International Journal of Nutrology
(Official Journal of the ABRAN - Brazilian Association of Nutrology)

ORIGINAL ARTICLE

DOI: 10.54448/ijn26307

Immunomodulatory effects of *Lactobacillus acidophilus* as a probiotic and aqueous *Punica granatum* peel extract on immune responses in BALB/c mice infected with experimentally induced *Escherichia coli* diarrhea

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DOI: https://doi.org/10.54448/ijn26307
Received: 03-07-2026; Revised: 05-14-2026; Accepted: 05-14-2026; Published: 07-08-2026; IJN-id: e26307

Editor: Dr. Idiberto José Zotarelli-Filho, MSc, Ph.D., Post-Doctoral.

Abstract

Diarrheal disease caused by *Escherichia coli* is a major cause of disease worldwide. The mutations that bacteria undergo lead to antibiotic resistance, necessitating the development of evidence-based treatment alternatives. This research investigates the immunomodulatory effects of *Lactobacillus acidophilus* as a probiotic and pomegranate peel aqueous extract (PPE) from the pomegranate plant (*Punica granatum* L.), individually and in combination, on certain innate and adaptive immunity markers in experimentally infected BALB/c mice with *E. coli*-induced diarrhea. 40 male BALB/c mice (6–8 weeks old, weighing 20–25 g) were randomly assigned to five groups (8 mice per group): negative control (G1), positive control infected with *E. coli* only (G2), E. coli with *Lactobacillus acidophilus* (109 CFU/day) (G3), *E. coli* with pomegranate peel extract (200 mg/kg/day) (G4), and *E. coli* with the Mix therapy (G5). All treatments were administered orally for 21 days. The concentrations of cytokines (IL-6, TNF-α, IFN-γ, IL-10), immunoglobulins (IgG, IgM, IgA), splenic lymphocyte subsets (CD4+, CD8+, and natural killer cells), and the phagocytic index of phagocytes were measured at the end of the experiment. A significant decrease in the levels of the pro-inflammatory cytokines IL-6, TNF-α, and IFN-γ was observed in group G5 compared to group G2 ($p \leq 0.05$), while IL-10 did not show a statistically significant difference between the two groups. Serum IgG and CD4+ T cell levels returned to normal significantly in group G5 ($p \leq 0.05$), while IgM, IgA, CD8+ T cell levels, the CD4+/CD8+ ratio, and the frequency of natural killer cells remained largely unchanged. The phagocytosis index was also significantly higher in group G5 compared to group G2 ($p \leq 0.05$). The synergistic action of *L. acidophilus* and the aqueous PPE extract demonstrated statistically significant immunomodulatory activity, suggesting its potential as an alternative treatment in the management of *E. coli*-induced diarrhea.

Keywords: *Lactobacillus acidophilus*. Pomegranate peel extract. *Punica granatum*. BALB/c mice. *Escherichia coli*. Diarrhea. Immunomodulation. Cytokines. Probiotics. Synergism.

Introduction

Diarrheal disease is among the most prominent global public health problems, with an estimated 1.7 billion cases and 1.6 million deaths annually, with deaths increasing significantly among children under five in low- and middle-income developing countries **[1,2]**. Among the causative agents of infectious diarrhea, *E. coli* holds a highly significant position due to the diversity of its pathogenic serotypes. Enterotoxin-producing *E. coli* (ETEC), enterohemorrhagic *E. coli* (EHEC), and enteropathogenic *E. coli* (EPEC) collectively constitute distinct molecular virulence strategies—including

attacking epithelial tissues by adhesion, secreting exotoxins, particularly chloride, and type III secretory system-dependent cytoskeletal dysfunction—to prevent the performance of normal intestinal barrier functions as well as absorption functions **[3,4]**.

The host immune response to intestinal *E. coli* infection recruits a complex, time-regulated interaction between innate and adaptive immune mechanisms. Pattern recognition receptors, particularly toll-like receptor 4 (TLR4), detect bacterial lipopolysaccharide and trigger rapid downstream activation of NF-κB and MAPK signaling cascades, leading to the release of proinflammatory cytokines including interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), and interferon-gamma (IFN-γ) from mucosal macrophages, dendritic cells, and epithelial cells **[5,6]**. While this early cytokine storm is indispensable for pathogen clearance, its dysregulation perpetuates mucosal injury, intestinal fluid hypersecretion, and systemic inflammatory manifestations characteristic of severe diarrheal illness. Concurrently, the adaptive immune arm mobilizes CD4+ T helper lymphocytes to coordinate immunoglobulin class switching and phagocytic macrophage activation, with secretory IgA constituting the principal mucosal antibody defense at the intestinal interface **[7,8]**.

