# The effect of Probiotic *Bacillus subtilis* HU58 on Immune function in Healthy Human

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ABSTRACT

**Objective:** Probiotic - *Bacillus subtilis* is known to modulate the immune function. The current study evaluates the role of *Bacillus subtilis* HU58 on immune function in healthy human and also for its safety and tolerability along with clinical variables.

**Material and Method:** An open-labeled trial was conducted in 18 apparently healthy subjects. Probiotic-*Bacillus subtilis* HU58 capsules were supplied by Synergia Life Sciences Pvt. Ltd. Each capsule contained 2 billion cfu of Probiotic-*Bacillus subtilis* HU58. These capsules were given once a day orally for 8 weeks. Blood biochemical and organ function tests including Proinflammatory cytokines (IL-6, TNF-α), NK Cell count, ESR, CBC, Lipid profile, HbA1c, Liver function test, S. Creatinine, Seropositivity (HIV, HCV, HBcAg) were studied at the baseline, at fourth week and at eight week. Prior to the study, the permission from Intersystem BioMedica Ethics Committee (ISBEC/NR-05/KM-VM/2016), Vile Parle, Mumbai was obtained. This study was registered with Clinical Trial Registry of India (CTRI) (CTRI/2016/12/007518). Informed written consent was obtained from the subjects before enrollment.

**Results:** At the end of 8th week it was observed that there is reduction in IL-6 by around 45 % and TNF-α by 55 % and continues to decrees. The reduction was found to be statistically significant (P<0.0001). Probiotic - *Bacillus subtilis* HU58 was well tolerated clinically and found to be safe as per the organ function tests in all the subjects. No serious adverse events were reported during the period of therapy.

**Conclusion:** This preliminary study has shown that Probiotic-*Bacillus subtilis* HU58 at a dose of 2 billion cfu once a day for 8 weeks was found to be well tolerated and without any side effects. However, the therapeutic efficacy needs to be evaluated further in a larger sample size and with disease specific indications.

**Key words:** Probiotic - Bacillus subtilis HU58, immune function, Proinflammatory cytokines (IL-6, TNF-α).

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## Introduction

*Bacillus* species have been used as probiotics for at least 50 years with the Italian product Enterogermina® registered 1958 in Italy as an OTC medicinal specialty. However the scientific interest in *Bacillus* species as probiotics has only occurred in the last 15 years and three principal reviews have covered the field. [1-3]

Of the species that have been most extensively examined are *Bacillus subtilis*, Bacillus clausii, Bacillus cereus, *Bacillus* coagulans and *Bacillus licheniformis.* Spores of these Bacillus species being heat-stable have a number of advantages over other non-spore-formers such as *Lactobacillus* spp., namely, that the product can be stored at room temperature in a desiccated form without any deleterious effect on viability. A second advantage is that the spores are capable of surviving the low pH of the gastric barrier [4,5] which is not the case for all species of Lactobacillus.[6] Hence a specified dose of spores can be stored for a longer period without refrigeration and the entire dose of ingested bacteria will reach the small intestine intact. It is generally accepted that they can stimulate innate immunity, competitively exclude potentially harmful pathogens, secrete antimicrobials and finally have the potential to produce beneficial nutrients.

One of such *Bacillus subtilis* strains HU58 which has been studied extensively in detail by Prof. Simon Cutting and his group, at the Royal Holloway College, University London and claimed to be more stable in acidic stomach environment, can grow and sporulate in anaerobic G.I. tract, Having high sporulation efficiency, form biofilms which enhance gut colonization, produces surfactant which enhances the gut adhesion.

Oral supplementation of living spores could provide a useful supply of beneficial bacteria for gut health, aiding nutrition and potentially stimulating the immune system. HU58 is a safe bacterium and this bacterium will not produce any toxins or substances that are deleterious to health.

Hence the current study is formulated in order to evaluate the effect of supplementation of *Bacillus subtilis* HU58 probiotics on immune function in healthy human subjects.

