg/ml before and after intervention in the treatment group ($p < 0.05$) while the difference was not significant ($p = ns$) in placebo group. In the CD patient in the treatment group serum IL-1 β level were 375.2 ± 36.4 pg/ml and 268 ± 32.2 pg/ml pre and post intervention ($p < 0.05$) while in the placebo group the difference was not significant ($p = ns$). Results are summarized in Table 1.

Serum serotonin and dopamine levels:

Before and after intervention, serum serotonin levels were 125.8 17.6 ng/ml and 110.30 12.4 ng/ml, respectively, in the treatment group and 121.5 16.50 ng/ml and 106.8 14.65 ng/ml, respectively, in the placebo group no significant difference. Before and after intervention, serum Dopamine levels were 9.25 3.22 pg/ml and 10.45 3.5 pg/ml, respectively, in the treatment group and 10.50 2.6 pg/ml and 11.4 3.6 pg/ml, respectively, in the placebo group with no significant difference. There was no significant difference in serum serotonin and dopamine levels in treatment and placebo groups. Results are summarized in Table 1.

Table 1: Serum Cytokines, serotonin and dopamine levels in pre and post intervention samples in treatment and placebo group

Cytokine (pg/ml)	<i>Bacillus clausii</i> UBBC-07 group			Placebo group		
	Pre	Post	P value	Pre	Post	P value
UC patients						
IL-10	21.6±4.8	31.5±5.2	<0.05	23.5 ± 3.9	22.6 ± 7.5	NS
IL-6	44.5 ± 5.6	31.5 ± 4.4	<0.05	45.4 ± 6.2	40.6 ± 4.2	NS
IL-17	39±6.3	24.5±4.5	<0.05	38.8± 6.2	41.9 ± 4.6	NS
IL-23	898.5 ± 54.6	805.6 ± 46.7	NS	906.8 ± 68.3	897.02 ±73.5	NS
IL-1β	384±45.5	246±38.4	<0.05	352.5 ± 67.6	386.7 ± 56.2	NS
TNF- α	65.6 ± 6.2	58.3 ± 5.6	NS	75 ±5.2	68.5 ± 9.5	NS
CD patients						
IL-10	18.4 ± 4.86	28.4 ±4.9	<0.05	22.2 ± 4.1	25.6 ± 6.6	NS
IL-6	44.4 ± 4.8	38.4 ± 7.2	NS	43.5 ± 5.6	46.7 ± 4.8	NS
IL-17	45.4±4.8	28.6±7.4	<0.05	41.4 ± 12.4	43.6 ± 6.8	NS
IL-23	902.6±48.5	834.6±40.8	NS	911.5 ± 66.4	875.6 ± 56.5	NS
IL-1β	375.2±36.4	268±32.2	<0.05	445 ± 58.2	284.5± 48.5	<0.05
TNF- α	78.4 ± 6.2	65.3 ± 8.2	NS	66.2 ±12.6	75.5 ± 10.8	NS
Serum serotonin and dopamine in both UC and CD patients						
Serotonin (ng/ml)	125.8±17.6	110.30±12.4	NS	121.5±16.50	106.8 14.65	NS
Dopamine (pg/ml)	9.25±3.22	10.45±3.5	NS	10.50±2.6	11.4±3.6	NS

Efficacy on disease symptoms

Data was collected and recorded for various symptoms of the disease as per standard protocol. Simple Clinical Colitis Activity Index (SCCAI) score (Walmsley RS et al. 1998) was used to quantify UC disease activity. The Crohn's Disease Activity Index (CDAI) score (Best et al 1976, Sandborn et al 2002) was used to assess CD. Reduction in SCCAI score indicates the decrease in the severity of symptoms of UC and reduction in the CDAI score indicates the decrease in the severity of symptoms of CD. The SCCAI score was decreased in 42.52 % and 31.58 % of the UC patients post intervention in treatment and placebo group respectively and

the difference was significant ($p < 0.05$). There was no significant difference in CDAI score between treatment and placebo groups.