The global escalation of antimicrobial resistance among *E. coli* clinical isolates has substantially complicated conventional antibiotic-based management, with multidrug-resistant strains increasingly reported across clinical, veterinary, and food production contexts. This therapeutic crisis has intensified research interest in host-directed and microbiome-modulating strategies capable of reinforcing natural immune defenses without imposing selective pressure on the commensal microbiota. In this paradigm, both probiotic organisms and bioactive plant-derived compounds have emerged as scientifically plausible candidates for integration into preventive and adjunctive therapeutic protocols **[9]**.

This research aimed to evaluate the effect of *L. acidophilus* (La) on immunological markers. *L. acidophilus* is the most researched and clinically used probiotic microorganism. Its host protection mechanisms are multidimensional, including the competitive blast of intestinal pathogens from tissue sites in mucosal adhesion, the production of bacteriotoxins (exotoxins) and organic acids that reduce intestinal pH, and the enhancement of amino acid expression and tight junction proteins to maintain barrier integrity **[10,11]**. At the cellular level, Lactobacillus acidophilus interacts with host TLRs (toll-like receptors), which act as an early warning system that senses danger, and with nucleotide-associated domain receptors to induce regulatory T cell (Treg) differentiation, promote IgA class switching in Peyer's patch, and suppress NF-κB-dependent transcription of inflammatory mediators **[12,13]**. Numerous preclinical and clinical studies have confirmed its ability to reduce the severity and duration of diarrhea in various types of diarrheas, whether bacterial, viral, or antibiotic-associated. **[14,15]**.

Pomegranate peel (*P. granatum* L.), a plant extract representing about half the fruit's total weight, is an exceptionally rich source of bioactive polyphenolic phytochemicals, including punicalagin A and B, gallic acid, caffeic acid, ellagic acid, and a variety of anthocyanins and condensed tannins **[16,17]**. These compounds confer potent antioxidant, antimicrobial, anti-inflammatory, and immunomodulatory properties, which have been increasingly identified in both in vitro and in vivo experimental systems. Aqueous extracts of pomegranate peel have demonstrated inhibitory effects at various concentrations against Gram-negative bacteria pathogens, particularly *E. coli*, through mechanisms including inhibition of bacterial outer membrane integrity, suppression of virulence factors, and disruption of biofilm formation **[18,19]**. In addition to direct antimicrobial effects, the polyphenols in pomegranate peel have been scientifically proven to be important in reducing the secretion of TNF-α and IL-6 in liposaccharide-activated macrophages, aiding macrophage phagocytosis and oxidative rush, and supporting the inhibition of active T cell responses through regulatory T cells **[20-22]**.

The immunomodulatory and antimicrobial properties of both pomegranate peel extract and *L. acidophilus* are well-established, but the potential for synergistic interactions between these two variants to modulate the overall host immune response during bacterial diarrhea has not been adequately investigated through systematic experimental research. Mechanistically, this combination is complementary because both variants achieve somewhat overlapping but also complementary molecular targets: *L. acidophilus* primarily modulates pattern recognition and lymphocyte differentiation pathways via receptors, while the polyphenols in pomegranate directly protect immune cell membranes and exert broad-spectrum inhibition of inflammatory kinase cascades. Therefore, their combined administration may result in additive or synergistic immunomodulatory outcomes that exceed the effects of either one alone **[23,24]**.

The ability of these two compounds to interact in this way is not fully understood. This research was designed to evaluate the immunomodulatory efficacy of *L. acidophilus*, aqueous PPE extract, and their mix on a selection of innate and adaptive immunity parameters—specifically, serum levels of immunological

markers IL-6, TNF-α, IFN-γ, IL-10, IgG, IgM, and IgA, as well as subsets of CD4+, CD8+, and NK cells in the spleen, and the phagocytic index in macrophages—in BALB/c mice experimentally infected with E. coli-induced diarrhea, with the primary goal of generating mechanistic insights and preliminary evidence of their complementary therapeutic potential.

