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CLINICAL TRIAL REPORT

Immunomodulatory Properties of Probiotic Complex ProbioSEB CSC3 in Healthy Adults: A Prospective, Interventional, Randomized, Double-Blinded, Parallel-Group, Placebo-Controlled Clinical Study

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Purpose: This study aimed to explore the clinical efficacy and safety of ProbioSEB CSC3 in modulating immune responses.

Patients and Methods: A prospective, interventional, randomized, double-blinded, parallel-group, placebo-controlled clinical study was conducted on 88-healthy subjects, divided into probiotic and placebo groups, who received ProbioSEB CSC3 and Maltodextrin, respectively. The primary endpoints were the total Wisconsin Upper Respiratory Symptom Survey (WURSS) score, overall severity of URTI symptoms, immunological parameters, and serum cortisol levels. The secondary endpoints were vital physical, hematological and biochemical parameters, adverse and/or serious adverse events (AEs/SAEs), tolerability, and Quality of Life (QoL).

Results: ProbioSEB CSC3 improved the WURSS and overall severity of the URTI symptom scores post-intervention. A significant improvement in the overall distribution of the illness duration (total symptom days/subject) was observed with probiotic intake compared to placebo (p=0.048,Log rank test). Furthermore, ProbioSEB CSC3 significantly improved immunoglobulin (Ig)-G, Ig-A, interferon-gamma (IFN-γ), interleukin (IL)-10, C-reactive protein (CRP), and cortisol levels ($p<0.01$). The overall QoL of the subjects in the probiotic group significantly improved ($p<0.01$). No AEs or SAEs leading to termination of the study were reported.

Conclusion: ProbioSEB CSC3 supplementation positively modulated the immune response and demonstrated efficacy against URTI and/or alleviated associated symptoms. The administered dose was well tolerated and found to be safe. Further clinical studies on larger populations, multiple centers, and prolonged intervention periods are necessary to validate the results and prophylactic role of probiotics in immunomodulation.

Keywords: ProbioSEB CSC3, *Heyndrickxia coagulans* LBSC, *Bacillus subtilis* PLSSC, *Alkalihalobacillus clausii* 088AE, immunomodulation, upper respiratory tract infections

Introduction

The immune system comprises cells, biomolecules, and processes, and is crucial in orchestrating the body's defense against foreign antigens and pathogens.[1,2] An unbalanced immune response can lead to disease, severe inflammation, and uncontrolled tissue damage. Thus, regulating the gut microbiota to modulate immunity could be an effective way to treat or prevent diseases and maintain a healthy lifestyle.[2] The use of probiotics is the most beneficial approach for restoring gut homeostasis and treating infections caused by antibiotic-resistant bacteria or viruses.[3] Notably, immunomodulation is believed to be one of the most plausible mechanisms for the beneficial effects of probiotics on health. Probiotics have a role in reducing gut permeability and promoting healing of enteric mucosa, enhancing local intestinal immune responses, especially immunoglobulin (Ig)-A synthesis, and restoring a balanced gut microbiota[4–6] Interestingly, the alterations in composition, biodiversity, or activity and function of gut microbiota affect the immunity and microbiota of

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the lungs and vice versa,[5] associating the gut microbiota with the respiratory tract, evolving the term "gut-lung axis." Consequently, probiotics are gaining popularity because of their ability to modulate immune responses, specifically in respiratory tract infections (RTIs). Further, probiotics can regulate and defend the body against allergies and infections.[7]

The immune regulation using probiotics is believed to be mediated by controlling the pro- and anti-inflammatory cytokines[8] and Treg cells.[9] Probiotics may enhance the interferon (IFN)-levels, activity and number of natural killer (NK)-cells, T-cells, and the level of specific antibodies in the lungs,[10] and Treg cells increase the production of anti-inflammatory cytokine IL-10.[3] Recent studies have shown the ability of probiotics to markedly boost plasma cytokine levels, the effectiveness of influenza vaccination and upper respiratory tract infections (URTI), overall quality of life (QoL) while reducing the virus titers and the frequency, duration of URTI, and increasing Ig-A levels.[11,12]

Lactobacillus and *Bifidobacterium* are the most commonly used probiotics explored for immunomodulation.[13] Bacillus species are believed to be gut commensals and have been applied as probiotics for prophylaxis of human gastrointestinal disorders, preventing recurrent RTIs.[14] *B. clausii* was reported to be beneficial in modulating cytokine profiles and inducing Treg cells with improved levels of IL-10 and TGF-β in children with allergies and recurrent respiratory infections.[15] Further, *H. coagulans* showed a significant decrease in the URTI symptom scores and cumulative number of days of disease symptoms in healthy adults. In addition, the salivary Ig-A and the NK-cell activity increased with the *H. coagulans* supplementation.[12] Similarly, *B. subtilis* stimulated immunity in elderly subjects to prevent upper/lower respiratory tract disorders.[14] All these species have proven excellent in modulating immunity and managing RTI symptoms. Correspondingly, studies have applied a combination of Bacillus spp. to demonstrate a synergistic effect in enhancing immunity. Considering the positive effect of *A. clausii, H. coagulans*, and *B. subtilis* on immunity modulation in previous studies, the present study has used ProbioSEB CSC3™ to achieve a collectively profound effect in modulating immunity. Moreover, ProbioSEB CSC3™ along with the enzymatic complex ImmunoSEB has shown a profound effect in managing COVID-19 symptoms for quicker recovery by enhancing immune functions.[16] Similarly, ProbioSEB CSC3 has been proven effective in managing post-COVID-19 fatigue, improving the functional status and QoL of patients.[17] However, a detailed study investigating the changes in disease symptoms through immunomodulation by such probiotic complexes is still unexplored. Considering the excellent efficacy of ProbioSEB CSC3 in managing COVID-19, the scope of such probiotic complexes in modulating immunity must be fully investigated.

Based on the immunomodulatory properties of these three specific strains, we hypothesized that a 5-week intervention of ProbioSEB CSC3 would result in a significant immunity modulation, improving the overall health and indicating the safety and efficacy of the test product in healthy adults. Accordingly, the present study is a prospective, randomized, double-blind, parallel-group, placebo-controlled, interventional trial that evaluated the immunomodulatory effect of the probiotic complex ProbioSEB CSC3™. The Wisconsin Upper Respiratory Symptom Survey (WURSS) and overall severity of URTI symptoms scores were used to test probiotic efficacy against URTI, and the corresponding immunological markers were evaluated as primary endpoints. Physical, hematological, and biochemical parameters, as well as Health-related QoL scores, PHQ-15, and SF-20 were evaluated as secondary endpoints. To the best of our knowledge, this study is the first to explore the efficacy of such a probiotic complex in the modulation of immunity by gauging the relevant symptom scores of URTI and their corresponding immunological parameters.

Materials and Methods

Investigational Product (IP)

ProbioSEB CSC3™, the IP, formulated as an oral capsule, contained *A. clausii* 088AE, *H. coagulans* LBSC, and *B. subtilis* PLSSC at a total strength of 5×10^9 CFU per capsule (475 mg per capsule). The placebo comprised maltodextrin-filled capsules (475 mg per capsule). IP and placebo, with the necessary quality and compliance standards, were supplied by Advanced Enzyme Technologies Ltd., Thane, India. The weight, size, color, packaging, physical appearance, and labeling of both products were identical, except for the unique coded batch numbers used for differentiation.