## Material and Method

### Study Design

The current study aims to evaluate the role of *Bacillus subtilis* HU58 on immune system in healthy volunteers. For this reason, an open labeled study was designed. Apparently healthy subjects were enrolled for the approved clinical site i.e. Kokan Hospital, Mumbai.

### Subjects

Prior to the study, the permission from Intersystem BioMedica Ethics Committee (ISBEC/NR-05/KM-VM/2016 dated 27th Feb 2016), Vile Parle, Mumbai was obtained. Total 22 healthy subjects were screened and of which 18 were recruited into study. 9 subjects were males and 9 subjects were females. 4 subjects were screen failure. Remaining 18 apparently healthy subjects were enrolled into study and evaluated by physical examination & clinical biochemistry at the end of 4th and 8th week. A written informed consent (approved by the ethics committee) was taken from all the subjects prior to the enrollment.

### The study procedure and assessments

Eighteen apparently healthy subjects after a proper history, examination and investigations were enrolled as per the selection criteria mentioned in the protocol (approved by the ethics committee). Blood investigations viz. Pro-inflammatory cytokines (IL-6, TNF-$\alpha$), NK Cell count, ESR, CBC, Lipid profile, HbA1c, Liver function test, S. Creatinine, Seropositivity (HIV, HCV, HBcAg) were done at base line, at fourth week and at the end of the study, i.e. at the end of 8th week. Pro-inflammatory cytokines were taken as efficacy markers and were not included in selection criteria.. The subjects were serially followed up at 1st week, 2nd week, 4th week and 8th week. A detailed physical (general and systemic) examination was done at the baseline and at every follow up visits. A pre-designed case record form (approved by the Ethics Committee) which included a page of adverse events was used. The subjects were given single capsule (2 billion cfu each) a day of Probiotic - *Bacillus subtilis* HU58 for 8 weeks. The symptoms (if any) were recorded in the Case Record Forms. The safety was assessed by clinical tolerability, adverse events and by any change in the levels of Pro-inflammatory cytokines (IL-6, TNF-$\alpha$) and NK Cell count. Any other effect during the therapy – beneficial or adverse – was also recorded.

### Methods for organ function tests

Complete blood counts were done by the PC 210 ERMA Blood Cell Counter. ESR was done by Wintrobe method. Liver function test and renal function test

were done by Biochemical method. Prothrombin time was done by coagulation method. TNF-α levels were done by EIA and IL-6 levels were done by ECLIA. The investigations were carried out in NABL accredited laboratory.

**Table 1: Mean values of TNF α and IL 6 of the subjects at baseline and the end of study (eight week) in males and females**

| TNF α (pg/ml) | | | | IL 6 (pg/ml) | | | |
|---|---|---|---|---|---|---|---|
| Males (n=9) | | Females (n=9) | | Males (n=9) | | Females (n=9) | |
| Baseline | 8th week | Baseline | 8th week | Baseline | 8th week | Baseline | 8th week |
| 54.2 | 30.09 | 106.559 | 47.849 | 57.8 | 27.47 | 104.72 | 48.76 |

### Interventional drug, dosage and compliance

Probiotic *Bacillus subtilis* HU58 was supplied by Synergia Life Sciences Pvt. Ltd., in the form of capsules (2 billion cfu) packed – 30 capsules per bottle. The capsules were dispensed in bottles to subjects at the time of enrollment and at an interval of every month. Subject ingested a capsule every morning after breakfast for 8 weeks. The compliance was judged by counting the capsules in the bottle at follow up visits. Subject was said to be compliant if he had consumed minimum 80% of the total dispensed capsules.