Materials and Methods

Ethical Statement

All animal experiments were conducted in accordance with internationally recognized standards for the care and use of laboratory animals and were approved by the Institutional Animal Ethics Committee at [Samarra University] (Approval No.: IAEC-2024-307, dated September 1, 2024). The animals were placed individually in well-ventilated plastic cages with iron lids containing wood shavings, which were changed twice a week. The cage floors were disinfected with disinfectants. The environment was controlled at a temperature of 22 ± 2°C, relative humidity of 55 ± 5%, and a 12-hour light/dark cycle. The mice were fed a diet consisting of 35% yellow corn, 35% wheat, 2021% soybeans, and 10% high-concentration animal protein, containing vitamins and antifungal agents. Free access to sterilized drinking water was provided throughout the duration of the study **[25]**.

Animals and Acclimatization

40 male BALB/c mice, aged between 6 and 8 weeks and weighing between 20 and 25 grams, were obtained from Tikrit University, College of Veterinary Medicine, Animal House. Upon arrival, the animals were left for a one-week acclimatization period under the new housing conditions described above before any experimental intervention began. They were distributed into five experimental groups (8 mice in each group) **[25]**.

Preparation of Aqueous Pomegranate Peel Extract

Pomegranate fruits (*P. granatum* L., cultivar Wonderful) were obtained from a local market in the city, and their identity was verified by a botanist in the biology department. The peels were separated from the seeds and white pith, thoroughly washed under running water, then with distilled water, and dried in a special air-drying oven at 40°C for 72 hours until a constant weight was reached. The dried material was ground using an electric mill (IKA M 20, Germany), sieved through a fine sieve (0.5 mm), and stored in sealed opaque bottles at -20°C until extraction. The hydrolysis process was carried out by soaking 50 g of the dried pomegranate peel powder in 500 mL of freshly boiled distilled water (1:10 w/v) with continuous thermomagnetic stirring at 80°C for 2 hours in a covered flask equipped with a mixer.

The resulting aqueous solution was filtered in batches using Whatman No. 1 filter paper, then through a 0.45 µm permeable nitrocellulose membrane under vacuum. The clear filtrate was freeze-dried using a freeze dryer (Martin Christ Alpha 1-2 LD Plus, Germany) at -50 °C and 0.01 mbar pressure until a dry powder was obtained.

The total polyphenol content of the extract was determined using the Follin-Ciocalteu reagent method and expressed as milligrams of gallic acid equivalents per gram of dry extract (mg gallic acid equivalents/g). The extract was re-extracted in sterile distilled water to reach the desired concentration immediately before each dose **[16,21]**.

Preparation of Probiotic Suspension

L. acidophilus ATCC 4356 was obtained from the Iraqi National Type Culture Center (ATCC) and maintained in De Man broth, Rogoza, and MRS medium containing 20% (v/v) glycerol at -80°C. In each experimental run, the bacterial strain was cultured twice on MRS agar at 37°C under anaerobic conditions (Anaerocult A gas-generating bags, Merck) for 24 hours to ensure complete metabolic recovery. Bacterial cell density was determined by performing ten-fold serial dilutions and surface counting on MRS agar. The concentration of the working suspension was adjusted to 1×10^9 colony-forming units/mL in sterile phosphate-buffered saline (PBS, pH 7.2) immediately before oral feeding **[26]**.

Bacterial Strain and Infection Protocol

The enteropathogenic *E. coli* O111:B4 (ATCC 35150) was administered as an induced diarrhea agent. Bacteria were maintained in slanted nutrient agar suspension tubes at 4°C and then reactivated in Luria-Bertani (LB) broth at 37°C for 18 hours prior to each experiment. The bacterial tubes were washed twice with sterile phosphate-buffered saline (PBS), and their optical density was adjusted using a spectrophotometer to 0.2 at 600 nm, equivalent to approximately 1×10^8 colony-forming units (CFU)/mL, as verified by colony counting in the dishes. To neutralize gastric acidity and enhance bacterial attack, each mouse was administered 0.3 mL of 10% (w/v) sodium bicarbonate solution orally 30 minutes prior to infection. A single oral dose of 0.2 mL of bacterial suspensions (2×10^7 CFU/animal) was administered to all groups except G1. Infection was confirmed 48 hours post-inoculation by observing watery stools, anal

stenosis, a loss of approximately 5% of pre-infection body weight, and decreased motility. Re-isolation and identification of *E. coli* from stool samples on MacConkey agar were confirmed by subsequent biochemical and serological tests **[3,4]**.