Ethics and Informed Consent

The Institutional Ethics Committee of Ashwin Multispecialty Hospital, Coimbatore, India [ECR/845/Inst/TN/2016] and Medstar Speciality Hospital, Bangalore, India [ECR/1324/Inst/KA/2019] reviewed and suggested modifications to the study protocol (if needed) and granted approval for the protocol prior to commencement of the investigation. The clinical trial was registered under the "Clinical Trial Registry of India" (CTRI) as per Indian regulations on 11/01/2023, and the registration number was CTRI/2023/01/048913 (https://ctri.nic.in/Clinicaltrials/pmaindet2.php?EncHid=NzcxNjk=andEnc=anduserName=) before enrolling any study subjects. The clinical protocol was designed according to the pertinent requirements of the ICH-GCP E6(R2), Declaration of Helsinki (2013) (64th World Medical Association General Assembly, Fortaleza, Brazil, October 2013),[18] New Drugs and Clinical Trial Rules (2019),[19] the Indian Council of Medical Research Guidelines for Biomedical and Health Research involving Human Subjects (2017),[20] and the Food Safety and Standards Authority of India guidelines. The approved protocol was strictly followed without any further amendments during the entire study period. All subjects were informed and made aware of the details of the clinical investigation, and the essential information associated with the study was explained orally and provided in written format in a suitable, understandable, and familiar language. After thoroughly understanding the details and information explained, along with the related objectives, probable health benefits, and risks, written informed consent was obtained from all the study participants. The details of the CONSORT checklist related to all the events and the details of the current clinical trial are presented in Table S1.

Selection of Subjects for the Study

A prospective, randomized, double-blind, parallel-group, placebo-controlled, interventional clinical trial was conducted in 88 subjects. The study sites were Eesha Multispecialty Hospital, Dasarahalli, Bengaluru, India, and Ashwin Multispecialty Hospital, Coimbatore, India. The clinical study involved two investigation arms (probiotic and placebo) and three visits to the clinical site by participants. The duration of probiotic intervention was 5 weeks (35 days), with efficacy analysis at baseline and after the intervention period, safety and tolerability assessment during each visit, and a final follow-up after a washout period of 21 days. The study was performed on registered subjects who were selected based on predefined inclusion and exclusion criteria.

Inclusion Criteria

The subjects enrolled were included in the trial based on the following inclusion criteria: (i) healthy male and/or female individuals aged 18–65 years at the time of consent, and (ii) individuals who were literate and willing to provide voluntary written informed consent for the study.

Exclusion Criteria

The enrolled subjects were excluded from the study based on the following criteria: (i) previous history of consumption of drugs, diet, or probiotic supplements related to immune function within two-weeks before screening; (ii) known hypersensitivity to the IP; (iii) known case of hypertension or diabetes mellitus; any history/presence of significant metabolic or autoimmune disease; (iv) history/presence of acute or chronic disorders of the cardiac, liver, kidney, or gastrointestinal system within the last 6-months of screening; (v) history/presence of any other acute or chronic disease requiring treatment, in the opinion of the investigator, which could jeopardize the study outcome; and (vi) Females who were breast-feeding/pregnant, or intended to become pregnant during the study.

Study Design, Randomization, and Treatment

The clinical trial consisted of preliminary screening of subjects and baseline analysis; a detailed schedule of events is provided in Table S2. Furthermore, the subjects included in the study were randomized using a random number in a 1:1 ratio to either the IP or ProbioSEB CSC3 arm (N=44) or placebo arm (N=44) using a random number table design. A randomized block design with block size of 6 to maintain the treatment balance (1:1 ratio) throughout recruitment. The randomization sequence has been generated by using simple randomization technique method. The treatment to be allocated and the procedures to be followed were continued thereafter (Figure 1). The clinical study had an intervention

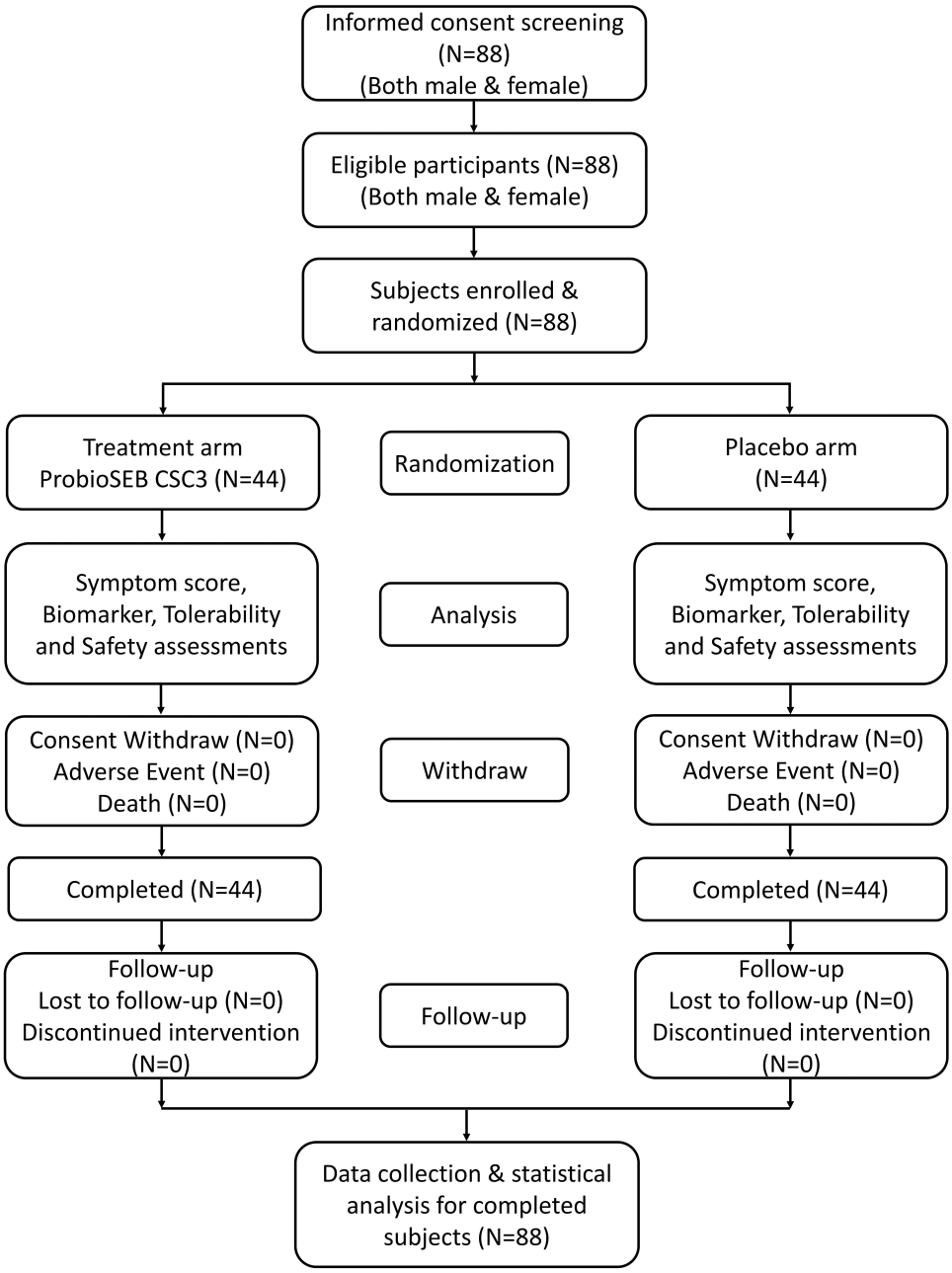

Figure 1 Flow diagram of the clinical study.

period of 5 weeks, followed by a washout period of 21 days and a follow-up visit. The recruited subjects, technicians, research staff, investigators, and physicians associated with the clinical investigation were blinded to whether the subjects were in the IP group or placebo until the completion of the study. The test arm received a ProbioSEB CSC3 capsule containing 5 billion CFU twice daily (morning and evening), while the control arm received a placebo containing a capsule of Maltodextrin (475 mg per capsule) for oral consumption.

Participants were asked to return the unused IP and placebo by the end of the trial. Participants were instructed not to consume curd/yogurt, raw fermented dairy products, raw fermented foods, other probiotic or probiotic medications during the study period. Supportive medications and treatments, if needed, were recommended and provided to the subjects by the concerned physician or investigator; however, prescribing antibiotics along with IP was not suggested.

The study protocol was strictly followed, and no changes or amendments were made to the approved study protocol once the trial commenced. Additionally, no interim analyses were performed during the study period.

