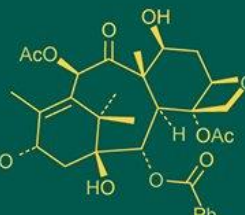


International Journal of Advanced Biochemistry Research



ISSN Print: 2617-4693
ISSN Online: 2617-4707
NAAS Rating (2026): 5.29
IJABR 2026; 10(6): 296-307
www.biochemjournal.com
Received: 28-03-2026
Accepted: 30-04-2026

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ImmunoSEB™ enhances immune resilience: Hematological, humoral, and microarray evidence in normal and immunosuppressed Wistar rats

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DOI: <https://www.doi.org/10.33545/26174693.2026.v10.i6d.8612>

Abstract

Background: Immune system is the primary defence against pathogens and internal ailments. In the modern era, immune competence is increasingly challenged and there is growing interest in safer, biologically derived alternatives. This study evaluates ImmunoSEB™, a proprietary blend of systemic enzymes, for its potential to modulate immune resilience.

Methods: 90-Wistar rats were allocated into nine groups to compare ImmunoSEB™ (50, 200, and 400 mg/kg) against normal and cyclophosphamide induced immunosuppressed controls. All rats, except those in the healthy control group, were vaccinated with *Brucella abortus* strain-19. Over 90 days, the study assessed hematological parameters, serum immunoglobulins (IgG, IgM, IgA), and splenic gene expression via cDNA microarray analysis.

Results: ImmunoSEB™ demonstrated potent immunomodulatory effects in both normal and immunosuppressed states. Hematological analysis showed that ImmunoSEB™ significantly increased total leukocyte counts, with the most pronounced elevation reaching from 6.75 to 19.7×10^3 per mm^3 (Group II). In immunosuppressed rats, ImmunoSEB™ restored lymphocyte counts from 68% to 89% (Group VII), surpassing the recovery observed with Levamisole. Humoral immunity was strongly stimulated, with IgG levels rising from 1.70 to 174.30 mg/dL (Group II), representing the most effective enhancement across treatment groups. Microarray profiling confirmed reversal of cyclophosphamide-induced suppression in 68 genes, restoring critical mediators such as IL-15, Cathepsin C, and Cysteinyl leukotriene receptor-1. It validates the dual role of ImmunoSEB™ as a restorative agent under immunosuppression and a fine-tuned enhancer of baseline immune response.

Conclusion: ImmunoSEB™ has helped in restoration of immune resilience under immunosuppression and an enhancer in normal physiology. It provides an alternative for immune modulation, effectively coordinating cellular and humoral defenses against environmental challenges.

Keywords: ImmunoSEB™, immunomodulation, Immunoglobulins, microarray analysis, Humoral immunity

Introduction

The immune system is the body's first line of defence against a wide range of outside pathogens and internal ailments, such as infectious microorganisms and cancerous cells. This well-organized group of cells, tissues and organs works together to preserve homeostasis and protect the system from diseases [1]. Immunomodulators are important for regulating the immune system because they can alter functions of the immune system to ensure it remains effective and targets appropriately. The ability to modulate immune activity is essential, as insufficient responses render the host susceptible to infection, while excessive or dysregulated responses may lead to tissue damage or autoimmune disorders [2]. Importantly, immune competence is not restricted to one organ; rather, it distributed throughout the body, with a large number of immune cells located in the gastrointestinal tract [3]. This widespread distribution underscores the fact that immunity is influenced not only by molecular and genetic factors but also by daily lifestyle choices such as diet, hydration, physical activity, stress management, and sleep quality. These elements collectively shape cytokine production and immune cell function [4, 5], highlighting the essential role of immune resilience in everyday health.

Immunomodulators can be divided into two main categories: immunostimulants and immunosuppressants.

Each category possesses a significant but complementary role. Immunostimulants enhance immune function, thereby enhancing the host's defence against infections and malignancies. Beta-glucans are naturally occurring polysaccharides from yeast and mushrooms that activate macrophages and other innate immune cells. Interferons are another example, they control antiviral defence mechanisms. Vaccines also fall into this category, as they prime adaptive immunity against specific pathogens [6, 7, 8]. In contrast, immunosuppressants, slow down the immune system so that it doesn't overstimulate or misdirected responses that could damage host tissues. Corticosteroids and methotrexate are commonly used in the treatment of autoimmune disorders characterized by immune system dysfunction leading to self-tissue destruction [9]. Collectively, both classes of immunomodulators are crucial in clinical practice, offering therapeutic strategies across a varied range of pathological conditions.

Among synthetic immunomodulators, Levamisole holds particular significance. Levamisole was first used as an anthelmintic agent in 1966 [10]. Later, it was found to exert numerous immunomodulatory effects and was added to the World Health Organization's list of essential medicines in 1988 [11]. Levamisole works through enhancing the activation of dendritic cells (DCs) and the production of cytokines like interleukin (IL)-12, IL-10, and interferon- γ , while concurrently suppressing pro-inflammatory mediators like tumor necrosis factor- α and IL-6. Both of these actions help stimulate the immune system and control inflammation, although the precise molecular pathways remain incompletely defined [12]. The extensive utilization of synthetic immunomodulators in infectious diseases and oncology emphasizes their significance as reference standards for the assessment of novel agents.

Beyond synthetic compounds, biologically derived immunomodulators, particularly enzymes, have gained increasing attention. Enzymes are very specific biocatalysts that control nearly all biochemical reactions in organisms. Biologically derived immunomodulators, particularly enzymes, have gained increasing attention beyond synthetic compounds. Exogenous enzyme supplementation has been shown to restore immune balance and enhance host defence in various experimental models. For example, supplementation with lactoferrin has been reported to improve antiviral responses in murine studies [13]. Other investigations have highlighted how enzyme mediated modulation of leukocyte activity can rebalance inflammatory pathways [14]. While selective activation of mast cell proteases via dipeptidyl peptidase I underscores the specificity of enzymatic regulation in modulating innate immune pathways [15]. These different functions highlight

the therapeutic potential of enzymes as immunomodulators. Moreover, their flexibility, specificity, and natural origin make them suitable agent for safer and more sustainable immunotherapeutic approaches.

The present study focus on ImmunoSEB™, a unique engineered blend of systemic enzymes. Its component enzymes has been reported to influence immune activity, and their combination is hypothesized to exert synergistic immunomodulatory effects [16, 17]. The rationale for investigating ImmunoSEB™ lies in its potential to modulate both innate and adaptive immunity, thereby enhancing host defence while maintaining immune equilibrium. Given the growing interest in enzyme-based therapeutics, ImmunoSEB™ represents a promising candidate for evaluation alongside established synthetic agents, particularly in the context of maintaining immunity that is essential for daily health.

In alignment with the established role of enzymes as immunomodulators, this investigation was designed to compare the immunostimulant potential of ImmunoSEB™ with that of Levamisole, a well-recognized synthetic agent. Using rats vaccinated against *Brucella abortus* strain 19, the study aimed to assess the capacity of ImmunoSEB™ to influence hematological parameters, lymphocyte activity, and immunoglobulin expression. Its effects under immunosuppressed conditions and preliminary transcriptomic changes in splenic tissue were also examined, thereby providing a comprehensive assessment of its immunomodulatory efficacy.