Table 2: Post intervention decrease in symptoms in the enrolled subjects for different physical, behavioral and psychological parameters

Physical, behavioural and psychological parameters	Post intervention decrease (% of subjects) in symptoms					
	UC patients			CD patients		
	Probiotic group	Placebo group	P value	Probiotic group	Placebo group	P value
Procrastination	37.14 %	28.20 %	<0.05	36.84 %	26.31 %	<0.05
Restlessness	28.20 %	20.51 %	<0.05	21.05 %	13.33 %	<0.05
Muscles stiffness	40.0 %	35.89 %	NS	36.84 %	40.0 %	NS
Heartburn	42.85 %	33.33 %	<0.05	36.84 %	26.66 %	<0.05
Headache	37.14 %	35.89 %	NS	31.57 %	26.66 %	NS
Shakiness or tremor	26.31 %	26.66 %	NS	42.85 %	30.76 %	<0.05
Sleep problem	42.85%	30.76%	<0.05	36.84 %	26.66 %	<0.05
Difficulty in completing work	34.28 %	30.76 %	NS	31.57 %	33.33 %	NS
Overwhelming	37.14 %	25.64 %	<0.05	31.57 %	20.0 %	<0.05
Trouble relaxing	34.28 %	23.07 %	<0.05	31.57 %	13.33 %	<0.05
Nervousness	31.42 %	30.76 %	NS	36.84 %	13.33 %	<0.05
Depression	25.71 %	25.64 %	NS	21.01 %	21.01 %	NS
Poor concentration	25.64 %	28.20 %	NS	31.57 %	26.31 %	NS
Quick temper	28.20 %	20.51 %	<0.05	21.05 %	13.33 %	<0.05

Physical, behavioral and psychological parameters

Before and after intervention the enrolled IBD subjects were assessed for the different physical, behavioral and psychological symptoms. Symptoms were assessed by scores, reduction in the score indicates the decrease in the severity of symptoms and rise in the score indicates the increase in the severity of the symptom. There was no significant reduction in the complaint of muscle stiffness, heartburn, headache, shakiness or tremor, difficulty in completing work, nervousness, depression, poor concentration after intervention in UC & CD patients in treatment group as compared to placebo group (p , ns). The complaint of sleep problems was reduced in 42.85% and 30.76% of UC patients in the treatment and placebo groups, respectively, with a significant difference between the groups ($p < 0.05$). Sleep complaints were reduced in 36.84 % and 26.66 % of CD patients in the treatment and placebo groups, respectively, with a significant difference between the groups ($p < 0.05$).

Procrastination was reduced in 37.14 % and 28.20 % of UC patients in the treatment and placebo groups, respectively, with a significant difference between the groups ($p < 0.05$). Procrastination was reduced in 36.84 % and 26.66 % of CD patients in the treatment and placebo group, respectively, with a significant difference between the groups ($p < 0.05$). Restlessness was reduced in 28.20 % and 20.51 % of UC patients in the treatment and placebo groups, respectively, with a significant difference between the groups ($p < 0.05$). Restlessness was reduced in 21.05 % and 13.33 % of CD patients, in the treatment and placebo groups, respectively, with a significant difference between the groups ($p < 0.05$). Results showed significant ($p < 0.05$) decreased in the complaint of sleep problem, complaint of procrastination, complaint of overwhelming, complaint of trouble relaxing, complaint of quick temper, complaint of restlessness in the post intervention in the probiotic treated group

Safety evaluations: No adverse events were noticed, recorded, or reported during or after intervention in the trial, which further supported the safety of *Bacillus clausii* UBBC-07.

Discussion

Probiotics are viable microorganisms that have a beneficial effect on health and have been used in different gastrointestinal (GI) conditions including IBD. The effects of probiotics are strains specific. *Lactobacillus* and *Bifidobacterium* are considered as good probiotic but in the harsh environment of the gut their survival is low (Keller et al., 2019). *Bacillus* species have potential probiotics properties (Ratna Sudha and Bhonagiri, 2012) and are considerably better than other probiotics in gastric conditions because of high heat resistance, better acid tolerance (Hyronimus et al., 2000; Lee et al., 2019). These *Bacillus* species may have beneficial effects in various disease including IBS, IBD, diarrhea, respiratory disorders, allergies, skin disorders, bacterial vaginosis and cancer (Pham et al 2008). The beneficial effects of these may exert through immune-modulation, competitive exclusion of pathogens and secretion of antimicrobial substances (Cunningham et al., 2021; Fuller, 1991; Indira et al., 2019; Rocchetti et al., 2021; Urdaci et al., 2004; Zommiti et al., 2020).