Endpoint Objectives: Efficacy and Safety Variables

Primary Endpoints

The primary endpoint evaluated in this study for efficacy assessment was the change in the WURSS–Daily Symptom Report score from baseline to the end of the study. The WURSS included the following evaluations: (i) total number of URTI symptoms per arm (total cases/arm); (ii) Average URTI symptoms per subject; (iii) total number of URTI symptoms per arm (total symptom days/arm; symptom days are the total number of days with at least one URTI Symptom experienced by the subject); (iv) average illness duration (total symptom days/subject); (v) URTI symptom days (total number of days with at least one symptom per subject); and (vi) overall severity of URTI symptoms. Furthermore, the URTI symptom evaluation included runny nose, plugged nose, sneezing, sore throat, scratchy throat, cough, hoarseness, head congestion, chest congestion, feeling tired, headache, body aches, and fever.

Additionally, the study evaluated immunological parameters, including serum IgM, serum Ig-G, and nasal Ig-A (Enzyme-Linked Immunosorbent Assay), and immune biomarkers, including NK cells (CD16+, CD56+), Th (CD3+), Tc (CD3+, CD4+, CD8+), and B cells (CD19+) (Flow Cytometry). Correspondingly, changes in cytokines such as IL-4 and IL-10, IFN-γ (ELISA, single cytokine), CRP (Immunoturbidimetric Assay), and serum cortisol levels (Chemiluminescent Immunoassay) were monitored from visits 1 to 3 with respect to baseline values in all study subjects.

Secondary Endpoints

Secondary endpoints were evaluated for safety and tolerability and included (i) percentage of subjects with AEs, (ii) number and percentage of subjects with changes in vital signs, (iii) number and percentage of subjects with changes in clinical laboratory parameters, (iv) Changes in HRQoL score that included the PHQ-15 and SF-20 scale, and (v) percentage of subjects able to tolerate the study product.

Statistical Analysis

Data were analyzed with a 95% confidence interval (CI) (significance level of 5%) and a minimum power of 80% using SAS software (Version 9.1). All generated datasets were processed using MS Excel-2016, and the findings are presented as mean±SE. Statistical analyses were performed using the GraphPad Prism software (version 9.5.1). For continuous data, a *t*-test was used, whereas Chi-Square analysis was used for categorical variables to compare the baseline demographic data between treatment groups. The normal distribution of the data was checked using the Kolmogorov–Smirnov Test. Paired and Independent t-tests were used to compare intragroup and intergroup differences in continuous variables, respectively, if the data are normally distributed; otherwise, suitable non-parametric tests– the Wilcoxon signed-rank test and the Mann–Whitney *U*-test were applied, respectively. Statistical significance was set at $p \leq 0.05$, unless otherwise specified.

Results

Study Flowchart and Baseline Characteristics

88-Healthy subjects fulfilling the predefined inclusion and exclusion criteria were deemed eligible and included in the study. The trial began in July (mid-week), 2023, and was completed in February (last week), 2024. The study subjects were randomized into two groups: the ProbioSEB CSC3 and control groups. The study population included 24-female (27.30%) and 64-male (72.70%). The average values of age, height, weight, and BMI of the overall study population at the baseline were 39.83±1.10 years, 167.07±0.79 cm, 64.29±0.64 kg, and 23.03±0.17 kg/m^2, respectively. The demographic details of the study participants at the baseline are presented in Table 1.

The age, height, and BMI of both arms at baseline were not significantly different ($p \geq 0.05$). None of the study subjects were excluded, and all enrolled individuals followed the study until completion. IP treatment was started at visit-1 (baseline, day-0) and ended at visit-3 (end of treatment: EOT, day 35±1). The clinical trial team, associated physicians, and principal investigator assessed the regulations of the study at each visit, along with all the safety and efficacy

Table 1 Demographic Details of Subjects Enrolled in the Study

Characteristics	ProbioSEB CSC3	Placebo	Overall	p-value (Inter Group)*
Number of subjects	44	44	88	1.000
Female [n, (%)]	10 (22.70%)	14 (31.80%)	24 (27.30%)	0.3384#
Male [n, (%)]	34 (77.30%)	30 (68.20%)	64 (72.70%)	
Race: Asian [n, (%)]	44 (100%)	44 (100%)	88 (100%)	1.0000
Age (years) [min/max]	39.84±1.52 [21/63]	39.82 ±1.62 [22/59]	39.83 ±1.10 [21/63]	0.9928
Height (cm) [min/max]	167.65±1.19 [150/184]	166.49±1.05 [152.0/180.0]	167.07±0.79 [150/184]	0.4668
Weight (Kg) [min/max]	64.61±0.91 [51.0/75.4]	63.98±0.92 [50.0/73.9]	64.29±0.64 [50/75.4]	0.6274
Body mass index (kg/m^2) [min/max]	22.98±0.22 [19.63/26.40]	23.08±0.26 [17.72/28.87]	23.03±0.17 [17.72/28.87]	0.7698

Notes: *p-value was measured using *t*-test analysis for continuous variables; # p-value was measured using Chi-squared analysis for the nominal variables.

variables and assays, in accordance with the regulations of the study at each visit, along with the predefined study procedures. The clinical trial was concluded after the completion of the target sample size and follow-up visit of the last enrolled subject as per the study procedure. All subjects in the ProbioSEB CSC3 group completed the study and reported no dropouts or discontinuation of IP. The clinical trial was concluded after the follow-up visit (visit-4) at the site on day 56±1 for any AEs/SAEs and URTI symptoms experienced by the participants, along with any impact on their QoL.

Evaluation of Symptom Scores

A significant decrease in the WURSS score was observed with the IP treatment (5.77±0.51 to 4.43±0.30) (p=0.007) compared to the placebo (5.93±0.58 to 5.48±0.49) (p=0.438). Additionally, intergroup analysis revealed that the difference in WURSS scores between the probiotic (−1.36; 95% CI:-2.30, −0.38) and placebo (−0.45; 95% CI:-1.63, 0.72) treatments was marginal and considerable (p=0.062) (Figure 2a). The overall severity of the URTI symptom scores was also determined (Figure 2b). Probiotic intervention caused a significant decrease in the symptom scores from 1.25 ±0.26 to 0.32±0.11 (p<0.001), whereas the placebo altered the scores from 1.25±0.31 to 1.05±0.26 (p=0.521). Intergroup analysis indicated that the changes in the overall severity of URTI symptom scores in the probiotic (−0.93; 95% CI: −1.46, −0.40) and placebo (−0.20; 95% CI: −0.84, 0.43) to were substantial but non-significant (p=0.358). Further, the total number of URTI symptoms and the number of URTI symptom days were 31.20±6.10 and 12.75±1.85 with the probiotic treatment, as compared to 50.02±9.41 and 18.95±2.65 with the placebo treatment, respectively (Table 2). Clearly, these values are considerably lower in the probiotic group. The median of average illness duration (total symptom days/subject) after supplementation with probiotics was 12-days [95% CI: 5, 17], and with placebo was 11.5 [95% CI: 10, 26] with a p-value=0.048 (Log rank test) [Chi-square:3.9; DF: 1].