**Table 2: Mean values of Blood Investigations of the subjects at baseline, fourth week and the end of study (eight week).**

| Investigations | Range | Unit | Female subjects | | | Male subjects | | |
|---|---|---|---|---|---|---|---|---|
| | | | 0 day | 4 wk | 8 wk | 0 day | 4 wk | 8 wk |
| Haemoglobin | 12.5-16.0 | gm% | 11.41 | 12.19 | 12.97 | 13.92 | 13.8 | 13.68 |
| RBC | 4.2-5.4 | m/cmm | 4.21 | 4.54 | 4.87 | 5.15 | 5.01 | 4.87 |
| WBC | 4000-10500 | /cmm | 9060 | 8307.5 | 7555 | 7344 | 6965 | 6453 |
| Total Bilirubin | 0.1-1.2 | mg/dl | 0.75 | 0.77 | 0.80 | 0.69 | 0.72 | 0.76 |
| SGPT | 9-36 | u/l | 15.33 | 18.18 | 21.03 | 21.7 | 19.3 | 16.9 |
| SGOT | 5-30 | u/l | 21.13 | 20.01 | 18.89 | 25.79 | 23.9 | 21.9 |
| Alkaline Phosphatase | upto 150 | u/l | 94.23 | 86.73 | 79.23 | 89.62 | 94.01 | 98.40 |
| Proteins | 6.2-8.0 | gm% | 6.76 | 6.80 | 6.84 | 6.92 | 6.96 | 6.99 |
| A:G ratio | 0.9-2.0 | gm% | 1.30 | 1.46 | 1.62 | 1.26 | 1.32 | 1.38 |
| Creatinine | 0.6-1.2 | mg% | 0.74 | 0.85 | 0.95 | 0.86 | 0.87 | 0.89 |
| CRP | 0-5 | mg/l | 2.63 | 2.54 | 2.44 | 3.36 | 2.67 | 1.98 |

## Results

At the end of 8th week it was observed that there is reduction in IL-6 by around 45 % (p=0.000122) and TNF-α by 55 % (p=0.000595) and continues to decrease (Table 1, Fig. 1). Biochemical investigations and the organ function tests were in normal limits at the baseline, at fourth week and at the end of eighth week (Table 2). Probiotic- *Bacillus subtilis* HU58 at a dose of 2 billion cfu once a day for 8 weeks was found to be well tolerated and without any side effects.

### Statistical Analysis

The statistical analysis of the IL-6 and TNF-α levels at 0 day, 4th week and 8th week was done by ANOVA method. The results of the statistical analysis are depicted in Table 3. Based on the results from ANOVA, it can be concluded that the reduction in IL-6 and

**Fig 1. Graphs showing decrease in TNF-α and IL6 from baseline to 8th week in both healthy males and females**

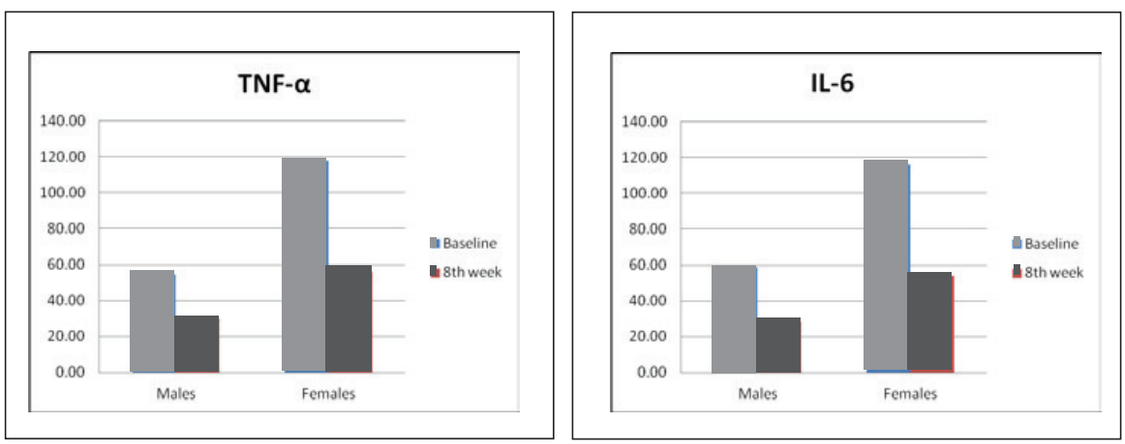

**Table 3: Statistical Analysis**

| SUMMARY | IL 6 levels | | | | TNF α levels | | | |
|---|---|---|---|---|---|---|---|---|
| Groups | Count | Sum | Average | Variance | Count | Sum | Average | Variance |
| 0 day | 18 | 1595 | 88.61 | 1533.899 | 18 | 1584 | 88.00 | 1918.824 |
| 4th week | 18 | 1078 | 59.89 | 773.9869 | 18 | 1094 | 60.78 | 962.3007 |
| 8th week | 18 | 762.7 | 42.37 | 414.5645 | 18 | 764.9 | 42.49 | 403.3888 |