Bacillus clausii UBBC-07 (MTCC 5472) is well characterized probiotic strain commercially available in different formulation (Lakshmi et al., 2017). Clinical studies have shown the effectiveness of *B. clausii* UBBC-07 in the treatment of acute diarrhea in adult (Sudha et al., 2013) and children (Sudha et al., 2019). Under in vitro GIT conditions *B clausii* UBBC-07 were also found capable of surviving and germinating (Ahire et al., 2020). Genetic makeup of *B. clausii* UBBC07 has proven absence of toxin genes, absence of transferable antibiotic traits (Upadrasta et al., 2016). Results of the present study showed that *B clausii* UBBC-07 was able to survive in GI tract of IBD patients. Colonization resistance of human microbiota is an important feature where indigenous non-pathogenic bacteria suppress the growth of pathogens and provide protection to host against colonization of harmful bacteria (Kho and Lal, 2018).

Changes into human gut microbiota may trigger infections and lifestyle disorder including IBD (Gagliardi et al., 2018; Vijay and Valdes, 2021). Results of metagenomic analysis showed that phylum Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria were more abundant in the patients of both the study group and this finding was in agreement that the Indian gut microbiome is dominated by these bacterial phyla (Gupta et al., 2019; Jain et al., 2018). After intervention, in the probiotic treated group, the abundance of Firmicutes was increased and abundance of Bacteroidetes was decreased. In post-intervention samples, the abundance of *Lactobacillus*, *Bifidobacterium*, and *Faecalibacterium* increased in the treatment group and decreased in the placebo group. *B clausii* UBBC-07 was able to increase

beneficial *Lactobacilli* in treatment group which is in agreement with another reported study where *Bacillus* species was able to increase the abundance of *Bifidobacteria* and *Lactobacillus* (Ara et al., 2002). Studies have reported that *Bacillus* species is capable of restoring the microbial imbalance (Hempel et al., 2012) by promoting the growth of *Lactobacillus* and *Bifidobacterium* (Cao et al., 2020). It is postulated that *Bacillus* species consume free oxygen in the intestine and inhibit redox processes, providing an anaerobic and acidic environment that is unfavorable to many pathogens (Cao et al., 2020). *Lactobacillus* and *Bifidobacteria* are among the first bacteria to colonize infants and have been linked to a variety of health benefits (Feng et al., 2015; Flint et al., 2012). An increase in abundance of genus *Faecalibacterium* in post intervention samples in treatment group and decrease in placebo group was observed which was in agreement with previously reported decreased abundance of *Faecalibacterium* in IBD (Becker et al., 2015; Wang et al., 2014). *Lactobacillus*, *Bifidobacterium*, and *Faecalibacterium* have been reported to induced up-regulation of the anti-inflammatory cytokine, and down-regulation of inflammatory cytokines in the host to modulate mucosal inflammation (Joossens et al., 2011; Sokol et al., 2008).

Consumption of *B. clausii* UBBC-07 was reported safe and effective in acute diarrhea in adults (Sudha et al., 2013) and children (Sudha et al., 2019). This is the first study to assess the efficacy of *Bacillus clausii* UBBC-07 on adult IBD patients. *B. clausii* UBBC-07 was able to reduce the severity of symptoms of disease to various degrees in IBD patients and these findings matched with those of another study, where a probiotic (VSL#3) lowered inflammatory cytokine expression and reduce the severity of symptom in UC patients (Tursi et al., 2010). Probiotics can reduce inflammation and disease symptoms by alteration of the mucosal immune system, competitive exclusion of pathogens, production of antimicrobial factors (Schlee et al., 2008). Probiotic intervention may improve GI symptoms due to antimicrobial properties (Didari et al., 2015; Gareau et al., 2010). Probiotics normalize bowel movements and reduce visceral hypersensitivity (Gareau et al., 2010; Korterink et al., 2014). Cytokines are important signals in the gut immune system that are known to and mediate local and systemic inflammation (Sanchez-Munoz et al., 2008). The GI microbiota is thought to be the primary cause of an improper host immune response (Kho and Lal, 2018).