Changes in Immunological Markers

Changes in immunological markers were evaluated to identify the role of ProbioSEB CSC3 supplementation in modulating immune responses (Table 3). A significant increase in the Ig-G levels was observed from 1.18±0.07 to 1.38±0.07 g/L after the treatment with IP (p<0.01), as shown in Figure 3a. Placebo treatment showed no significant difference in IgG levels after the treatment period relative to the corresponding baseline values (p=0.434). The intergroup analysis revealed a significant difference in IgG levels after the treatment period, which was considerably higher with probiotic treatment than with placebo treatment (p=0.008). A similar trend was observed with the Ig-A levels (Figure 3b), where a significant increase in the probiotic group was observed at EOT (160.39±4.60 mg/dL) from the start of treatment (SOT) (152.09±5.29 mg/dL; p<0.001). Notably, a significant increase, although comparatively less than that of the probiotic treatment, was observed

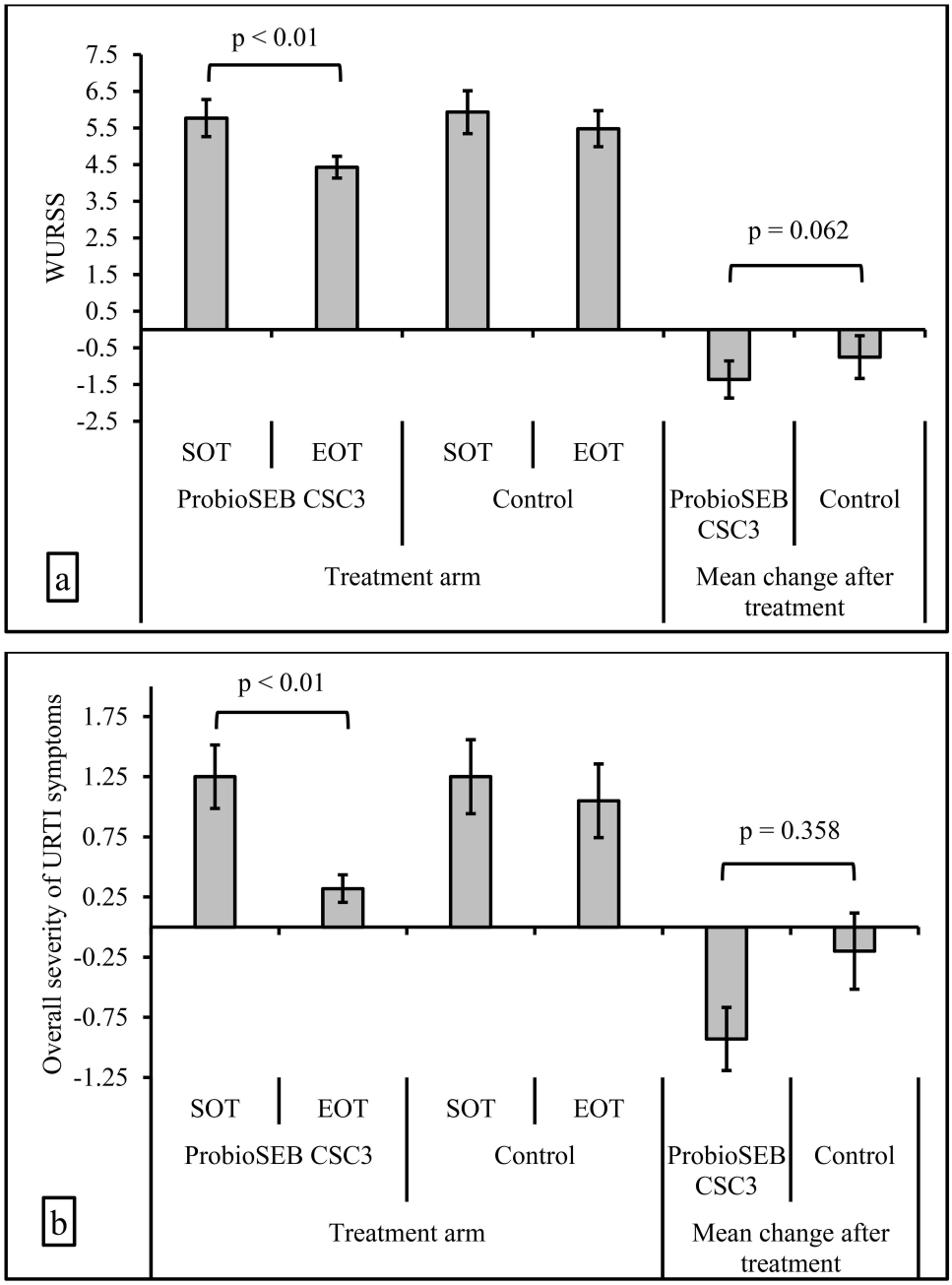

Figure 2 Effect of supplementation of ProbioSEB CSC3 and placebo on Changes in (**a**) Wisconsin Upper Respiratory Symptom Survey (WURSS) scores, and (**b**) Overall severity of URTI symptoms at the start of treatment (SOT) to end of treatment (EOT).

in the placebo group at EOT from SOT ($p<0.01$). However, a significant difference in the mean change in IgA levels was observed between the probiotic and placebo groups ($p=0.0003$), with greater changes reported in the probiotic group.

The IFN-γ levels showed a significant increase ($p<0.01$) after the probiotic treatment (1.22±0.05 to 1.53±0.07 pg/mL), while the placebo treatment showed no significant increase in the IFN-γ values by the EOT (Figure 4a). The increase in IFN-γ levels by EOT in the probiotic group led to a significant intergroup difference ($p=0.003$) between the probiotic and placebo groups. Furthermore, the probiotic treatment significantly increased the IL-10 levels (1.69±0.06 to 1.98±0.06 pg/mL; $p<0.001$). Conversely, the placebo group showed a slight decrease in IL-10 levels after EOT ($p=0.9250$), corresponding to a significant difference in the change in IL-10 levels between

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Table 2 Summary of URTI Symptoms per Arm from Baseline (Day-1) to End of Study (Day-56) ITT/PP Population, and Kaplan-Meier Estimate for Average Illness Duration (Total Symptom days/Subject)

Study Population	ProbioSEB CSC3 (N = 44)	Placebo (N = 44)	p-value (Intergroup)
Total number of URTI symptoms (Average±SE) [Min, max]	31.20±6.10 [0, 168]	50.02±9.41 [0, 208]	0.098
Average URTI symptoms (Average±SE) [Min, max]	0.57±0.11 [0, 3.05]	0.90±0.17 [0, 3.71]	0.099
Total number of URTI symptom days (Average±SE) [Min, max]	12.75±1.85 [0, 50]	18.95±2.65 [0, 56]	0.059
Average illness duration (total symptom days/subject) (Median)	12 [95% CI: 5, 17]	11.5 [95% CI: 10, 26]	0.048 (Log rank test) [Chi-square:3.9; DF:1]

Table 3 Changes in Immunological Cells and Markers of Subjects After Intervention with the ProbioSEB CSC3 and Placebo at the Start of Treatment (SOT) to End of Treatment (EOT). Results Expressed as Mean±SE

Immunological Markers and Cells	ProbioSEB CSC3			Placebo			p-value (Inter Group)
	SOT	EOT	EOT-SOT	SOT	EOT	EOT-SOT	
Ig M (mg/dL)	108.61±3.12	116.93±3.40	8.32±0.95	112.41±3.92	119.16±4.11	6.75±2.08	0.496
IL-4 (pg/mL)	1.47±0.04	1.62±0.05	0.16±0.04	1.42±0.05	1.48±0.05	0.06±0.05	0.261
NK (CD16, CD56) cells population (cells/μL)	351.93±9.05	364.75±10.04	12.82±4.45	357.75±10.52	369.68±10.85	11.93±3.05	0.212
Tc (CD3+) (cells/μL)	1072.84±40.73	1063.93±42.64	−8.91±7.91	1041.82±36.48	1013.14±37.02	−28.68±11.54	0.082
Tc (CD3+, CD8+) (cells/mcL)	564.66±32.80	592.05±33.85	27.39±12.15	567.91±37.01	574.18±34.17	6.27±4.96	0.204
B (CD19) (cells/μL)	283.68±10.16	313±10.84	29.32±6.55	277.82±9.15	304.18±10.04	26.36±6.66	0.401
IL-10/IFN-γ	1.44±0.06	1.37±0.06	−0.07±0.05	1.43±0.05	1.34±0.05	−0.09±0.04	0.768

probiotic and placebo treatments ($p<0.0001$) (Figure 4b). The ratio IL-10/IFN-γ in the probiotic group was slightly higher than the placebo group, amounting to a non-significant difference.