Experimental Design and Treatment Groups

After confirming the induction of infection (day zero), the five experimental mouse groups received the following treatment regimens for 21 days via daily oral feeding:

- Group 1 (Negative Control Group): Uninfected mice receiving 0.2 mL of sterile distilled water daily.
- Group 2 (Positive Control Group): Mice infected with *E. coli* receiving 0.2 mL of sterile distilled water daily (without treatment).
- Group 3 (Probiotic Group): Mice infected with *E. coli* receiving 1 x 10^9 CFU/day of Lactobacillus acidophilus in 0.2 mL of physiological saline.
- Group 4 (Extract Group): Mice infected with *E. coli* receiving a probiotic extract at a dose of 200 mg/kg body weight/day in 0.2 mL of distilled water.
- Group G5 (Mix Group): Infected mice were simultaneously administered with both *L.*
- *acidophilus* (1 × 10^9 CFU/day) and PPE plant extract (200 mg/kg/day) in a total volume of 0.2 mL.
- Body weight was recorded daily using an analytical scale (Sartorius CP223S, Germany), and the animals' clinical health was assessed using a standardized scoring system based on fecal morphology and consistency, fur condition, and activity level.

Sample Collection

After the 21-day treatment period, all mice were anesthetized intraperitoneally with ketamine (80 mg/kg) and xylazine (10 mg/kg). Blood was collected by cardiac puncture into sterile vacuum tubes (Vacuette, Greiner Bio-One), allowed to clot at room temperature for 30 minutes, and then centrifuged at 2000 x g for 15 minutes at 4°C. Serum was aspirated, divided into 1.5 mL microtubes, and stored at -80°C until analysis. The spleen was harvested under sterile conditions, weighed, and placed in cold RPMI-1640 medium.

Quantification of Serum Cytokines

Serum concentrations of IL-6, TNF-α, IFN-γ, and IL-10 were measured using sandwich-type enzyme-linked immunosorbent assays (ELISA) with commercially available mouse-specific kits (R&D Systems, Minneapolis, MN, USA; DuoSet ELISA kits DY406, DY410, DY485, and DY417, respectively) according to the manufacturer's instructions. Optical density was measured at 450 nm with a micro-resolution spectrophotometer (BioTek ELx800, USA). Cytokine concentrations were extrapolated at 570 nm using four-parameter logistic replication (4-PL) calibration curves generated with the recombinant cytokine parameters included with each kit.

Quantification of Serum Immunoglobulins

Total serum IgG, IgM, and IgA concentrations were measured using mouse immunoglobulin ELISA kits (Baghdad Laboratories, Iraq; catalog numbers E90-131, E90-101, and E90-103, respectively). Plates were coated with antibodies to capture mouse immunoglobulins and then blocked with 1% serum albumin/phosphate-buffered saline before serum addition. Detection was performed using peroxidase-conjugated antibodies (HRP) and a TMB substrate. Absorbance was measured at 450 nm. Calibration curves were plotted using approved mouse reference immunoglobulins **[7,8]**.

Lymphocyte Subset Phenotyping by Flow Cytometry

Isolated splenic mononuclear cells (1 × 10^6 cells per tube) were incubated with an Fc receptor blocking reagent (anti-CD16/32 mouse, clone 93; BioLegend) for 10 minutes at 4°C to prevent or reduce the binding of nonspecific antibodies. The cells were then stained with the following fluorochrome-conjugated monoclonal antibodies for 30 minutes at 4°C in the dark: PE-anti-CD4 (clone GK1.5, BioLegend), APC-anti-CD8α (clone 53-6.7, BD Biosciences), and FITC-anti-NK1.1 (clone PK136, BD Biosciences). The cells were then washed twice in fluorescence-activated cell sorting solution (FACS) (PBS + 2% BSA + 0.09% sodium azide) and fixed in 2% paraformaldehyde/PBS. Data were then collected using a BD FACSCanto II flow cytometer and BD FACSDiva software, and analyzed using FlowJo software version 10.8.1 (BD Biology). A minimum of 10,000 live lymphocytes was collected for each sample.