Changes in the serum levels of anti-inflammatory cytokine and pro-inflammatory cytokines is reported in GI disorders (Bennet et al., 2016). *B. clausii* UBBC-07 was able to up-regulate the secretion of serum IL-10. An inactivation of IL-10 leads to increased release of IL-12 and IFN- γ . Low level of IL-10 has been observed in inflamed tissues and granulomas of IBD. The observed results of our study indicated that *B. clausii* UBBC-07 has significantly

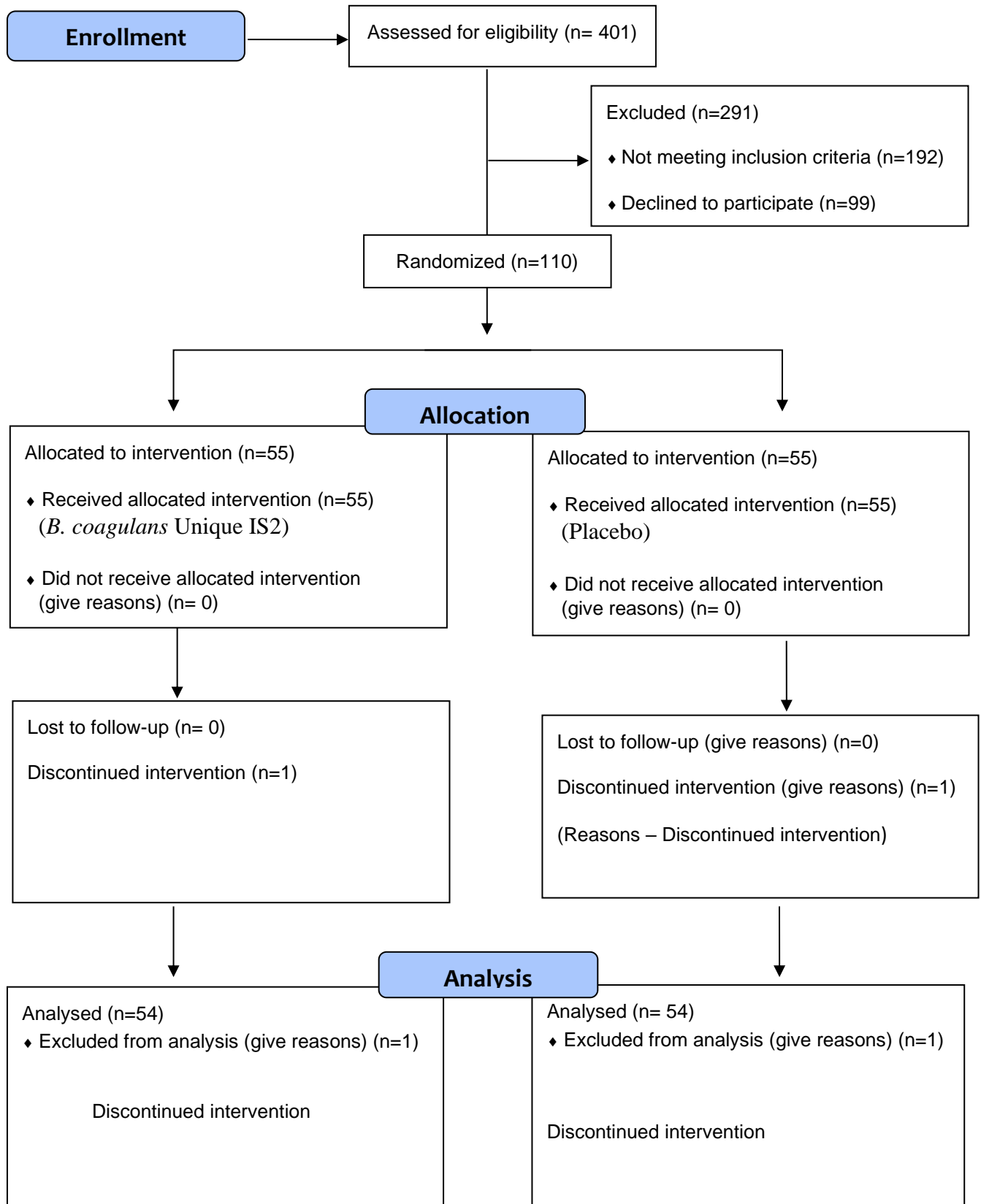
decreased the level of IL-6 in IBD patients. IL-6 is a pleiotropic pro-inflammatory cytokine which induces the terminal differentiation of B cells, enhances the secretion of immunoglobulin, and also elevates the secretion of acute-phase proteins. Literature reported that the expression of IL-6 is found to be expressed in high level in the patients of IBD indicates its role in the intestinal inflammation of IBD. Clinical studies reported the expression of IL-6 as an indication in the pathogenesis of mesangial proliferative glomerulonephritis, multiple myeloma, rheumatoid Arthritis (Liu et al., 2019; Weber et al., 2016).