Meanwhile, in terms of CRP levels, a significant decrease in the probiotic group (1.69±0.07 to 1.49±0.06 mg/L; $p=0.017$) was observed as opposed to a significant increase in the placebo group (1.72±0.03 to 1.91±0.04 mg/L; $p=0.042$) after the treatment periods relative to their corresponding baseline levels (Figure 5a). There was a significant difference in the changes in CRP levels between the probiotic and placebo groups ($p<0.002$; Figure 5a), indicating a substantial reduction in inflammation. Finally, a significant decrease in cortisol levels was observed by EOT from SOT with the probiotic intake ($p=0.002$), whereas no changes were observed in the placebo group ($p=0.192$) (Figure 5b). The inter-group analysis revealed a significant difference in the mean change in cortisol levels between the IP and placebo groups ($p=0.003$).

However, a higher change in IgM levels and Tc cells was observed after EOT in the probiotic group than in the placebo group, but failed to amount to any significance. Notably, NK cell activity improved in both groups, but was slightly higher in the probiotic group.

Safety Assessment: Effect on Physical, Hematological, and Biochemical Parameters and QoL

The effects of IP and placebo supplementation on the secondary endpoints were evaluated to assess the safety and tolerability of the product and its impact on the QoL of the subjects. The safety of IP was assessed for tolerance, AEs/SAEs, and systemic biomarkers such as physical, hematological, and biochemical parameters. The PHQ-15 questionnaire measured somatic symptoms such as stomach pain, back pain, pain in the limbs, menstrual cramps, dizziness, and

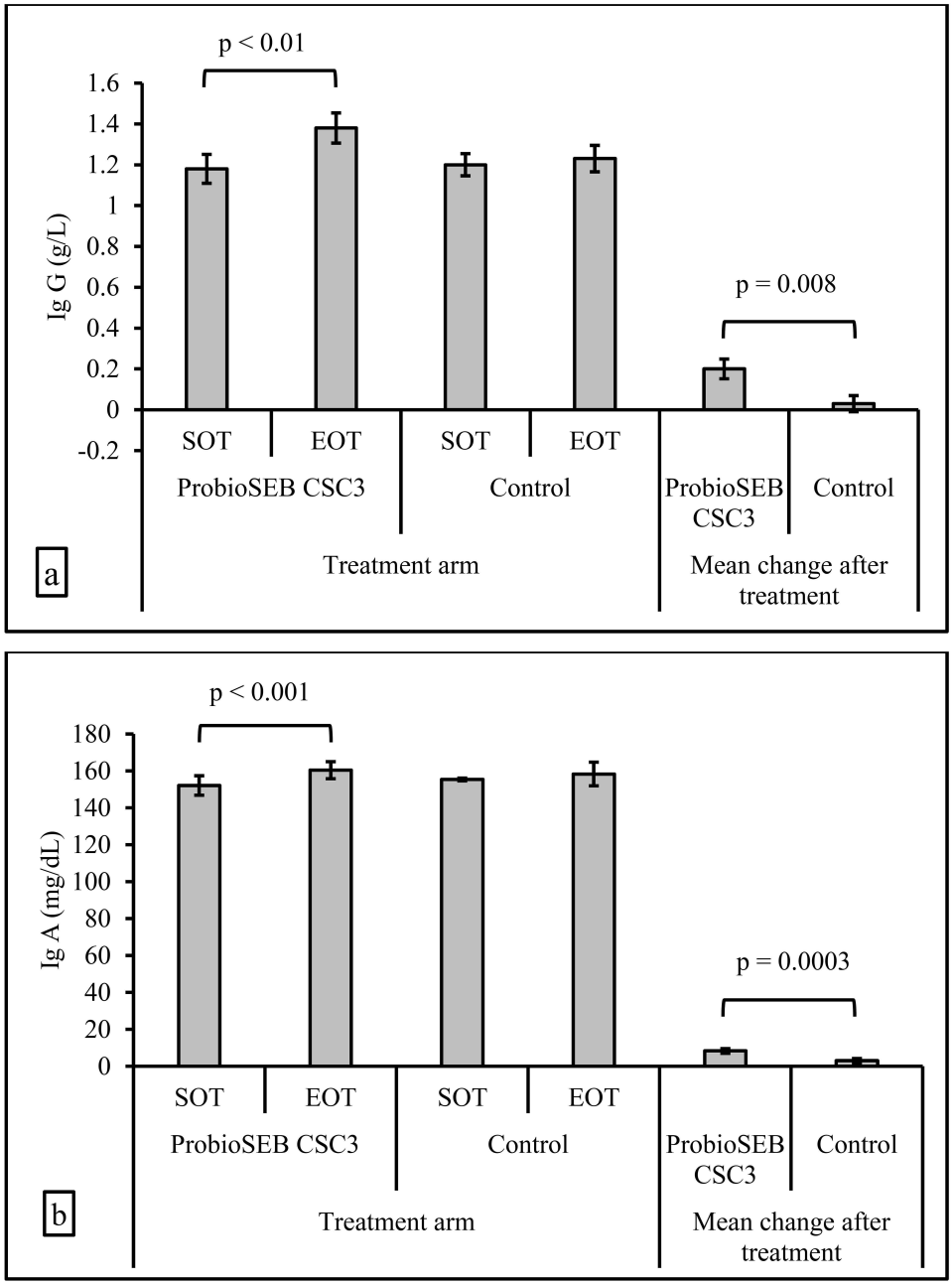

Figure 3 Effect of supplementation of ProbioSEB CSC3 and placebo on changes in levels of immunoglobulins. (**a**) Ig-G levels; (**b**) Ig-A levels.

shortness of breath. The SF-20 questionnaire includes six scales that measure the effect of health on physical functioning, bodily pain, role functioning, general health, social functioning, and mental health. Overall, the PHQ-15 and SF-20 scores were significantly improved after probiotic treatment ($p<0.01$ and $p<0.001$, respectively) (Table 4). In particular, health perception ($p<0.001$) was significantly increased, and pain ($p<0.001$) was significantly reduced after probiotic treatment. In contrast, placebo treatment showed no significant improvement in the quality of life of subjects. Furthermore, the investigational product was well-tolerated by all participants included in the study. For ProbioSEB CSC3, 68.2% and 31.8% of subjects showed very good and good tolerability, respectively, whereas 56.8% and 43.2% of subjects showed very good and good tolerability of placebo, respectively.

Out of the 88-enrolled subjects, 25-subjects each from the IP and placebo groups reported at least one AE. In all, 75-AEs were reported during the study, of which 33-AEs were reported in the probiotic group and 42-AEs in the placebo