Materials and Methods

Laboratory Animals

Ninety healthy Wistar rats of either sex, weighing between 80-100 g were used in this study. All experimental procedures were performed at Raj Biotech India Pvt. Ltd. (RBIPL), Satara, MH, India, and conducted in accordance with Good Laboratory Practices (GLP), and the SOP (Standard Operating Procedures) of RBIPL. Animals were housed in polypropylene cages, separated by sex and grouped as outlined in Table 1. They were maintained under controlled laboratory conditions with free access to drinking water and lab animal diet (Rodent pellet diet manufactured by Nav Maharashtra Chakan Mills Ltd.). The room temperature was kept between 21-27 °C with relative humidity around 70%. Prior to the experiment, all rats were dewormed using Albendazole (Albendos, DOSCH Pharmaceuticals, Mumbai) at a dose of 0.75 mg/200 g body weight for three consecutive days. After seven days acclimatization period following deworming, the rats were randomly assigned to the respective experimental groups for the assessment of immunomodulatory activity.

Table 1: Group wise classification of lab animals

Group No.	Group	Immunosuppression	Drug/Dose	Vaccine- <i>Brucella abortus</i> strain-19
I	Healthy control	-	-	-
II	Normal	-	50 mg/kg	0.1 mL/rat; S/C
III	Normal	-	200 mg/kg	0.1 mL/rat; S/C
IV	Normal	-	400 mg/kg	0.1 mL/rat; S/C
V	Immunosuppressed control	Cyclophosphamide 5 mg/kg; 3 injections on alternate days	-	0.1 mL/rat; S/C
VI	Immunosuppressed	Cyclophosphamide 5 mg/kg; 3 injections on alternate days	50 mg/kg	0.1 mL/rat; S/C
VII	Immunosuppressed	Cyclophosphamide 5 mg/kg; 3 injections on alternate days	200 mg/kg	0.1 mL/rat; S/C
VIII	Immunosuppressed	Cyclophosphamide 5 mg/kg; 3 injections on alternate days	400 mg/kg	0.1 mL/rat; S/C
IX	Immunostimulant (Levamisole treated)	Cyclophosphamide 5 mg/kg; 3 injections on alternate days	Livamisol 2.5 mg/kg; S/C; Weekly	0.1 mL/rat; S/C

Investigational product and Experimental Design

The investigational product, ImmunoSEB™, was provided Specialty Enzymes and Probiotics, Chino, CA, USA. A total of ninety healthy Wistar rats were randomly allocated into nine groups, each including five male and five females (Table 1). The animals from all the groups received their designated treatment for 90 days. Animals in Group I served as the normal control and were maintained on a standard diet without any additional treatment. All rats in Group II to IX received *Brucella abortus* strain-19 vaccine at a dose of 0.1ml per rat, administered subcutaneously. Groups II, III, and IV received ImmunoSEB™ at doses of 50 mg/kg, 200 mg/kg, and 400 mg/kg body weight, respectively, without induction of immunosuppression. Group V served as the immunosuppressed control, in which no test product was administered. Immunosuppression was induced in Group VI, VII, and VIII using cyclophosphamide at 5 mg/kg body weight, delivered subcutaneously in three injections on alternate days. Additionally, these groups received ImmunoSEB™ at doses of 50 mg/kg, 200 mg/kg, and 400 mg/kg body weight, respectively. Moreover, Group IX was treated with Levamisole (reference immunostimulant), administered subcutaneously at 2.5 mg/kg once weekly for four consecutive weeks.

Blood samples from each group were pooled and collected at baseline (day 0) and subsequently at 15 days interval throughout the study, specifically on days 15, 30, 45, 60, 75, and 90, for systematic immunological evaluation. The collected blood samples were used for measurement of hematological and immunological parameters including leucocyte, T-lymphocyte and immunoglobulin count. In addition, microarray analysis of splenic RNA was performed using cDNA microarray analysis.

Hematological analysis

Total leukocyte count (TLC)

The total leukocyte counts were determined using the improved Neubauer hemocytometer as described by Sastry^[18]. Briefly, blood samples were diluted 1:10 in Türk's solution for white blood cells estimation. The diluted samples were charged into the improved Neubauer hemocytometer and allowed to settle. Under 400× magnification, cells were counted manually in the appropriate grid areas of the chamber. The results were expressed as cells per mm³ of blood.

Lymphocyte count

Lymphocyte counts were determined using the method described by Sastry^[18]. Briefly, blood samples were collected into EDTA coated tubes to prevent coagulation. A thin blood smear was prepared on a clean glass slide, air-dried, and fixed with methanol. The smear was then stained using Leishman's stain to differentiate lymphocytes from other leukocyte populations. Under oil immersion (100× objective), leukocytes were counted, and the proportion of lymphocytes was expressed as a percentage.

Immunological assessment

Immunological assessment was performed to evaluate serum concentrations of Ig-A, Ig-M, and Ig-G. Quantification of immunoglobulins was carried out by nephelometry using diagnostic kits developed by Boehringer Mannheim Lab Diagnostics (Germany). Briefly, serum samples were collected aseptically and centrifuged to remove cellular

debris. The clarified serum was then subjected to nephelometric analysis, wherein antigen-antibody complexes formed between immunoglobulins in the sample and specific antisera scatter incident light. The intensity of scattered light was measured at a fixed angle using the nephelometer, and the signal obtained was directly proportional to the concentration of immunoglobulins present. Standard calibration curves were generated using reference sera provided with the diagnostic kits, and sample concentrations were calculated accordingly. Results were expressed in milligrams per decilitre (mg/dL).

Microarray analysis

On the basis of hematological and immunological assessments, the dose group that exhibited the most effective response in terms of leukocyte, lymphocyte, and immunoglobulin modulation was selected for microarray analysis.

Spleen tissue (200 mg) was collected from the selected group and transported in RNA Later (Ambion Inc., USA) at 4±2 °C. Samples were stored at -20±2 °C until RNA extraction, which was performed after 30 days. RNA was isolated using RNeasy reagent (Qiagen Inc., USA) following homogenization by grinding in liquid nitrogen. The quantification and quality assessment of RNA was evaluated through gel electrophoresis and spectrophotometric analysis.

For microarray analysis, cDNA labeling was performed using the TSA Micromax Indirect Labeling Kit (PerkinElmer, USA) according to the manufacturer's instructions. Labeled cDNAs were quality-controlled as instructed by the manufacturer.

Eight Rat 5.7k oligonucleotide microarray chips (University of Cincinnati, Genomics and Microarray Laboratory), each containing approximately 5,700 rat genes, were used for the analysis. Prehybridization was performed by immersing slides in prehybridization buffer (5× SSC, 0.1% SDS, 1% BSA) at 42±2 °C for 30 min, followed by ten washes in filtered double-distilled water and drying with isopropanol. Hybridizations were conducted overnight on prehybridized slides by mixing labeled cDNAs in hybridization buffer Q (provided in the Micromax kit). Washes and development followed manufacturer recommendations.

Slides were scanned using a GenePix Personal 4100A scanner (Axon Instruments, USA), and raw image files were processed using GenePix Pro software to extract fluorescence intensity values. To minimize technical variability, signal data underwent further enhancement using proprietary analytical tools (GeneWizard, Bias Finder, and Bias Fixer). Normalization was conducted both intensity-wise and grid-wise to correct for systematic biases across the array. Gene expression ratios were subsequently calculated, and statistical significance was tested. Replicate spots consistently yielded reproducible results, strengthening the reliability of the dataset.