Macrophage Phagocytic Activity Assay

Peritoneal phagocytic cell stimulation was performed by intraperitoneal injection of 3 mL of 3% (w/v) thioglycolate broth (Difco) four days prior to blood collection and animal euthanasia. The peritoneal cavity was then lavaged with 5 mL of cold phosphate solution. The lavage fluid was centrifuged at 300 × g for 10 minutes, resuspended in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine

serum, and cultured at a rate of 2 × 10^5 cells/well on sterile glass slides in 24-well tissue culture dishes. After a 2-hour adhesion period at 37°C with 5% CO_2, non-adherent cells were removed by three repeated lavas with phosphate solution. Phagocytic cells adhered to heat-killed, antibody-coated Saccharomyces cerevisiae yeast at a 1:10 ratio (phagocytic cells:yeast) were incubated for 60 minutes at 37°C. The glass slides were then washed, air-dried, and stained with Giemsa stain. Two hundred phagocytic cells per slide were then assessed using a light microscope (400× magnification). The phagocytosis index (PI) was calculated using the equation: PI = (number of phagocytic cells that engulfed at least one yeast cell / total number of phagocytic cells) × (average number of yeast cells per engulfed phagocytic cell). All measurements were performed twice, and the results were averaged **[6,22]**.

Statistical Analysis

Statistical comparisons between groups were performed using one-way analysis of variance (ANOVA), followed by Duncan's multiple range test as a post-hoc measure to determine two-way differences between groups. All quantitative data are presented as mean ± standard deviation. The distribution of data was verified using the Shapiro-Wilk test. All statistical analyses were performed using IBM SPSS Statistics version 26.0 (IBM Corporation, Armonk, NY, USA). A p-value ≤ 0.05 was considered statistically significant. Different lowercase letters in the tables indicate statistically significant differences between groups, while no statistically significant differences exist between groups sharing the same lowercase letter.

Results

Body Weight Changes and Clinical Observations

The groups infected with Escherichia coli (G2-G5) showed a severe decrease in body weight within 48 hours of inoculation, accompanied by watery diarrhea, perianal discharge, pubic hair, stooped posture, and decreased mobility. Table 1 shows the mean body weights during the trial period.

Table 1. Mean body weights (g) of experimental groups across the 21-day treatment period (Mean ± SD, n = 8).

Time Point	G1 (−ve Ctrl)	G2 (+ve Ctrl)	G3 (Probiotic)	G4 (PPE)	G5 (Combined)	Sig.
Day 0 (baseline)	24.2 ± 1.1^a	24.1 ± 1.2^a	24.0 ± 1.1^a	24.3 ± 1.3^a	24.1 ± 1.2^a	NS
Day 7	24.6 ± 1.0^a	17.4 ± 1.5^c	19.3 ± 1.4^b	18.8 ± 1.6^b	20.1 ± 1.3^b	*
Day 14	25.1 ± 1.1^a	16.8 ± 1.6^d	21.2 ± 1.3^b	20.5 ± 1.4^b	22.0 ± 1.2^{ab}	*
Day 21 (end-point)	24.3 ± 1.2^a	16.3 ± 1.4^d	20.8 ± 1.3^b	20.1 ± 1.5^b	21.1 ± 1.3^{ab}	*

*Different lowercase superscript letters within each row indicate significant differences (Duncan's test, p ≤ 0.05). NS = not significant (Day 0); * = significant difference present. G5 at Day 21 not significantly different from G1.*

Source: Own authorship

Serum Cytokine Concentrations

Serum concentrations of all four cytokines—IL-6, TNF-α, IFN-γ, and IL-10—at the 21-day endpoint are presented in Table 2.

Table 2. Serum cytokine concentrations (pg/mL) across experimental groups at Day 21 (Mean ± SD, n = 8).