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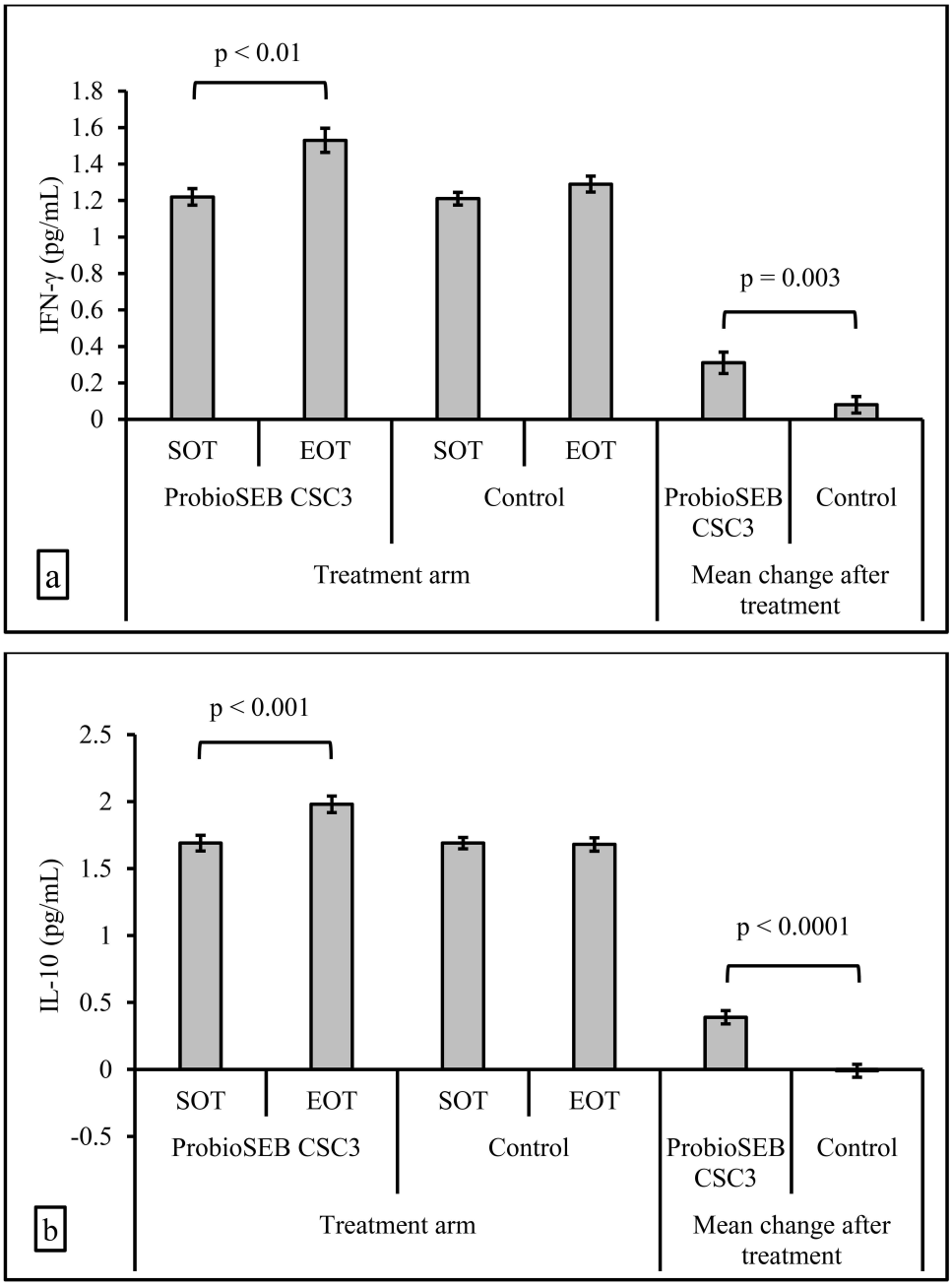

Figure 4 Effect of supplementation of ProbioSEB CSC3 and placebo on levels of interferon (IFN) and interleukins (IL). (**a**) Changes in IFN-γ levels; (**b**) Changes in IL-10 levels.

group. All AEs were of "mild" grade and "unrelated" to the study formulation. A total of 3 subjects in the test arm and 4 subjects in the placebo arm experienced the adverse events that needed concomitant medications. The adverse events experienced by these subjects were diarrhea, constipation, and loose bowel, which were unrelated to the study formulation. Furthermore, no reports related to deaths leading to the termination of the clinical study have been documented.

Accordingly, the physical parameters of the participants, including body temperature, systolic and diastolic blood pressure, pulse rate, and respiration rate, were monitored at each visit (Table S3). IP and placebo treatments showed no significant changes in these parameters, and the values remained within the normal biological range. Furthermore, hematological parameters, including complete and differential blood counts, platelets, and biochemical parameters, including SGOT, SGPT, serum creatinine, BUN, RBS, total cholesterol, total albumin, and globulin, were also evaluated

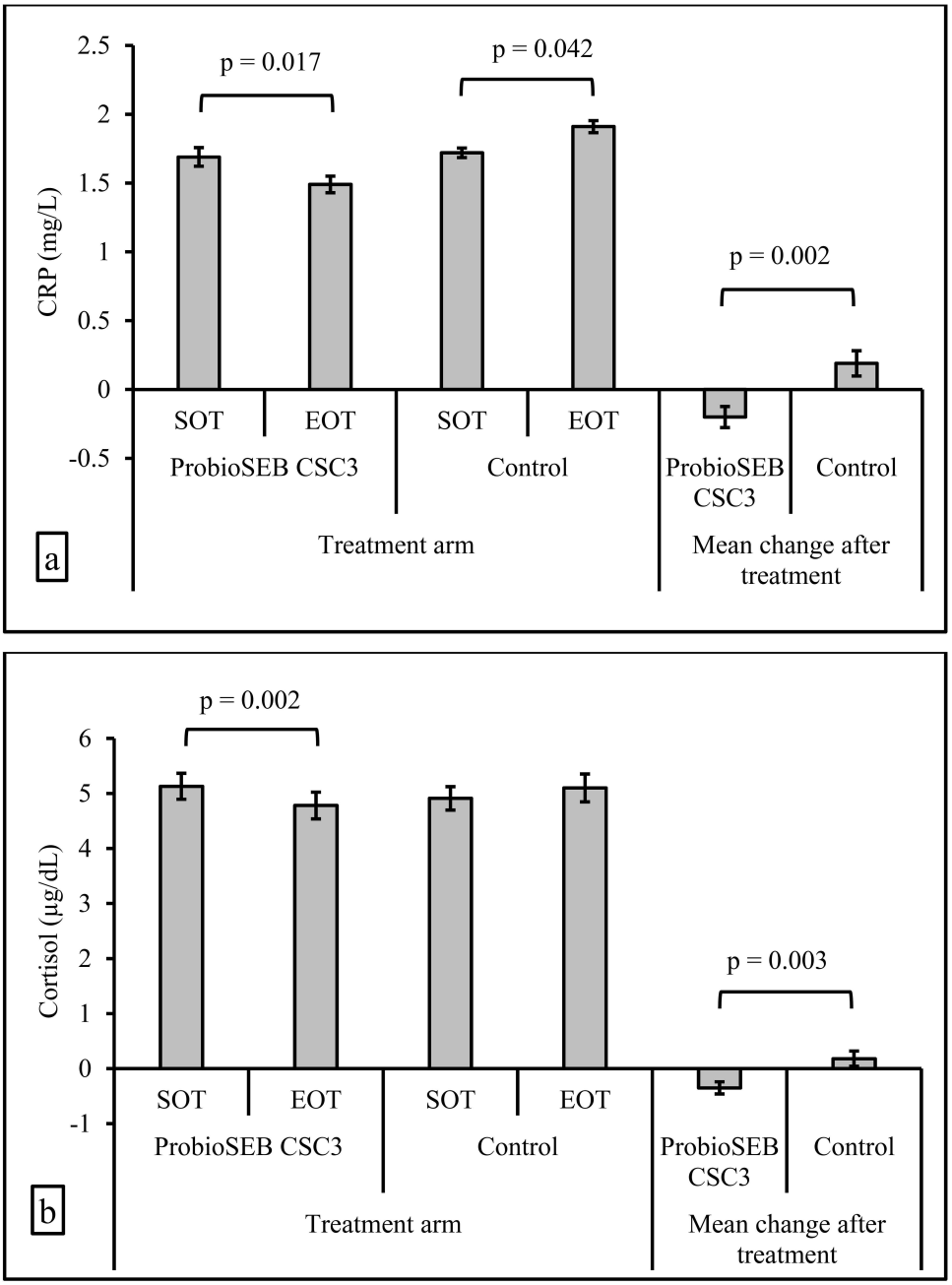

Figure 5 Effect of supplementation of ProbioSEB CSC3 and placebo on (**a**) CRP levels; (**b**) Cortisol levels.

(Table S4). No significant changes were observed in any of these parameters in either group, and the values were within the standard reference range.

Discussion

In the present study, supplementation with ProbioSEB CSC3 showed a prominent effect in modulating immunity and maintaining healthy QoL. Consequently, to evaluate the function of probiotics in modulating and maintaining healthy immunity, the effectiveness of probiotics against URTIs was determined by monitoring disease symptoms. Specifically, the QoL and severity of URTI in the study participants were assessed quantitatively using the WURSS, which evaluates the severity of URTI symptoms. Along with the severity of the URTI, the number of corresponding symptom days and, thus, the URTI duration in the subjects was also evaluated.