Statistical Analysis

The data of various hematological and Immunological parameters were analysed by using two way analysis of variance (ANOVA) as per the method suggested by Sheth *et al.*^[19]. For microarray analysis, gene expression ratios were calculated, and statistical significance was assessed using Student's t-test.

Results

A total of ninety Wistar rats were included in the experimental investigation to evaluate the immunostimulant effect of ImmunoSEB™ administration at different dose levels (50 mg, 200 mg and 400 mg/kg body weight) in both normal and immunosuppressed animals. The baseline body weights of rats across the nine groups ranged from 119.3 to 133.8 gm. No significant differences were observed between the control and treatment groups at any interval during the study or at termination.

The mean daily feed intake of rats in the nine groups over the 90 day experimental period ranged from 13.50 to 41.60

gm across all groups, with no statistically significant variation observed.

Effect of ImmunoSEB™ on total leucocyte count (TLC)

The leucocyte counts (mean values) of ninety rats in nine groups were recorded at 15 days interval throughout the study. Statistical analysis revealed significant differences in the mean leucocyte counts of all groups when compared with their respective pre-treatment (day 0) values. The mean leucocyte counts of rats in different groups are presented in Table 2.

Table 2: Effect of feeding of ImmunoSEB™ on Total leucocyte count (TLC) of rats

Description	Group	Day 0 (SOT)	Day 15	Day 30	Day 45	Day 60	Day 75	Day 90 (EOT)
Healthy control	I	6.65	13.25	10.1	12.6	20.4	18	19.6
ImmunoSEB™ 50 mg/kg	II	6.75	13.5	12.45	14.7	12.4	14	19.7
ImmunoSEB™ 200 mg/kg	III	10.45	17.3	11.35	19.55	16.1	15	20
ImmunoSEB™ 400 mg/kg	IV	7.25	12.25	14.6	12.15	18.7	17	18.9
Immunosuppressed control	V	6.6	11.6	14.95	13.15	17.8	15	17
Immunosuppressed + ImmunoSEB™ 50 mg/kg	VI	6.15	9.4	12.7	14.55	19.6	11	12.1
Immunosuppressed + ImmunoSEB™ 200 mg/kg	VII	5.45	4.3	16.75	17.3	18.7	16	13.6
Immunosuppressed + ImmunoSEB™ 400 mg/kg	VIII	8.35	11.35	14.25	14.3	15.55	14	13.2
Immunostimulant (Levamisole treated)	IX	7.8	14.7	10.75	14.05	18.45	9	16.3

After 90 days of treatment, mean leucocyte count ranged between 12.10 and 20.00×10^3 per mm^3 across the groups. The untreated control group exhibited a significant elevation from 6.65×10^3 per mm^3 at baseline to 19.6×10^3 per mm^3 . Similar increases were also noted in Groups II, III, and IV, with values rising from 6.75 to 19.7×10^3 per mm^3 , 10.45 to 20.0×10^3 per mm^3 , and 7.25 to 18.9×10^3 per mm^3 , respectively. Similarly, the immunosuppressed non treated group (Group V) also showed significant increase in mean leucocyte count, increasing from 6.6×10^3 per mm^3 to 17.0×10^3 per mm^3 . Furthermore, in the immunosuppressed and treated groups, significant elevations were observed-Group VI increased from 6.15 to 12.1×10^3 per mm^3 , Group VII from 5.45 to 13.6×10^3 per mm^3 , and Group VIII from 8.35 to 13.2×10^3 per mm^3 . Group IX, which received the known immunostimulant Levamisole, also demonstrated a significant increase from 7.8×10^3 per mm^3 to 16.3×10^3 per mm^3 relative to pre-treatment values. Notably, Group II showed the highest elevation (from 6.75 to 19.7×10^3 per mm^3), representing approximately a three-fold increase compared to baseline.

Although leucocyte counts in Group VI, VII and VIII (immunosuppressed and ImmunoSEB™ treated) were elevated relative to their pre-treatment levels, no statistical

difference was observed when compared with Group IX (Levamisole treated).

Effect of ImmunoSEB™ on lymphocyte count

Lymphocyte counts in the nine experimental groups showed distinct patterns over the treatment period. In the healthy control group, lymphocyte count increased from 78% to 85%. Although slight reduction in the lymphocyte counts in group II and Group III rats 87% to 81% and 86% to 78%, respectively and slight elevation in the lymphocyte counts of Group IV rats 77% to 79% was observed these variations are not statistically significant. Non-significant elevation from 77% to 87% in the lymphocyte counts was also observed in Group V rats. However, the lymphocyte counts of rats from Group VI, VIII and IX (79% to 69%, 71% to 69%, and 86% to 79%, respectively) was observed to be reduced which were not statistically significant. Statistically significant increase from 68% to 89% in the lymphocyte counts of Group VII rats was observed when compared with its baseline values and also when compared with the Group V (immunosuppressed rats) and Levamisole treated rats (Group IX). Table 3 provides the complete lymphocyte data for each treatment group.

Table 3: Effect of feeding of ImmunoSEB™ on T-Lymphocyte counts of rats

Description	Group	Day 0 (SOT)	Day 15	Day 30	Day 45	Day 60	Day 75	Day 90 (EOT)
Healthy control	I	46	50	51	51	50	50	51
ImmunoSEB™ 50 mg/kg	II	50	56	53	54	59	52	53
ImmunoSEB™ 200 mg/kg	III	50	55	54	59	55	56	57
ImmunoSEB™ 400 mg/kg	IV	43	50	54	55	54	56	58
Immunosuppressed control	V	55	50	51	52	53	52	51
Immunosuppressed + ImmunoSEB™ 50 mg/kg	VI	50	51	53	52	53	54	59
Immunosuppressed + ImmunoSEB™ 200 mg/kg	VII	46	44	46	54	52	52	58
Immunosuppressed + ImmunoSEB™ 400 mg/kg	VIII	42	46	49	48	54	50	55
Immunostimulant (Levamisole treated)	IX	51	55	49	55	52	51	53

Taken together, these findings indicate that ImmunoSEB™ supplementation significantly increased lymphocyte count in immunosuppressed rats, where no significant changes were observed in normal rats. As lymphocytes are the primary mediators to drive adaptive immunity, the findings suggest that ImmunoSEB™ supplementation enhances adaptive immunity response more effectively under immune suppression condition than in normal animals. In contrast, Levamisole was associated with reduction in lymphocyte counts, highlighting the comparatively greater immunomodulatory potential of ImmunoSEB™ in

immunosuppressed condition.

Effect of ImmunoSEB™ on Serum Immunoglobulin Levels

Serum IgG Levels

The mean IgG levels of rats in all experimental groups showed discrete variations during the study. In the healthy control group, a statistically non-significant reduction was observed with IgG levels declining from 34.80 to 31.70 mg/dL after 90 days, compared to pre-treatment values. The complete data are presented in Table 4.

Table 4: Effect of feeding of ImmunoSEB™ on IgG level of rats.