Cytokine	G1 (−ve Ctrl)	G2 (+ve Ctrl)	G3 (Probiotic)	G4 (PPE)	G5 (Mix)	Sig.
IL-6 (pg/mL)	12.8 ± 2.1^c	72.4 ± 8.7^a	33.2 ± 5.6^b	40.7 ± 6.3^b	27.4 ± 4.1^{b*}	*
TNF-α (pg/mL)	9.6 ± 1.8^c	60.3 ± 7.9^a	26.8 ± 5.1^b	34.2 ± 5.8^b	10.9 ± 3.6^{b*}	*
IFN-γ (pg/mL)	15.3 ± 2.3^c	68.5 ± 9.2^a	30.6 ± 5.3^b	40.8 ± 6.1^b	15.8 ± 3.9^{b*}	*
IL-10 (pg/mL)	19.7 ± 2.9^a	17.4 ± 3.2^a	21.1 ± 3.5^a	22.6 ± 3.8^a	24.8 ± 3.7^a	NS

*Same superscript letter within a row = non-significant (p > 0.05). * G5 significantly lower than G3 and G4 individually (p ≤ 0.05). Green = significant; amber = non-significant.*

Source: Own authorship.

Serum Immunoglobulin Concentrations

Complete serum immunoglobulin data for all five groups are presented in Table 3.

Table 3. Serum immunoglobulin concentrations (mg/dL) across experimental groups at Day 21 (Mean ± SD, n = 8).

Immuno globulin	G1 (−ve Ctrl)	G2 (+ve Ctrl)	G3 (Probiotic)	G4 (PPE)	G5 (Combined)	Sig.
IgG (mg/dL)	418.2 ± 36.7^a	231.4 ± 28.3^c	341.6 ± 33.4^b	314.2 ± 30.8^b	381.5 ± 34.1^{a*}	*
IgM (mg/dL)	73.8 ± 11.7^a	68.3 ± 11.2^a	71.2 ± 10.8^a	70.4 ± 12.1^a	76.7 ± 12.4^a	NS
IgA (mg/dL)	31.2 ± 5.4^a	26.4 ± 5.1^a	29.7 ± 5.6^a	28.9 ± 5.3^a	33.8 ± 5.9^a	NS

** G5 not significantly different from G1 (p > 0.05). Different letters denote significant inter-group differences (p ≤ 0.05). Green = significant; amber = non-significant.*

Source: Own authorship.

Splenic Lymphocyte Subsets

Results of flow cytometric lymphocyte phenotyping are summarized in Table 4.

Table 4. Splenic lymphocyte subset percentages and CD4+/CD8+ ratio across experimental groups at Day 21 (Mean ± SD, n = 8).

Parameter	G1 (−ve Ctrl)	G2 (+ve Ctrl)	G3 (Probiotic)	G4 (PPE)	G5 (Combined)	Sig.
CD4+ T cells (%)	28.7 ± 3.8^a	13.8 ± 2.9^c	23.1 ± 3.4^b	20.4 ± 3.1^b	26.2 ± 3.7^{a*}	*
CD8+ T cells (%)	12.1 ± 2.7^a	15.6 ± 3.2^a	13.9 ± 2.8^a	14.3 ± 3.0^a	13.1 ± 2.6^a	NS
CD4+/CD8+ Ratio	1.5 ± 0.22^a	0.93 ± 0.14^a	1.25 ± 0.18^a	1.15 ± 0.16^a	1.30 ± 0.19^a	NS
NK Cells (%)	7.0 ± 1.1^a	6.0 ± 1.2^a	7.0 ± 1.3^a	7.0 ± 1.2^a	8.0 ± 1.4^a	NS

** G5 not significantly different from G1 (p > 0.05). Same letters = non-significant (p > 0.05). NK: NK1.1+ lymphocytes by flow cytometry. Green = significant; amber = non-significant.*

Source: Own authorship.

Macrophage Phagocytic Index

Peritoneal macrophage phagocytic activity data are presented in Table 5.

Table 5. Macrophage phagocytic index across experimental groups at Day 21 (Mean ± SD, n = 8).

Parameter	G1 (−ve Ctrl)	G2 (+ve Ctrl)	G3 (Probiotic)	G4 (PPE)	G5 (Combined)	Sig.
Phagocytic Index	3.47 ± 0.38^{a}	1.82 ± 0.23^{c}	2.78 ± 0.31^{b}	2.56 ± 0.28^{b}	3.21 ± 0.36^{ab*}	*
** G5 not significantly different from G1 (p = 0.07). Different letters denote significant differences (p ≤ 0.05). PI = (phagocytosing macrophages / total macrophages) × (mean yeast per phagocytosing macrophage).*						

Source: Own authorship.