IL 6 levels

| Source of Variation | SS | df | MS | F | P-value | F crit |
|---|---|---|---|---|---|---|
| Between Groups | 19619.00704 | 2 | 9809.504 | 10.80957 | 0.000122 | 3.178799 |
| Within Groups | 46281.65167 | 51 | 907.4834 | | | |
| Total | 65900.6587 | 53 | | | | |

TNF α levels

| *Source of Variation* | SS | df | MS | F | P-value | F crit |
|---|---|---|---|---|---|---|
| Between Groups | 18877 | 2 | 9438.256 | 8.62069 | 0.000595 | 3.178799 |
| Within Groups | 55837 | 51 | 1094.838 | | | |
| Total | 74713 | 53 | | | | |

TNF-$\alpha$ levels by the end of eight week is statistically significant (P<0.0001).

### Tolerability and Safety

Probiotic *Bacillus subtilis* HU58 capsules were clinically tolerated well by all the subjects. No serious adverse events were reported during the period of therapy. The biochemical investigations were in normal limits at the baseline, at fourth week and at eight week.

### Symptomatology

The subjects who were complaining of flatulence, abdominal discomforts were reduced to significant extent by the end of study. The subjects have also experienced changes in the consistency of stools and reduction in the difficulty in defecation.

### Compliance

Drug intake during 8 weeks of trial was monitored by counting the number of capsules remaining in the bottle at the end of every month from the date of dispensing the bottle. The subject compliance was very good.

## Discussion

Probiotics have been shown to have an effect on the immune system of body. Intestinal epithelial cells are active participants in the gut immune response. They can mediate gut-derived systemic inflammatory processes through the production of proinflammatory cytokines, such as IL-6 and TNF-$\alpha$, are crucial for the recruitment and activation of various immune cells.[7] The inflammation is associated with weakened gut barrier integrity via enhancement of paracellular permeability to macromolecules, causing increased absorption of certain toxins as well as possible invasion of pathogenic bacteria.[8] IL-6 and TNF-$\alpha$ are also important factors involved in the regulation of the acute-phase response to injury and infection. Increase of IL-6 and TNF-$\alpha$ concentration is observed after an inflammatory response. Various studies have shown that probiotic strains seem to be effective in reducing pro-inflammatory cytokine production in such cases of inflammatory response.

In an in vitro study on intestinal mucosal extracts isolated from Crohn's patients were co-cultured with L. casei DN 114 001 and production of IL-6 and TNF-$\alpha$ was significantly reduced thereby restoring immune homeostasis.[9]. In another in vitro study,[10] the bone marrow-derived murine dendritic cells (DC) were exposed to probiotic strains and resultant culture supernatants were analyzed for IL-6, IL-10, IL-12, and TNF-alpha. The production of IL-12, IL-6 and TNF-$\alpha$ was inhibited which is indicative of DC maturation.

In an in vitro study the anti-inflammatory effect of probiotic strain was studied on non tumorigenic porcine intestinal epithelial cell line (IPEC-J2) and primary culture of porcine hepatocytes. To mimic inflammation, lipopolysaccharide (LPS; 1 and 10 μg/mL) was applied. Production of IL-8 and IL-6 was measured as a marker of inflammatory responses. The IL-6 concentration was significantly reduced ($P < 0.01$) by the probiotic strain in the co culture. Hence it is proved to be effective in the treatment of intestinal inflammation.[11]

In an in vitro study,[12] it has been shown that in presence of probiotic strains the deleterious effects of TNF-alpha and IFN-gamma on human intestinal epithelial function can be prevented. In another in vitro study,[13] using a human colonic microbiota model, it has been shown that specific probiotic bacteria decrease colonic lipopolysaccharide concentrations which were significantly correlated with TNF-$\alpha$ and IL-1$\beta$ concentrations thereby reducing the pro-inflam-

matory tone.