Description	Group	Day 0 (SOT)	Day 15	Day 30	Day 45	Day 60	Day 75	Day 90 (EOT)
Healthy control	I	34.8	34.3	16.1	152.5	18.3	7.8	31.7
ImmunoSEB™ 50 mg/kg	II	1.7	4.8	25.6	28.7	5.2	24.3	174.3
ImmunoSEB™ 200 mg/kg	III	3.5	34.8	21.7	16.9	10	35.6	38.7
ImmunoSEB™ 400 mg/kg	IV	8.7	13.9	26.9	4	13.5	18.7	23.9
Immunosuppressed control	V	0	26.5	10.9	8.7	16.1	9.1	30
Immunosuppressed + ImmunoSEB™ 50 mg/kg	VI	5.6	14.8	23.5	31.7	10.9	6.1	10.4
Immunosuppressed + ImmunoSEB™ 200 mg/kg	VII	10	4.8	5.2	10	4.9	3	26.9
Immunosuppressed + ImmunoSEB™ 400 mg/kg	VIII	8.3	24.3	17.8	16.9	7.8	14.3	39.5
Immunostimulant (Levamisole treated)	IX	5.6	26.9	9.6	3.9	5.2	8.7	143.9

In contrast, statistically significant elevations in IgG levels were recorded in Groups II, III, and IV, increasing from 1.70 to 174.30 mg/dL, 3.50 to 38.70 mg/dL, and 8.70 to 23.90 mg/dL, respectively, both relative to their baseline values and to the control group. Group V (immunosuppressed control) also showed a significant increase from 0.00 to 30.00 mg/dL. Among immunosuppressed and treated groups, significant elevations were observed in Group VI (5.60 to 10.40 mg/dL), Group VII (10.00 to 26.90 mg/dL), Group VIII (8.30 to 39.50 mg/dL), and Group IX (5.60 to 143.90 mg/dL) compared to their pre-treatment values. Overall, the elevation in IgG levels highlights the

immunostimulant effect of ImmunoSEB™. Notably, the increase in Group II rats was the most pronounced among treatment groups, suggesting that low dose ImmunoSEB™ (50 mg/kg) can effectively stimulate humoral immunity in both normal and immunosuppressed rats.

Serum IgM levels

The IgM profile values for each experimental group showed distinct changes during the study. In the healthy control group, a significant reduction was observed with IgM levels decreasing from 66.3 to 27.3 mg/dL after 90 days. The complete data are presented in Table 5.

Table 5: Effect of feeding of ImmunoSEB™ on IgM level of rats.

Description	Group	Day 0 (SOT)	Day 15	Day 30	Day 45	Day 60	Day 75	Day 90 (EOT)
Healthy control	I	66.3	79.7	70.1	66.6	36.6	32.7	27.3
ImmunoSEB™ 50 mg/kg	II	55.1	78.6	85.8	57	54.2	47.9	39.1
ImmunoSEB™ 200 mg/kg	III	80.2	121.2	69.3	61.4	45.3	89.3	35
ImmunoSEB™ 400 mg/kg	IV	75.1	107.4	88.4	10.9	49.5	56.5	43
Immunosuppressed control	V	78.7	99.8	77.2	44.9	51.2	97.4	45.1
Immunosuppressed + ImmunoSEB™ 50 mg/kg	VI	41.9	123	94.8	52.9	43.6	39.3	43.7
Immunosuppressed + ImmunoSEB™ 200 mg/kg	VII	91.4	97.9	55.5	68.7	42.9	35.8	52.1
Immunosuppressed + ImmunoSEB™ 400 mg/kg	VIII	88.7	121.3	81.4	41.9	85.9	35.6	55.7
Immunostimulant (Levamisole treated)	IX	68.9	83	57	31.3	35.4	41.6	42.7

In Groups II, III, and IV (ImmunoSEB™ treated normal rats), IgM levels fluctuated but ultimately declined significantly from 55.1 to 39.1 mg/dL, 80.2 to 35.0 mg/dL, and 75.1 to 43.0 mg/dL, respectively, compared to their pre-treatment values. Group V also showed a reduction from 78.7 to 45.1 mg/dL, demonstrating the suppressive impact of cyclophosphamide on immune function. Among immunosuppressed and treated groups, Group VI showed a modest elevation (41.9 to 43.7 mg/dL), whereas Groups VII (91.4 to 52.1 mg/dL), VIII (88.7 to 55.7 mg/dL), and IX (Levamisole treated, 68.9 to 42.7 mg/dL) demonstrated significant reductions relative to baseline values. Overall, the data indicate that ImmunoSEB™ supplementation produced limited improvement in IgM

levels, with only Group VI showing a slight recovery. The results suggest that ImmunoSEB™'s immunostimulant effect on IgM was less pronounced compared to its impact on IgG and IgA.

Serum IgA levels

The mean IgA levels of rats in all nine experimental groups are presented in Table 6. In the healthy control group, IgA levels declined from 9.3 to 5.6 mg/dL at the end of the experiment compared to pre-treatment values.

In Groups II, III, and IV, IgA levels showed fluctuations but eventually decreased from 6.8 to 6.1 mg/dL, 9.9 to 5.5 mg/dL, and 11.2 to 11.5 mg/dL, respectively. Group V (immunosuppressed control) also demonstrated a reduction

from 12.5 to 11.9 mg/dL, reflecting the effect of cyclophosphamide induced immunosuppression. Among immunosuppressed and treated groups, Group VI and VIII showed a slight increase (5.1 to 5.8 mg/dL and 6.7 to 8.7 mg/dL, respectively), while Groups VII (11.9 to 9.8 mg/dL) exhibited modest decline compared to its pre-treatment value. Group IX (Levamisole treated) demonstrated an increase from 3.8 to 8.7 mg/dL.

Overall, the data suggest that ImmunoSEB™ supplementation produced variable effects on IgA levels,

with modest recovery in some immunosuppressed groups (VI and VIII) and a notable increase in the Levamisole group. Table 7 provides a comparative overview of changes in immunoglobulin levels (IgG, IgM, and IgA) across the experimental groups, illustrating differences between baseline and post-treatment values. These findings indicate that ImmunoSEB™ has potential to support humoral immunity, though its effect on IgA was less consistent than that observed for IgG.

Table 6: Effect of feeding of ImmunoSEB™ on IgA level of rats.

Description	Group	Day 0 (SOT)	Day 15	Day 30	Day 45	Day 60	Day 75	Day 90 (EOT)
Healthy control	I	9.3	10.5	12.5	14.7	8.1	8.9	5.6
ImmunoSEB™ 50 mg/kg	II	6.8	14.7	23.4	16.4	19.2	16.1	6.1
ImmunoSEB™ 200 mg/kg	III	9.9	19.1	15.9	15.8	8.1	22.9	5.5
ImmunoSEB™ 400 mg/kg	IV	11.2	13.1	23	1.3	11.1	17.6	11.5
Immunosuppressed control	V	12.5	15.9	18.5	16.8	7.3	42.3	11.9
Immunosuppressed + ImmunoSEB™ 50 mg/kg	VI	5.1	23.6	13.9	13.2	8.5	6.9	5.8
Immunosuppressed + ImmunoSEB™ 200 mg/kg	VII	11.9	16.3	12.1	30.7	7.1	3.2	9.8
Immunosuppressed + ImmunoSEB™ 400 mg/kg	VIII	6.7	26.9	9.1	11	14.2	3.8	8.7
Immunostimulant (Levamisole treated)	IX	3.8	14.1	11.4	7.1	4.8	10.5	8.7

Table 7: Effect of feeding of ImmunoSEB™ on serum immunoglobulins level of rats.