We have observed the reduction in the level of IL-17 and IL-23 in the treatment group. IL 17 is an important pro-inflammatory cytokine produced by a T-cell subset. IL-23 amplifies the Th17 cell responses and leads to gut inflammation. It is evident that IL-23 helps in enhancing the synthesis of both Th1 and Th17-cytokines in IBD. We have observed the decreased secretion of serum IL-1 β in the treatment group. IL-1 β is a pro-inflammatory cytokine and an increased production of IL-1 β may lead to inflammation. We have observed a decrease in the secretion of serum TNF- α in the treatment group. TNF- α is a pro-inflammatory cytokine which enhanced expression of IL-2, IL-1 β and IL-6 (Liu et al., 2019; Mavropoulou et al., 2020). It has been reported that the macrophages of IBD patients are having the high expression of TNF alpha and leads to the intestinal inflammation. Clinical studies have reported an improvement in CD patients treated with anti-TNF- α therapy such as infliximab, adalimumab and certolizumab pegol (Sanchez-Munoz et al., 2008). Observed results suggested that probiotic *Bacillus clausii* UBBC-07 has the potential to modulate the secretion of cytokines and gut inflammation.

CONCLUSIONS

Bacillus clausii UBBC-07 showed good survival in IBD patients without any reported adverse event. An increase in the abundance of phylum Firmicutes and decrease in the Bacteroidetes was observed after intervention in the probiotic treated group. Significant increase was observed in the bacterial genera *Lactobacillus*, *Bifidobacterium* and *Faecalibacterium* in post intervention sample in the probiotic treated group. A significant increase in the IL-10 and variable decreased in the secretion of IL-6, IL-1 β , TNF- α , IL -17 and IL -23 was observed in probiotic treated group. Probiotic strain *B clausii* UBBC-07 shown efficacy in IBD patients by modulating gut microbiota, cytokines, decreased in the symptoms of IBD and improvement in the psychological parameter to various degrees.

‘CONSORT Flow Chart’



Material and Methods

- **Study design:** A double blind, randomized, placebo-controlled study was conducted with intervention of 4 week to assess the efficacy of *Bacillus clausii* UBBC-07 (MTCC 5472) in IBD patient. The randomization was generated by the nQuery clinical trial design sample size software in a 1:1 ratio. It was comprised of before intervention baseline visit (visit-1, week 0 / day 0), and post intervention visit (visit-2, 1 week after completion of intervention).
- **Study site and ethical approval:** The study was conducted in the tertiary care hospital after ethical approval from Institute Ethics Committee of All India Institute of Medical Sciences, New Delhi, India (Ref – IEC.478/07.10. 2016.OP-7). Before enrollment in the study, a written informed consent from each enrolled patient.
- **Subject selection:** Adult patients of ulcerative colitis (UC) and Crohn's disease (CD) IBD of age between 18–60 years under standard medical treatment (SMT) were enrolled in the study. To quantify UC disease activity Simple Clinical Colitis Activity Index (SCCAI) score (Walmsley et al., 1998) was used. SCCAI score is a validated symptom based clinical scoring index which is used by the clinicians to quantify UC disease activity and has a good correlation with disease activity indices. SCCAI score along with other symptoms of all the enrolled UC patients were assessed before and after intervention and UC patient with Mild to moderate severity were included in this study. The Crohn's Disease Activity Index (CDAI) is a validated scoring method to assess disease severity which has been developed and used for long (Best et al 1976). In CD severe disease is defined as a CDAI >450, moderate Crohn's is CDAI 220–450, mild Crohn's is CDAI 150–220, and clinical remission is defined as a CDAI <150 (Sandborn et al., 2002). In this study the CD patient with mild to moderate severity were included. 5-aminosalicylic acid (5-ASA) - Sulfasalazine (3 grams/day) or Mesalamine 800 mg orally 3 times a day was SMT for the enrolled patient in this study.
- **Inclusion criteria:** (a) Subject clinically diagnosed with Ulcerative Colitis (UC) or, Crohn's Disease (CD), (c) UC or CD patients of the age between 18–60-year of either sex (d) patient ready to give written consent to participate in the study, (e) subject receiving SMT and attending out patient department (OPD) of the hospital.
- **Exclusion criteria:** (a) Known case of any gastrointestinal disease other than IBD, (b) known case of any carcinoma (c) known case of immunodeficiency disorder, (d) subject is on any probiotic drug or consumed any probiotic in the last one month, (e) subject not taking food through oral route.