In a study conducted by Mardem Machado de Souza et al, the authors fed experimental colitis model rats with Probiotic strain for 6 days. Animals receiving enemas containing probiotics (0.19±0.03 mg/dL; $p<0.01$) presented a significant decrease in serum IL-6 levels. This result support probiotics can reduce the inflammatory response of experimental colitis by reducing the cytokines response. Probiotics, by blocking the IL-6 receptor animal colitis models can play an important weapon in the treatment of colitis.[14]

In a randomized, double-blind, placebo-controlled trial to evaluate the impact of oral probiotics on serum levels of endotoxemia and cytokines in peritoneal dialysis (PD) patients, it was seen that the levels of serum TNF-$\alpha$, IL-5, IL-6, and endotoxin significantly decreased after six months of treatment in comparison to placebo. This helps to preserve residual renal function in PD patients.[15]

In yet another randomized, double-blind, placebo-controlled trial, the effect of probiotics was determined on inflammatory markers in 40 critically ill-patients in Intensive Care Unit [16]. A significant difference in IL-6 ($P = 0.003$) was seen over the treatment period as compared to placebo group thereby reducing the inflammation.

In traditional foods, species of *Bacillus subtilis* are well known for their ability to produce and secrete enzymes such as amylses and proteases. In addition, *Bacillus subtilis* has other potential health benefits including anti-coagualtion,[17] enhancing the immunological system,[18] and helping to prevent urinary tract infections (UTI) in the elderly etc.

Recent studies have shown that HU58 is one of the strains of *Bacillus subtilis* identified so far, that can form spores, germinate into vegetative cells, and grow in the anaerobic conditions of the human gastro-intestinal tract,[19,20,21] thereby being able to create and maintain a useful microbial flora in gut. When taken orally, spores of *Bacillus subtilis* HU58 are extremely stable in the acidic conditions of the stomach. As such they can transit the stomach unscathed and can germinate and grow in the small intestine.

*Bacillus subtilis* species has commonly been used in traditional foods in Japan for thousands of years and is considered safe for human consumption. In Europe it is designated QPS (Qualified Presumption of Safety) and in the USA as GRAS (Generally Regarded As Safe). Bacillus subtilis has shown to be safe and without any side effects at a dose up to 1010 spores per day.[3] Also acute and sub chronic (repeated dose) toxicity studies conducted on *Bacillus subtilis* HU58 has shown that it is safe even at very high dosage (Data on file).

In the line with above mentioned studies, in the current study also, the consumption of Bacillus subtilis HU58, 2 billion spores for eight weeks decreased the levels of IL-6 by around 45 % and TNF-$\alpha$ by around 55%. IL-6 stimulates the inflammatory and autoimmune processes in many diseases. Hence there is an interest in developing anti-IL-6 agents as therapy against many of these diseases. TNF-$\alpha$ promotes the inflammatory response, which, in turn, causes many of the clinical problems associated with autoimmune disorders such as rheumatoid arthritis, ankylosing spondylitis, inflammatory bowel disease, psoriasis etc. These disorders are many a times treated by using a TNF inhibitor. The capacity of reducing the levels of IL-6 and TNF-$\alpha$ by *Bacillus subtilis* HU58 in our current study shows the therapeutic potential of role of this probiotic in the inflammatory and auto-immune disorders. However this needs to be verified and evaluated by conducting the study on larger sample size considering specific disease condition and also further with randomized placebo controlled or cross over studies.

## Conclusion

Probiotic *Bacillus subtilis* HU58 at a dose of 2 billion cfu once a day for 8 weeks was found to be well tolerated and has a potential for improving the immune system of human beings. However, the therapeutic efficacy needs to be evaluated further in a larger sample size and in disease specific trial with placebo controlled and cross over studies.

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