Description	Group	IgG			IgM			IgA		
		Day 0 (SOT)	Day 90 (EOT)	Difference	Day 0 (SOT)	Day 90 (EOT)	Difference	Day 0 (SOT)	Day 90 (EOT)	Difference
Healthy control	I	34.8	31.7	-3.1	66.3	27.3	-39	9.3	5.6	-3.7
ImmunoSEB™ 50 mg/kg	II	1.7	174.3	172.6	55.1	39.1	-16	6.8	6.1	-0.7
ImmunoSEB™ 200 mg/kg	III	3.5	38.7	35.2	80.2	35	-45.2	9.9	5.5	-4.4
ImmunoSEB™ 400 mg/kg	IV	8.7	23.9	15.2	75.1	43	-32.1	11.2	11.5	0.3
Immunosuppressed control	V	0	30	30	78.7	45.1	-33.6	12.5	11.9	-0.6
Immunosuppressed + ImmunoSEB™ 50 mg/kg	VI	5.6	10.4	4.8	41.9	43.7	1.8	5.1	5.8	0.7
Immunosuppressed + ImmunoSEB™ 200 mg/kg	VII	10	26.9	16.9	91.4	52.1	-39.3	11.9	9.8	-2.1
Immunosuppressed + ImmunoSEB™ 400 mg/kg	VIII	8.3	39.5	31.2	88.7	55.7	-33	6.7	8.7	2
Immunostimulant (Levamisole treated)	IX	5.6	143.9	138.3	68.9	42.7	-26.2	3.8	8.7	4.9

Microarray analysis

The current study used Rat 5.7k oligonucleotide microarray chips to compare the gene expression profiles between ImmunoSEB™ and Levamisole (reference) fed immunosuppressed and normal rats, with the aim of identifying differentially regulated genes in spleen tissue related to immune response. On the basis of hematological and immunological assessments, the dose group that exhibited the most effective response in terms of leukocyte, lymphocyte, and immunoglobulin modulation was selected for microarray analysis. Accordingly, the 200 mg/kg ImmunoSEB™ group, which demonstrated the most pronounced immunostimulatory effects, was chosen for further gene expression profiling.

Differential Gene Expression in Cyclophosphamide Induced Immunosuppression

The microarray data analysis revealed that immunosuppression of rats with cyclophosphamide resulted in differential regulation of nearly 250 splenic genes. Among these, transcript level of 205 genes got decreased at least 2-folds and 146 genes got upregulated to a similar extent, as compared to non-treated controls. Multiple cytokine and signaling mediators were considerably

reduced, including *Cysteinyl leukotriene receptor 1* (AB052685), which regulates leukotriene driven inflammatory responses, and *Interleukin-15* (NM_013129), a cytokine essential for T-cell and NK-cell activation. Several inflammatory effectors also demonstrated reduced expression, such as *Cytokine-induced neutrophil chemoattractant 2* (D21095), *Galanin* (J03624), and *Cathepsin C* (NM_017097), indicating impaired activation of innate immune cells.

Receptors associated with adaptive immunity were similarly affected. Expression of *Fc receptor, IgG low affinity III* (L08446) and *TNF receptor superfamily member 8/CD30* (NM_019135) was reduced, indicating weakened antibody mediated responses and altered NF-κB signaling. In contrast, cyclophosphamide treatment enhanced the expression of *MHC class II molecules* (AF084934, Y00480) and *Ly-49 stimulatory receptor 3* (AY102036), which may result in compensatory increment of antigen presentation. However, the immune system under cyclophosphamide is presumably still suppressed, since the majority of pro-immune genes, including multiple cytokines and receptors were downregulated.

Taken together, these alteration in gene expression denotes that cyclophosphamide has exerted broad

immunosuppressive effects by effecting cytokine signaling, inflammatory mediators and receptor pathway, while selectively modifying antigen presentation mechanism.

Differential Gene Expression in Immunosuppressed Rats Treated with ImmunoSEB™

The microarray data analysis of ImmunoSEB™ treated group demonstrated reversal of multiple transcriptional changes that were induced by cyclophosphamide, showing results comparable to that of reference immunomodulator Levamisole. Upon treatment of the immunosuppressed rats with Levamisole, expression of 42 of the 146 up regulated genes, were brought down and 149 of the 205 of these down regulated genes went up. While in the ImmunoSEB™ treated rats, expression of 70 of these 146 up regulated genes, were brought down and 68 of the 205 down regulated genes went up resulting in a reversal of cyclophosphamide Immunosuppression. This bidirectional modulation of gene expression suggests that ImmunoSEB™ effectively counteracted cyclophosphamide induced immunosuppression. Table 8 provide an overview of the splenic gene expression changes induced by Levamisole and ImmunoSEB™ treatment.

Marker genes associated with the cytokine signaling and inflammatory responses, including *Cysteinyl Leukotriene receptor 1* (AB052685), which mediates leukotriene driven inflammatory constriction of microvascular smooth muscle; *Interleukin-15* (NM_013129), a cytokine critical for T-cell and NK-cell activation and proliferation; *Cytokine-induced neutrophil chemoattractant 2* (D21095), a chemokine that recruits neutrophils to sites of inflammation; *Galanin* (J03624), a neuropeptide that negatively regulates thymocyte proliferation and modulates immune and endocrine responses; *Lymphotoxin A* (NM_001013144), a macrophage derived cytokine capable of inducing cell death of certain tumor cell lines; and *Cathepsin C* (NM_017097), a lysosomal cysteine protease involved in inflammatory and immune responses, were all downregulated under immunosuppression but returned to normal level after ImmunoSEB™ treatment, parallel to the upregulation observed with Levamisole. Similarly, receptors involved in adaptive immunity and effector function, such as the *Fc receptor, IgG low affinity III* (L08446), which mediates binding of IgG immune complexes, and *TNF receptor superfamily member 8/CD30* (NM_019135), a receptor regulating lymphoblast growth and NF-κB activation, also returned to baseline levels following ImmunoSEB™ administration. This restoration was comparable to the recovery observed with the reference immunomodulator Levamisole. Moreover, B-cell mediated immune genes such as *B-cell receptor associated protein 37* (U7592), involved in B-cell signalling, were not affected by either ImmunoSEB™ or Levamisole, indicating that both agents lack effectiveness in enhancing antibody-mediated

immunity. Collectively, these results specify that ImmunoSEB™ produced immune responses similar to that of Levamisole and have the capability of restoring cytokine and inflammatory responses to baseline, while imparting distinct effects on antigen presentation, suggesting selective tuning of innate immune responses.

Effect of ImmunoSEB™ on Genes Normally Expressed Under Immunosuppression

Microarray profiling revealed that ImmunoSEB™ modulates the expression of genes which remain normally active even under immunosuppressed conditions, thereby exerting immunomodulatory effects as summarized in Table 8. During ImmunoSEB™ supplementation, a significant enhancement of cytokines was observed-with upregulation of genes such as *interleukin 1 alpha* (IL1A) and *interleukin 1 receptor-like 1* (IL1RL1), underlining a shared role in T-cell activation and inflammatory signaling. However, ImmunoSEB™ administration did not induce *interleukin 2 receptor alpha* (IL2RA), *interleukin 2 receptor beta* (IL2RB), or *interleukin 4* (IL4), suggesting a distinct modulation of receptor-mediated signaling, unlike Levamisole.