- **Sample size:** SAS software was used to calculate the sample size. To detect the presence of a proportion difference, the assumption was made that a minimum of 116 subjects to be screened and 94 to be recruited to evaluate the primary endpoint. When the overall response is minimum 30% at a significant level of 0.05 this will provide 80% power to reject the null hypothesis.
- **Randomization:** Subjects were randomized into two arms (*Bacillus clausii* UBBC-07 - 2 billion cfu twice in a day or placebo twice in a day). Randomization was conducted using opaque sealed envelopes that were indistinguishable between groups. The codes were kept blinded and given to the enrolled subjects based on the randomization numbers.
- **Enrolment of subjects:** After screening as per inclusion and exclusion criteria, a total of 110 subjects were recruited. During baseline visit (day 0) complete medical history, medications, physical examination and vital signs including pulse rate, respiratory rate, blood pressure and temperature were assessed during hospital visit. FDA / DCGI / FSSAI approved probiotic strain *Bacillus clausii* UBBC-07 (MTCC 5472) was used as an intervention agent in this study. Enrolled subjects were given probiotic (*Bacillus clausii* UBBC-07, 2 billion-CFU/capsule) or placebo (identical to the probiotic capsule but contained only excipient, maltodextrin) twice in day for 4 weeks. The compliance of drug and dose were followed by observation and telephonic follow-up.
- **Data analysis:** In this paper we are reporting the results of the study on IBD patients. Data of 54 subjects were analyzed in each group. In probiotic treated group 19 patients of CD and 35 of UC were included. In placebo group 15 patients of CD and 39 of UC were included. The Stata statistical software (Version 14, USA) was used for statistical evaluations. Subject assessment was evaluated as frequency distribution and significance was assessed with chi-square test. A p value < 0.05 was considered as statistically significant.
- **Outcome measures:** The outcomes were measured by (i) detection of probiotic *Bacillus clausii* UBBC-07, (ii) alteration in gut microbiota, (iii) alteration in concentration of serum cytokines, (iv) alteration in serum concentration of dopamine and serotonin, (v) improvement in disease symptoms, (vi) assessment of physical and psychological parameters. For the evaluation of physical, behavioral and psychological parameters all the enrolled subjects were asked to answer the questionnaire as per the Hopkins Symptom Checklist (HSCL): A self-report symptom inventory (Derogatis et al., 1974; Kleppang and Hagquist, 2016). The parameters included were headache, heartburn, muscle stiffness, shakiness, sleep problem, procrastination, overwhelming, difficulty in completing work, nervousness, restlessness, poor concentration, feeling of depression, quick temper and

trouble relaxing. These symptoms were assessed based on scores. The rise in the score indicating an increase in the severity of the symptom and drop in the score indicating the decrease in the severity of symptoms.