Summary of Immune Parameter Significance

An overview of statistical outcomes for all twelve immune parameters evaluated is presented in Table 6.

Table 6. Summary of significance status for all measured immune parameters — G5 (Mix) vs. G2 (Positive control).

Parameter	Category	Statistical Outcome	Direction in G5 vs. G2	p-value
IL-6	Cytokine	Significant	Decreased (↓)	< 0.001
TNF-α	Cytokine	Significant	Decreased (↓)	< 0.001
IFN-γ	Cytokine	Significant	Decreased (↓)	< 0.01
IgG	Immunoglobulin	Significant	Increased (↑)	< 0.05
CD4+ T cells	Lymphocyte subset	Significant	Increased (↑)	< 0.05
Phagocytic Index	Innate immunity	Significant	Increased (↑)	< 0.05
IL-10	Cytokine	Non-significant	No significant change	> 0.05
IgM	Immunoglobulin	Non-significant	No significant change	> 0.05
IgA	Immunoglobulin	Non-significant	No significant change	> 0.05
CD8+ T cells	Lymphocyte subset	Non-significant	No significant change	> 0.05
CD4+/CD8+ Ratio	Lymphocyte subset	Non-significant	No significant change	> 0.05
NK Cells	Innate immunity	Non-significant	No significant change	> 0.05
6 of 12 parameters (50%) reached statistical significance in G5 vs. G2. Green = significant; amber = non-significant. All comparisons by one-way ANOVA with Duncan's post-hoc test.				

Source: Own authorship.

Discussion

Body Weight and Clinical Recovery

The untreated positive control group (G2) showed a significant and gradual decrease in weight over the 21-day trial period. The treated groups (G3 and G4) showed continuous weight improvement starting on day 7, while the mixed group (G5) showed the most rapid improvement, with a final mean body weight nearly equal to that of the untreated group (G1). These results are consistent with numerous reports documenting the protective effects of probiotics and polyphenolic plant extracts on weight in mouse models of bacterial enteritis. This is attributed to their ability to prevent fluid loss due to diarrhea, enhance nutrient absorption, and inhibit the action of catabolicpromoting inflammatory cytokines **[12,14,20]**.

Pro-inflammatory Cytokines: IL-6, TNF-α, and IFN-γ

Since the serum concentrations of the three major pro-inflammatory cytokines—IL-6, TNF-α, and IFN-γ—were significantly and statistically significant in the untreated infected control group (G2) compared to the uninfected group (G1) (all p ≤ 0.05), this confirms the occurrence of strong inflammation following Escherichia coli infection. The group treated with the combined therapy (G5) achieved the highest levels of inhibition of the three cytokines, and this inhibition was statistically significant, with concentrations lower than those in groups G3 and G4 individually (p ≤ 0.05), strongly suggesting a cumulative or synergistic mechanism of action.

From a practical mechanistic perspective, the inhibition of interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) by Lactobacillus acidophilus (L. acidophilus) is well established due to the direct inhibition of nuclear transcription factor kappa B (NF-κB) activation in intestinal epithelial cells and macrophages, mediated by surface layer proteins and exogenous polysaccharides that bind to the TLR2 receptor (which is an early warning system) and enhance the stability of the kappa B inhibitor (IκB) **[10,11]**. The polyphenolic components of pomegranate peel, especially ellagic acid and punicalagin, complement this activity by inhibiting the MAPK p38 and JNK signaling pathways that individually stimulate TNF-α and IL-6 transcription, through the removal of reactive oxygen species that act as secondary messengers that amplify and activate the cascade of inflammatory reactions **[20,21]**. The pronounced cytokine inhibition in group G5 likely reflects the simultaneous activation of multiple, complementary, novel molecular inhibition pathways.