In contrast, ImmunoSEB™ downregulated two rat MHC class II genes (*Rat MHC RT1 class II E-beta chain mRNA, 3' end*, and *Rat MHC class II RT1.u-D-alpha chain mRNA*) as compared to Levamisole, which had no measurable effect on these genes. This downregulation indicated potential reduction of antigen presentation, thereby balancing its otherwise stimulatory effects.

Microarray analysis showed that ImmunoSEB™ influences the expression of genes which remain normally active even during immunosuppression, producing immunomodulatory effects comparable to those observed under suppressed conditions. A key finding was the upregulation of *interferon-gamma* (IFN-γ), indicating that ImmunoSEB™ acts primarily through induction of IFN-γ, which subsequently activates the NF-κB pathway. This activation is further supported by the modulation of *Lymphocyte antigen 68* (Ly68), which helps in lymphocyte adhesion and homeostatic regulation. Together, these factors coordinate the recruitment and activation of immune cells, defining ImmunoSEB™'s role in immunomodulation.

Safety and Tolerability

Throughout the study, rats in the control group as well as those subjected to different treatment schedules did not exhibit any changes in behaviour or visible adverse reactions. Although mortality was observed in two rats at the highest dose level (400 mg/kg), post-mortem examination did not reveal any drug related pathological changes, indicating that ImmunoSEB™ was generally safe and well tolerated across the tested dose range.

Table 8: Comparison of genes differentially regulated by Levamisole and ImmunoSEB™ in spleen tissue of rats

Gene symbol	Gene name	Gene function	Immune-suppressed condition	Levamisole treatment	ImmunoSEB™ treatment
CYSLTR1	Cysteinyl leukotriene receptor 1	Receptor for cysteinyl leukotrienes mediating constriction of the microvascular smooth muscle during an inflammatory response (Mellor <i>et al.</i> , 2003)	Down	Up	Normal
RT1-B β	Rat MHC RT1 class II E-beta chain	Involved in the presentation of foreign antigens to the immune system (Dlaske <i>et al.</i> , 2009)	Up	Normal	Down
Ly49s3 (Klra3)	Ly-49 stimulatory receptor 3	Receptor for non-classical MHC Class I-encoded target ligands (Dai <i>et al.</i> , 2018)	Up	Normal	Down
Cxcl3 (CINC-2)	Cytokine induced neutrophil chemoattractant 2	Acts as a chemoattractant for neutrophils; involved in the inflammatory response (Wu <i>et al.</i> , 1994)	Down	Up	Normal
GAL	Galanin	Binds G-protein coupled receptors galr1, galr2, and galr3; involved in negative regulation of immature thymocyte proliferation; plays a role in regulation of the immune system (Trejter <i>et al.</i> , 2002)	Down	Up	Normal
FCGR3A	Fc receptor, IgG, low affinity III	Receptor that binds immunoglobulin gamma (Maenaka <i>et al.</i> , 2001)	Down	Up	Normal
IL15	Interleukin 15	Human homolog is a cytokine involved in the regulation of T and natural killer cell activation and proliferation (Wang & Zhao, 2021; Van Acker, 2016)	Down	Up	Normal
CTSC	Cathepsin C	Acts as a cysteine dipeptidyl aminopeptidase; may play a role in immune and inflammatory response (Shen <i>et al.</i> , 2021)	Down	Up	Normal
TNFRSF8 (CD30)	TNF receptor superfamily member 8 (CD30 receptor)	Regulates gene expression through activation of NF-kappa-B (Mir <i>et al.</i> , 2000)	Down	UP	Normal
RT1-D α	Rat MHC class II RT1.u-D-alpha chain	Antigen presentation (Dlaske <i>et al.</i> , 2009)	UP	Normal	Down
LTA	Lymphotoxin A	Cytokine that binds to TNFRSF1A/TNFR1 and TNFRSF1B/TNFR. It is mainly secreted by macrophages and can induce cell death of certain tumor cell lines (Hockendorf <i>et al.</i> , 2025)	Down	Normal	Up
BCAP37	B-cell receptor associated protein 37	B cell signalling (Kraus <i>et al.</i> , 2004)	Up	Down	Down
IL1A	Interleukin 1 alpha	Pro-inflammatory cytokine produced by activated macrophages (Di Paolo <i>et al.</i> , 2009)	Normal	Up	Up
IL1RL1	Interleukin 1 receptor like 1	Involved in helper T-cell function (Xu <i>et al.</i> , 2022)	Normal	Up	Up
IL4	Interleukin 4	Participates in B-cell activation processes as well as of other cell types (Hofman <i>et al.</i> , 1988; Heeb <i>et al.</i> , 2020)	Normal	Up	Normal
IL2RB	Interleukin 2 receptor, beta chain	This beta subunit is involved in receptor mediated endocytosis and transduces the mitogenic signals of IL2 (Doi <i>et al.</i> , 1989)	Normal	Up	Normal
IL2RA	Interleukin 2 receptor, alpha chain	Alpha chain of the interleukin 2 receptor; human homolog homodimerizes to form low affinity receptor (Allouche <i>et al.</i> , 1990)	Normal	Up	Normal
IL13	Interleukin 13	Synergizes with IL2 in regulating interferon-gamma synthesis (Minty <i>et al.</i> , 1993)	Normal	Normal	Up
Ly68	Lymphocyte antigen 68	Play role in innate immune response against pathogens and toxic cell debris (Yu & Liu, 2019)	Normal	Normal	Up
RT1-Db1	Rat MHC RT1 class II E-beta chain mRNA, 3' end	Involved in the presentation of foreign antigens to the immune system (Dlaske <i>et al.</i> , 2009)	Normal	Normal	Down

Discussion

Immunomodulators are the agents that regulate immune system's response against different stimuli. This modulation can take the form of either immunostimulation, enhancing immune activity, or immunosuppression, inhibiting immune reactions. Both phenomena carry important clinical consequences. Immunosuppression has therapeutic applications in organ transplantation and cytotoxic therapy, but it also induces immunodeficiency, responsible for increasing susceptibility to opportunistic infections. In contrast, immunostimulation enhances host defence mechanisms, which induce resistance against invading pathogens. Markedly, depending on dose, a single agent

may have both immunostimulatory and immunosuppressive effects, hence the broader term immunomodulators is used for the agents that modify immune function^[20].

Immunosuppression can result from exposure to halogenated hydrocarbons, certain drugs, pesticide residues, heavy metals, mycotoxins, air pollutants, and some feed additives. In today's era of heightened environmental health hazards, protection against unavoidable immunotoxicants is essential^[21, 22].

ImmunoSEB™ is a novel preparation of naturally derived enzyme blend, formulated from microbial and botanical sources, reported to exhibit immunostimulant properties. The present study reveals that intervention of

ImmunoSEB™ produced significant immunomodulatory effects in both normal and immunosuppressed rats. The substance was well tolerated throughout all dose ranges examined, with non-significant changes in body weight and feed intake. This supports the safety aspect of ImmunoSEB™ for long-term administration.