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Table 4 Changes in Quality of Life, Social Functioning, Role Functioning, Mental Health, Health Perception, and Pain of Subjects After Intervention with the ProbioSEB CSC3 and Placebo at the Start of Treatment (SOT) to End of Treatment (EOT). Results Expressed as Mean±SE

Parameters	ProbioSEB CSC3			Control			p-value (Inter Group)
	SOT	EOT	EOT-SOT	SOT	EOT	EOT-SOT	
PHQ-15 score	3.14±0.33	1.27± 0.26	−1.86±0.46	2.75±0.26	2.93±0.37	0.18±0.44	<0.001
SF-20 score (Physical functioning)	77.47±1.81	86.37±2.05	8.90±2.60	80.12±1.84	76.90±1.93	−3.22±2.12	<0.001
Role functioning by visit	90.91±2.58	96.59±1.54	5.68±2.92	94.32±1.97	92.61±2.08	−1.70±2.21	0.075
Social functioning	88.18±1.63	95.45±1.28	7.27±2.26	91.36±1.51	90.45±1.66	−0.91± 1.83	0.015
Mental health	97.18±0.84	99.27±0.35	2.09±0.96	98.54±0.52	98.00±0.69	−0.45±0.63	0.066
Health perception	83.27±2.07	94.18±1.50	10.90±2.58	87.53±1.31	85.17±1.93	−2.37±2.36	<0.001
Pain	30.91±2.47	11.82±2.63	−19.09±3.67	27.73±2.36	26.36±3.03	−1.36±3.70	<0.001

A significant decrease in the WURSS score indicated the effectiveness of ProbioSEB CSC3 in alleviating the URTI symptoms ($p<0.01$). Furthermore, although non-significant, probiotic treatment showed better efficacy over placebo supplementation in reducing the WURSS score. Recently, WURSS scores were evaluated to assess the efficacy of *H. coagulans* in managing URTI symptoms in humans. A significant decrease in the scores was reported for *H. coagulans* compared with the placebo group. Furthermore, the cumulative number of days of symptoms was significantly lower in the probiotic group than in the placebo group, suggesting that the probiotic could manage URTI symptoms.[12] These results align with the outcome of the present study, where the WURSS symptom scores for URTI and the number of days with symptoms were considerably lower after ProbioSEB CSC3 treatment than after placebo treatment. While the Kaplan-Meier median for average illness duration was slightly higher in the test group (12.0 vs 11.5 days), the overall recovery curves were significantly different ($p < 0.05$). This indicates that the probiotic influenced the entire duration of the illness across the population, not just the 50th percentile. Further, in this healthy population, most infections were mild, which might have caused a clustering of recovery around the 11–12 day mark. Additionally, for a healthy population with mild infections, a 0.5-day difference at this point could be essentially random variation or noise. Further, in this healthy population, the total average symptom days were substantially lower in the test group (12.75 days) compared to the placebo (18.95 days). This discrepancy, where the mean is considerably improved despite a similar median, suggests that the probiotic was effective at reducing frequency of prolonged URTI episodes.

Evaluating the immunological parameters is imperative to gain insights related to probiotics in modulating immunity and preventing or alleviating URTI symptoms. Several studies have shown that probiotics modulate immune responses by identifying specific patterns in microbes, triggering molecules with nod- and toll-like receptors, and consequently, reducing allergic responses in the host.[15,21–25] In the present study, no significant changes were observed in IgM, IL-4, NK, Th, Tc, and B-cells in the placebo or probiotic groups after treatment. Although non-significant, changes in IgM levels and Tc cells were higher in the probiotic group than in the placebo group. However, the NK cell population increased in both the probiotic and placebo groups and was slightly higher in the probiotic group. This finding supports the hypothesis of the current study on modulation of immunity using probiotic supplementation, which is further corroborated by the alleviation of URTI symptoms. Moreover, NK-cells cells play a vital role in innate immune response.[26] NK cells provide substantial defense against viral infections, and poor NK cell activity could contribute to the development of infections in healthy elderly individuals.[27]

Furthermore, reflecting on the fact that NK cells are a major source of IFN-γ, the increase in NK cell activity followed by the increase in IFN-γ could supplement the immune-enhancing effect of intake of probiotic yogurt in healthy subjects.[26] These observations are consistent with the results of the present study, in which the probiotic group showed significantly higher IFN-γ levels relative to their baseline values, justifying the change in URTI symptom scores through

modulation of immunity. Additionally, the placebo showed no change in IFN-γ levels, clearly demonstrating the sharp distinction between the efficacy of ProbioSEB CSC3 and placebo in positively modulating immune function. In addition to NK cells, T and B-cells are cells produce IFN-γ. It is known that T and B-cells have a key role in adaptive immunity.[7] Furthermore, Th-1 cells produce IL-2, IFN-γ, TNF-α, and IL-12, whereas the Th-2 cells generate IL-4, IL-5, IL-6, and IL-10 and upregulate humoral immunity.[28] IL-12 is considered to promote Th-1 immunity, directly activating CD56+ NK-cell-mediated cytotoxicity.[29] Overall, the present study outcomes agree with the above notion of probiotics inducing cytokines to trigger immune responses.

Primarily, probiotics induce maturation of dendritic cells to restore the balance between Th-1 and Th-2 cells. This ratio is attainable by either producing IL-12 and IFN-γ or by suppressing the response of Th-2 cells via downregulating IL-4 levels and upregulating IgA, IgE, and IgG1.[28,30] Further, stimulating IL-10[31] and TGF-β6 suppresses the response of Th-2 cells and enhances Treg cell activity.[24] Noteworthily, *B. clausii* was found to stimulate Th-1 and Treg immunity, promote IL-12, IFN-γ, IL-10, and TGF-ß synthesis, and downregulate Th-2 response, inhibiting IL-4 production, in allergic children with respiratory infections.[6] The supplementation of *B. coagulans* in healthy subjects led to increased CD3+ and CD69+ cells, and IL-6, IL-8, and IFN-γ levels after exposure to the influenza virus.[32] In a similar study, treatment with a Broncho-Vaxom, a mixture of eight probiotics, diminished levels of IL-4 and IL-13, and increased IFN-γ levels after 8-week treatment.[22] While, our study demonstrated a significant impact on IFN-γ, other parameters including NK cells, IgM, IL-4, and Total T-cell counts, did not reach statistical significance ($p > 0.05$). This contrasts with the findings in previous studies. However, it is noteworthy that those studies often focused on populations with active clinical conditions. In our healthy adult cohort, the lack of significant change in these markers suggests that ProbioSEB CSC3 may have a more targeted effect on the cytokine signaling pathway (IFN-γ) rather than inducing broad changes in total cellular populations (Total T cells/Tc cells). Additionally, the present data show a selective Th1 activation (increased IFN-γ) rather than a reciprocal Th1/Th2 shift. Again, it might be due to the fact that the participants in this study were healthy. In a healthy homeostatic state, the baseline levels of IL-4 are already low, unlike in an allergy or asthma study (where IL-4 is pathologically high). Thus, there might not have been enough scope for the probiotic to further suppress IL-4 levels, which can be considered consistent with the homeostatic maintenance. Therefore, the probiotic's effect was manifested as Th1 strengthening (increasing IFN-γ) rather than Th2 suppression.

On the other hand, a significant increase in IL-10 levels ($p<0.001$) was observed in the probiotic group, unlike the placebo group, which showed a slight decrease in IL-10 levels. This effect of probiotics has been corroborated in other studies. A meta-analysis reported reduced levels of IL-6 and IL-12 but increased levels of IL-10 with probiotic treatment for preventing URTI.[33] Specifically, *B. clausii* has been reported to modulate cytokine profile and increase IL-10 and TGF-β levels in allergic children with respiratory infections.[15] *H. coagulans* supplementation led to an increase in the IL-10 levels in both the probiotic and placebo groups, suppressing the symptoms of URTI.[12] In another study exploring the immunomodulatory mechanism of *B. subtilis* in macrophages, levels of IL-10 were found to increase.[34] Further, the ratio of IL-10 to IFN-γ was only slightly higher in the probiotic group compared to that in the placebo group. While the individual concentrations of IL-10 and IFN-γ showed significant increases in the probiotic group compared to the placebo group, the IL-10/IFN-γ ratio showed no significant difference between the two groups. This result shows that the magnitude of the immune response increased significantly, but the balance remained perfectly intact, indicating that the probiotic-induced immunomodulation was strictly proportional, balancing the homeostasis of the healthy individuals.