- **Safety evaluation:** Safety of the given probiotic was assessed by reporting of adverse event by the enrolled subject. During hospital visit, monitoring of vital signs including heart rate, respiratory rate, temperature and blood pressure were measured.
- **Sample collection and processing:** A stool sample and a blood sample were collected from each enrolled subject before and after intervention. A fresh stool sample was collected in a sterile container and a blood sample in a plain vial was collected from each enrolled subject. After collection, the stool samples were aliquoted and processed for microbial identification and bacterial DNA isolation. Blood samples were also processed for serum separation. Serum was separated from the whole blood samples collected in the plain vial by centrifuging at 3000-4000 rpm for 5-10 min. The serum samples were used for cytokine assays and stored at -80°C till further use.
- **Microbial detection:** Mueller Hinton (MH) broth, MH agar (Difco Laboratory, Detroit, MI) and Chrome *Bacillus* agar (Hi Media) were used to detect *Bacillus* strain. The stool sample were incubated for 24 hours at 37°C in MH broth and then plated on MH agar plate and Chrome *Bacillus* agar to isolate the *Bacillus clausii* UBBC-07. To detect *Lactobacillus* species de Man, Rogosa and Sharpe (MRS) broth and agar (Difco Laboratory, Detroit, MI) was used. The stool sample were incubated for 48 hours at 37°C in MRS broth in Anaerobic Glove Box (Anaerobic Workstation-Whitley DG250-DonWhitley Scientific, United Kingdom) in anaerobic condition and then plated on MRS agar plate. Standard microbial culture and biochemical method and Matrix-assisted laser desorption/ionization- (MALDI- and the mass analyzer is time-of-flight (TOF) analyzer (bioMérieux Inc, USA) were used to identify the isolated colonies were identified. The isolated organisms were also checked by molecular method.
- **Molecular detection:** *Bacillus clausii* was checked by 16S rDNA sequencing using direct PCR using Thermal Cycler (Applied Biosystems, USA) with the in-house designed primers: forward 5'- CCTTGACGGTACCTCACCAC -3' and reverse 5'- AAGCCCAATCTCTTGGGTGG -3' with the product size 299bp. The sequence similarity of primer was also checked using BLAST match (NCBI) which was 98%. After standardization bacterial DNA samples were amplified using standard PCR and PCR product was checked by the electrophoresis and Gel Doc System (BioRad, USA). For positive control a known strain of *Bacillus clausii* UBBC-07 was used.

- **DNA isolation for metagenomic analysis:** Enrolled patients were asked to collect a fresh stool sample in provided sterile container on both pre and post intervention visit. Total bacterial DNA from stool sample was extracted using QIAamp DNA Stool Mini Kit (Qiagen) with some modification (Bamola et al., 2017). Extracted DNA samples were checked and quantified by Nanodrop (TECAN Nano quant) and DNA samples of UC patients were processed for metagenomic analysis.
- **Sequencing:** Metagenomic analysis was performed on Illumina MiSeq® sequencing system (Illumina, San Diego, CA, USA). The analysis was carried out as per reported methodology (Ahmed et al., 2020). V3-V4, hyper variable regions of 16S rRNA were amplified by V3-V4F (CCTACGGGNGGCWGCAG) and V3-V4R (GACTACHVGGGTATCTAATCC) primers. PCR amplification of DNA was carried out by KAPA HiFi HotStart Ready Mix as per standard protocol. The amplicons were purified using Ampure beads to remove unused primers and product was amplified with Illumina primers to generate sequencing libraries. Qubit dsDNA assay kit and Illumina Miseq with 2x300PE sequencing kit were used for libraries preparation and sequencing (Ahmed et al., 2020). FastQC and MultiQC were used to check sequence data quality. Only QC passed reads were used in mothur for pairing and a known reference and UCHIME algorithm was used for chimeric sequence identification. Operational Taxonomic Unit (OTUs) and abundance was calculated using Silva v.132 database. Chao1 and ACE, Shannon, Simpson, and Fisher indices were used for richness and relative abundance. To assess the difference among OTUs abundance between groups, Kruskal-Wallis rank sum test was used.
- **ELISA for Cytokines, serum serotonin and dopamine:** A blood sample was drawn from each enrolled subject and serum was separated. The separated serum samples were assayed for the concentration of IL10, IL6, IL 1 β , TNF, IL17, IL23, dopamine and serotonin as per manufacture's instruction (Fine Test, Fine Biotech Co. Ltd). In brief, (i) test samples and standards were added in 96 well coated plates, (ii) plates were incubated at 37°C for 90 min and wells were washed with wash buffer, (iii) secondary antibody were added, (iv) plates were incubated at 37°C for 60 min and wells were washed with wash buffer, (v) HRP was added (vi) plates were incubated at 37°C for 30 min and multiple washings were done, (vii) TMB substrates were added to visualize HRP reaction, (viii) absorbance was recorded at 450 nm using Nanodrop, Nanoquant Infinite M 200 Pro -microplate reader (Texan, Austria GmbH).

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