The increase in total leukocyte count observed in our study revealed a notable trend across most treated groups, particularly at lower dosages, where the increment was approximately three times higher compared to baseline. This finding clearly reflects that ImmunoSEB™ is capable of inducing innate immune system responses even at lower dosage. Moreover, ImmunoSEB™ administration resulted in partial restoration of total leukocyte count in immunosuppressed rats, which is comparable to the effect shown by Levamisole, a known immunomodulator.

Effect of ImmunoSEB™ treatment on leukocyte count from our study are consistent with previous studies conducted for proteolytic enzymes and lactoferrin, which have shown modulation and activation of leukocyte activity. Barth *et al.* [14] reported that bromelain stimulated a significant proliferative response for peripheral blood mononuclear cells (PBMCs). Bromelain treatment was related with increased secretion of macrophage/monocyte associated cytokines and expansion of interferon gamma (IFN-γ) producing CD4 + T-cells. These findings underscores the ability of bromelain to activate macrophages and monocytes, thereby stimulating leukocyte mediated immune responses. The leukocyte elevation observed with ImmunoSEB™ corresponds with these effects, which suggests that proteolytic enzymes within the formulation of ImmunoSEB™ may act via similar mechanisms of leukocyte activation.

Similar results were observed by Zimecki *et al.* [16], who demonstrated that enzyme administration stimulated thymocyte differentiation into CD4 + helper T-cells. Furthermore, enzyme treatment exerted immunomodulatory effects by promoting leukocyte differentiation and functional maturation. In line with these outcomes, De la Rosa *et al.* [23] reported that enzyme treatment was capable of chemoattracting monocytes. Conclusively, enzyme treatment enhanced both innate and adaptive immunity by promoting the maturation of dendritic cells and recruitment of neutrophils and macrophages, indicating that administration of enzyme directly contributes to leukocyte recruitment and activation.

Beyond leukocyte modulation, ImmunoSEB™ exerted a prominent effect on lymphocyte count as well. In normal rats, non-significant changes in lymphocytes were observed, indicating that formulation does not overstimulate these cells under normal conditions. On the other hand, ImmunoSEB™ administration in cyclophosphamide induced immunosuppressed rats showed a substantial increment in lymphocyte counts. This restoration was significant as well as greater than that observed in Levamisole treated rats. The ability of ImmunoSEB™ to restore lymphocyte counts is consistent with earlier reports on enzyme based immunomodulators. Shimizu *et al.* [13] reported that bovine lactoferrin intervention before murine cytomegalovirus infection, protected mice from mortality. This antiviral effect was correlated with significant increase in natural killer (NK) cell activity, which was diminished due to NK cell depletion. Furthermore, the antiviral effect was absent in athymic nude mice but transfer of spleen cells

from enzyme treated donors restored the effect significantly. These findings highlights that T lymphocytes not only facilitate direct adaptive responses but also stimulate NK cell activation. Such cooperative interaction between lymphocytes and innate immune cells suggest a mechanistic basis for the observed restoration of lymphocyte counts with ImmunoSEB™, specifically under immunosuppressed conditions.

Additionally, Shau *et al.* [24] found that enzyme treatment significantly enhanced NK and lymphokine-activated killer (LAK) cell cytotoxicity *in vitro*. This amplification occurred at very low concentration and was independent of iron binding or RNase activity of lactoferrin, suggesting direct modulation of immune system. These findings aligns with those of Engwerda *et al.* [25], who investigated the modulation of lymphocytes from bromelain. Their study reported that bromelain indirectly stimulated T-cell proliferation by the enhancement of costimulatory activity of accessory cells such as B cells and antigen-presenting cells. In contrast, direct inhibition of cytokine signalling was also observed by reduction of IL-2, which indicates the simultaneous enhancement and inhibition of T-cell proliferation, underscoring immunomodulatory effect of bromelain.

Apart from its involvement in regulating leukocyte and lymphocyte numbers, ImmunoSEB™ had a measurable impact on humoral immunity, indicated by the serum immunoglobulin profile. The most significant impact was on IgG levels, where treatment resulted in substantial increase in both normal and immunosuppressed rats. This increase in IgG formation is especially essential because IgG is the main antibody class in systemic circulation and plays a crucial role in long-term immune defence [26]. Moreover, the restoration of IgG levels in immunosuppressed conditions highlights ImmunoSEB™'s ability to restore competence of the adaptive immune response when baseline antibody production is reduced.

The ability of ImmunoSEB™ to enhance IgG formation is consistent with previous reports on enzyme supplementation based immunomodulation. Boyaka *et al.* [27] demonstrated that oral administration of enzyme in mice modulated both mucosal and systemic immune responses, including modulation of serum IgG subclasses. These findings emphasize that enzyme supplementation can influence immune responses toward enhanced antibody production, thereby supporting systemic humoral immunity.

The immunostimulatory action of ImmunoSEB™ in enhancing IgG formation is supported by another study of enzyme supplementation. Debbabi *et al.* [28] reported that oral administration of lactoferrin enhanced both mucosal and systemic immune responses. The immunoglobulin levels of intestinal secretion in lactoferrin fed groups were found much higher than control group. Moreover, IgG and IgA secretion was induced in Peyer's patches and spleen, accompanied by increase lymphocyte production. Such findings provide a mechanistic basis for the observed restoration of IgG levels with ImmunoSEB™, and supports its role in sustaining long-term immune defence.

Regarding IgM, the effect of ImmunoSEB™ supplementation appeared less consistent as compared to IgG. Across most groups, IgM levels showed a downward trend, with the exception of Group VI, which exhibited a modest recovery after the treatment. Considering that IgM is typically the first antibody produced during primary

immune responses [29], these findings suggest that ImmunoSEB™ may not strongly influence early phase humoral activity but instead favours the maturation and class-switching of B cells toward IgG production [30]. This selective stimulation of IgG over IgM, suggest the restoration capability of ImmunoSEB™ towards adaptive immune system.

Furthermore, the impact on IgA levels was variable, with modest increase in certain immunosuppressed groups and marked elevation in the Levamisole-treated cohort. Since IgA is critical for mucosal defence, these results indicate that ImmunoSEB™ may provide partial support for mucosal immunity, though its effect is less consistent compared to IgG enhancement. However, the observed recovery in certain groups suggests that ImmunoSEB™ has the capacity to contribute to broader humoral regulation in dose dependent manner, under immunosuppressed conditions. Additional findings from previous studies also supports the role of oral supplementation in IgA level enhancement. Debbabi *et al.* [28] showed that enzyme supplementation in mice model increased IgA secretion, as a part of mucosal immunity, hence confirming its immunomodulatory effect. Similarly, Boyaka *et al.* [27] demonstrated that Peyer's patch cells from mice administered lactoferrin intra-gastrically were able to produce high levels of IgA after *in vitro* stimulation, underscoring the capacity of lactoferrin to activate mucosal immune pathways.