Ig-G levels increased significantly with probiotic supplementation, unlike the placebo, which showed no change. This finding is consistent with previous studies on other probiotics, reporting that supplementation with *L. plantarum* for 12 weeks significantly increased Ig-G1 levels, suggesting that Th-cell activity was promoted. Further, Ig-G1 was also considered to be associated with optimal activation of complement.[26] In another study focused on immunity modulation using K8-probiotic in health workers exposed to COVID-19, the serum level of Ig-G was found to be significantly higher compared to the placebo.[35,36] The results suggested that the K8-probiotic supplement helped sustain humoral immunity produced by the vaccine.[35,36] In a similar study, the specific antibody assays against SARS-CoV-2 infection revealed that Ig-G levels were significantly higher in the probiotic group over placebo.[9] This increase in Ig-G levels was ascribed to the possibility of the IP supplementation stimulating specific immunity against the infections, which could also be the case in the present study.

The salivary IgA concentrations were significantly higher in the probiotic group than in the placebo group. A significant increase in the salivary Ig-A levels was observed post-treatment with *H. coagulans* over the placebo.[12] Further, these results suggested that IFN-α may contribute to inducing NK-cell activity and producing salivary IgA.[12] In addition, as discussed earlier, NK-cell activity and Ig-A levels are involved in URTI symptom alleviation, possibly the reason for the lower symptom scores for URTI using ProbioSEB CSC3 treatment over placebo. Additionally, other in vivo and in vitro studies have confirmed that *H. coagulans* increases NK cell activation and IgA production in the intestine, spleen, and bone marrow.[12,37] Coherently, the increased NK cell activity and salivary IgA levels observed in the present study could be attributed to the significant increase in IFN-γ levels with IP supplementation.

Furthermore, probiotics can significantly reduce the serum levels of CRP,[38] supporting the significant decrease in CRP levels in the probiotic group observed in the present study. A significant decrease in CRP levels with ImmunoSEB + ProbioSEBCSC3 supplementation, and this decline in inflammatory markers indicated resolution of infection and faster clinical improvement.[16] Thus, the above studies suggest that any possible inflammation initiated in the probiotic group during the treatment period might have promptly resolved to detect any URTI symptoms, justifying the significantly low CRP levels at EOT. Conversely, the placebo group showed a significant increase in CRP levels, which might be due to exposure to URTI coupled with the inefficiency of placebo treatment in modulating the immune response. Consequently, the severity of inflammation and the corresponding CRP levels were elevated over time in the placebo group.

Furthermore, cortisol levels were significantly reduced in the probiotic group, as opposed to an insignificant but substantial increase in the placebo group by EOT. A similar observation was reported with *H. coagulans* supplementation for URTI symptom alleviation.[12] Further, this decrease in cortisol levels was associated with increased NK-cell activity; specifically, low cortisol levels were reported to result in high NK-cell activity,[39] consistent with the results observed in the present study. Cortisol is a marker for stress assessment, and studies have reported that the release of stress-associated cortisol hormones could compromise immune response and induce susceptibility towards URTIs like the common cold.[40] Previous studies have reported the ability of probiotics to regulate gut microbiota to improve immunity, and it is linked to psychiatric and neurological disorders through the "gut-brain axis".[41] This association of immunity against infections and stress parameters can be further corroborated through PHQ-15 and SF-20 scores, which were found to significantly improve after the probiotic treatment over placebo. These measures of somatic symptom severity, functional status, mental health, and pain in subjects indicated that their overall QoL changed during treatment. Specifically, the health perception, pain, and social functioning were significantly improved with the probiotic supplementation. This effect can be correlated with the reduced inflammation and cortisol levels, and regulated gut microbiota observed with the test supplementation.

Moreover, the safety risk assessment revealed that ProbioSEB CSC3 was well-tolerated by all subjects studied. Safety was examined by evaluating physical, hematological, and biochemical parameters, which indicated that the values of these parameters did not vary significantly in either arm ($p \geq 0.05$). These clinically relevant and important findings indicate that ProbioSEB CSC3 is safe for oral consumption. In addition, at the specified dose of 10 billion CFU/day, the AEs reported by the participants or physician during the treatment or follow-up period were mild and unrelated to the study formulation. Further, no serious AEs were observed leading to termination of the study treatment, which reflects the tolerability and safety of ProbioSEB CSC3 for oral administration and therapeutic use. Previously, ProbioSEB CSC3 was also found safe and effective in the resolution of acute allergic rhinitis symptoms,[42] recovery of COVID-19 patients as a supplementary therapy,[16] and resolving post-COVID fatigue.[17] *A. clausii* 088AE at a dosage of 6-billion CFU/day for 7-days was found to be safe and effective in treating antibiotic-associated diarrhea.[24] Similarly, *H. coagulans* LBSC was found to be safe and effective at a dosage of 6-billion CFU/day for 80 days in treating irritable bowel syndrome.[23] Finally, *B. subtilis* was also found clinically safe and well tolerated to modulate immunity function in healthy patients at a dosage of 2-billion CFU/ day for 8-weeks.[43] Collectively, these studies support the safety and tolerability of ProbioSEB CSC3 in humans.

The present study has demonstrated a positive effect of ProbioSEB CSC3 in modulating the immunity. However, a few limitations need to be addressed. First, the study was conducted on healthy subjects and thus, further research is warranted to determine if these benefits translate to populations that already have allergies or infections to induce a major effect in these biomarkers and other parameters. Second, the moderate study duration may not capture the long-term

persistence of the observed Th1-shift, suggesting that future trials should include a large population with extended follow-up periods to evaluate the sustained effect of the intervention.

Conclusion

Evidence is slowly emerging in favor of probiotics as one of the approaches for maintaining or restoring immune balance, thereby preventing or treating diseases. The present study validates and supports the transpiring role of probiotics in immunity modulation for human health management. ProbioSEB CSC3 (a combination of *Alkalihalobacillus clausii* 088AE, *Bacillus subtilis* PLSSC, and *Heyndrickxia coagulans* LBSC) exerted beneficial effects by altering immune functions to confer overall health to individuals. Thus, it has the potential to cause immunological changes against the most common infectious diseases, such as URTIs. Supplementation with 10 billion CFU/day ProbioSEB CSC3 was well tolerated and safe for consumption. Probiotic treatment showed potential for symptom alleviation of common infections, such as URTI, which was corroborated by the positive response in immunological parameters. Enhanced NK cell activity and, correspondingly, improved IFN-γ, Ig-G, and Ig-A levels could be highlighted as a possible mechanism of action of ProbioSEB CSC3 against respiratory infections. Anticipating the need for effective and reliable methods to control and manage highly infectious respiratory diseases that affect large populations globally, the use of such probiotic complexes should be further investigated to include varied study parameters and conditions. This approach could assist in gaining broader insights into exploring their potential to a full extent. Accordingly, studies involving large-scale interventional trials with prolonged follow-up are needed.

Human and Animal Rights

No animals were used in the study.

Data Sharing Statement

The datasets used or analyzed in the study are available from the corresponding author upon reasonable request.

Consent for Publication

Written informed consent was obtained from all the participants. None of the vulnerable subject participated in the study.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

All authors are paid employees of Advanced Enzymes Technologies Limited, Thane, India, which has a corporate affiliation with Specialty Enzymes and Probiotics, USA. Specialty Enzymes and Probiotics, USA, had no role in the study design or the actual conduct of the study. The authors report no other conflicts of interest in this work.

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