In addition to the hematological and immunoglobulin responses, the microarray profiling provides mechanistic evidence for the immunomodulatory action of ImmunoSEB™. Cyclophosphamide induced immunosuppression was characterized by downregulation of cytokines, inflammatory mediators, and receptor pathways, confirming the suppression of both innate and adaptive immunity [31]. Markedly, genes such as *Cysteinyl leukotriene receptor 1*, *Interleukin-15*, *Cytokine-induced neutrophil chemoattractant 2*, *Galanin* and *Cathepsin C* were prominently reduced, this reflects impaired activation of T-cells, NK-cells, and neutrophils. This transcriptional suppression aligns with the observed decline in leukocyte and lymphocyte counts, therefore validating the immunosuppressive state.

Cyclophosphamide inhibited essential cytokines, including *Interleukin-15*, *Cysteinyl leukotriene receptor 1*, *Cytokine-induced neutrophil chemoattractant 2*, *Galanin*, and *Cathepsin C*. These genes are important for activating T-cells and NK-cells, causing inflammation through leukotrienes, introducing neutrophils to the site of infection, controlling thymocytes, and lysosomal proteases activity [32, 33, 34, 35]. Their suppression elucidates the compromised innate and adaptive immune responses observed. ImmunoSEB™ supplementation restored their expression, thereby reactivating cytokine signaling and innate immune activation. This reversal aligns with the findings of Sorimachi *et al.* [36], which indicated that lactoferrin stimulates macrophages to secrete TNF- α , IL-8, and nitric oxide. The cytokine release pattern detailed in that study resembles our findings of re-established cytokine gene expression, indicating that ImmunoSEB™ may operate via macrophage activation pathways comparable to lactoferrin. Miyauchi *et al.* [37] provide additional evidence that enzyme treatment enhances neutrophil phagocytic activity. Our data showing the normalization of *Cathepsin C* and *Lymphotoxin A* genes aligns with this, suggesting that ImmunoSEB™

restores neutrophil and macrophage activity [38, 39].

Cyclophosphamide additionally impaired adaptive immunity, as shown by the lower levels of *Fc receptor IgG low affinity III* and *TNF receptor superfamily member 8/CD30* [40]. Supplementing with ImmunoSEB™ brought these genes back to normal, showing that antibody-mediated effector functions and NF- κ B signaling were restored. This restoration is consistent with the findings of Shau *et al.* [24], which demonstrated that enzyme treatment increases the cytotoxicity of NK cells and lymphokine-activated killer (LAK) cells. Additionally, Zimecki *et al.* [16] showed that lactotransferrin helps CD4-CD8-thymocytes mature into helper T-cells. This is consistent with how ImmunoSEB™ supplementation restores *Interleukin-15* signaling, which is important for T-cell differentiation [41].

Microarray profiling also revealed that ImmunoSEB™ selectively modulates genes which remain normally expressed even under immunosuppressed conditions, exerting immunomodulatory effects by altering major pathways. In this context, ImmunoSEB™ downregulated rat MHC class II genes, suggesting a balancing mechanism to prevent excessive antigen presentation [42]. This selective modulation resembles the findings of Coudert *et al.* [17], which indicated that papain treatment elevated cell surface antigen expression in leukemic cells, thus modulating immunogenicity. Our findings indicate that ImmunoSEB™ optimizes antigen presentation by aligning the effects of stimulatory cytokines with controlled antigen exposure. This selective modulation may stop the immune system from becoming over active while keeping it responsive.

Moreover, ImmunoSEB™ increased immune activation by upregulation of *interleukin-1-alpha* (IL1A) and *interleukin-1-receptor-like-1* (IL1RL1) genes. Both of these genes play a direct role in T-cell activation and inflammatory signalling [43]. Accordingly, our findings illustrate that ImmunoSEB™ amplifies inflammatory pathways by coordinating cytokine gene upregulation with T-cell activation. This effect is further supported by Engwerda *et al.* [25], who reported that bromelain supplementation stimulated T-cell receptor and CD28-mediated proliferation, and Shimizu *et al.* [13], who demonstrated that lactoferrin administration augmented NK cell activity in a T-cell-dependent manner. Together, these studies provide evidence that enzyme supplementation can stimulate T-cell activation, thereby supporting the mechanistic link between ImmunoSEB™ administration, increased IL1A and IL1RL1 expression, and enhanced inflammatory responses.

Protease activity is also essential for immune regulation. Wolters *et al.* [15] demonstrated that dipeptidyl peptidase I (DPPI) is crucial for the activation of mast cell chymases, thereby selectively modulating the release of inflammatory mediators. Our observation that ImmunoSEB™ restored *Cathepsin C* genes corresponds to this finding, indicating that protease activation is a crucial mechanism in generating immune response [44]. Complementing this observation, Tenma *et al.* [45] showed that restoring dipeptidyl peptidase 4 (DPP4) boosts immunity by enhancing NK-cell recruitment and cytokine signaling. This is consistent with the observation that ImmunoSEB™ increased IFN- γ expression and NF- κ B pathway genes, thereby promoting NK-cell activation and enhancing immune cell adhesion [46].

Furthermore, ImmunoSEB™ significantly impacted humoral immunity by effectively activating IgG proliferation [47], while showing a partial effect on IgM and a

variable modulation of IgA. This immunoglobulin profile aligns with the findings of Boyaka^[27] and Debbabi^[28], who showed that enzyme-based immunomodulation increases IgG and IgA, whereas IgM is inconsistently influenced. Microarray analysis of ImmunoSEB™ support this by demonstrating that B-cell receptor-associated protein 37 remained unchanged, suggesting that ImmunoSEB™ does not directly augment antibody receptor signalling but rather functions upstream via cytokine cascades^[48].

The microarray analysis collectively shows that ImmunoSEB™ has a broad spectrum of immunomodulatory effect by reactivating suppressed cytokine and receptor pathways, stimulating innate immune mediators, and selectively tuning antigen presentation. ImmunoSEB™ functions as both a restorative agent under immunosuppression and a fine-tuned enhancer of genes that remain normally expressed even under immunosuppressed conditions ensuring balanced immune activation across different physiological states. Its mechanisms appear to converge on lymphocyte activation, cytokine secretion, protease regulation, and NK/T-cell differentiation, ultimately leading to systemic recovery of both cellular and humoral immunity.

Conclusion

The study concludes that ImmunoSEB™, a natural enzyme blend, is a potent and safe immunomodulator that effectively maintains immune equilibrium against environmental stressors. By significantly boosting IgG levels and restoring leukocyte counts in immunosuppressed and normal subjects, the supplementation proved more effective than the clinical standard, Levamisole. Its ability to reverse the suppression of 68 key genes (such as IL-15, Cathepsin C, and Cysteinyl leukotriene receptor 1, etc.) validates its dual role as both immune restorative agent for those with compromised immunity and a fine-tuned enhancer for normal physiology.

Acknowledgments

Authors would like to thank Specialty Enzymes and Probiotics, Chino, USA, for providing the supplement, and Advanced Enzymes Technologies Ltd., Thane for funding the study. The authors would also like to thank Dr. Abhijeet B. Muley and Dr. Tanushree Sarkar for continuous assistance in manuscript writing.

Conflict of interest

AR is a paid employee of Advanced Enzymes Technologies Limited, Thane, India that has a corporate affiliation with Specialty Enzymes and Probiotics, USA. VLR is a chairperson of Advanced Enzymes Technologies Limited. Specialty Enzymes and Probiotics, USA had no role in study design and actual conduct of the study

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