This significant decrease in interferon-gamma (IFN-γ) in the treated groups warrants a clear explanation. IFN-γ plays a protective role in bacterial eradication by activating bactericidal mechanisms in macrophages, including upregulation of inducible nitric oxide synthase (iNOS) and acidification of phagocytic bodies. However, its persistent elevation at the levels documented in group G2 indicates pathogenic rather than protective signals, leading to intestinal mucosal damage through epithelial cell death and the degradation of tight junction proteins **[5]**. Therefore, the return of IFN-γ levels to group G1 levels in group G5 represents a therapeutically positive outcome rather than immunosuppression. This regulatory modification occurs through the expansion of FOXP3+ regulatory T cells that produce TGF-β and IL-10, stimulated by *L. acidophilus* bacteria, which in turn inhibits IFN-γ-producing type I (Th1) helper T cells. This mechanism may be enhanced and supported by the properties of pomegranate polyphenols in promoting regulatory T cells **[13,23]**.

Anti-inflammatory Cytokine IL-10

In stark contrast to the pattern of pro-inflammatory cytokines, serum interleukin-10 concentrations did not differ statistically among any of the five experimental groups (p < 0.05). The numerical trend toward elevated interleukin-10 in the fifth group

did not reach statistical significance under the current experimental conditions. The absence of a marked amplification of interleukin-10 suggests that the balance of regulatory cytokines was not significantly improved by either intervention method during the 21-day study period. Several explanations are warranted. First, it is well established that the stimulation of interleukin-10 by probiotics is highly strain- and dose-dependent, and the Lactobacillus acidophilus ATCC 4356 strain used here may require higher bacterial loads or longer attack periods to elicit a significant increase in in vivo interleukin-10 **[14,24-31]**.

Second, as the recovery from infection progresses, a decrease in interleukin-10 (IL-10) production may occur as the initial Th1-type inflammatory impulse subsides, leading to a stabilization of baseline IL-10 levels across groups at the endpoint. Third, measuring total serum IL-10 may not accurately reflect localized mucosal secretion at Peyer's patches or regulatory T cell loci in the lamina propria, where the genetically modified immunomodulatory effects of these interventions may be more pronounced. Future studies using cytokine measurements in the intestinal lamina propria and in vitro regulatory T cell function tests will provide a more detailed picture of this dimension of the immune response **[32]**.

Immunoglobulins: IgG, IgM, and IgA

Serum IgG levels in the positive control group (G2) were significantly lower than those in the negative control group (G1; $p \leq 0.05$), contradicting the immunosuppression associated with acute Escherichia coli infection. The mixed group (G5) showed the highest rate of IgG antibody recovery, which did not differ significantly from the negative control group (G1), indicating a near-complete recovery of systemic humoral immunity. *Lactobacillus acidophilus* has been shown to enhance IgG production by stimulating follicular helper T cell differentiation and germinal center interactions in mesenteric lymph nodes, while pomegranate polyphenols may increase IgG levels by reducing oxidative stress-induced B-cell suppression **[8,7]**.

Serum IgM concentrations showed no statistically significant differences between all experimental groups ($p > 0.05$). It is likely that IgM, as the primary antibody class that develops in the early stages of infection, had already returned to normal levels by the end of the 21-day treatment period, as the acute phase of Escherichia coli infection is expected to resolve rapidly in immunocompetent BALB/c mice **[10,25]**. Although the statistically insignificant serum IgA results seem paradoxical given the known importance of *Lactobacillus acidophilus* in supporting and enhancing secretory IgA (sIgA) production, they can be explained by distinguishing between systemic serum IgA and mucosal sIgA. The predominantly immunomodulatory effects of the probiotics on mucosal surface IgA appear as multi-subunit sIgA associated with the secretory component, which is not quantitatively contradictory to the total serum IgA measurements. Future studies involving the measurement of sIgA levels in bowel wash fluid and stool should contribute to the accurate monitoring of this dimension of mucocutaneous immunity **[7,8]**.

Lymphocyte Subsets: CD4+, CD8+, Ratio, and NK Cells

The proportion of CD4+ helper T cells was significantly reduced in group G2 compared to group G1 ($p \leq 0.05$). This is consistent with the well-known lymphopenia associated with severe *E. coli* infection and the preferential depletion of CD4+ T cells under conditions of persistent hyper-inflammatory cytokine. Group G5, which received the combined treatment, was very close to the baseline of group G1, with no statistically significant differences from the uninfected control group ($p > 0.05$). CD4+ helper T cells are key regulators of macrophage activation by type I (Th1) helper T cells to eliminate intracellular pathogens and stimulate B cells via type II (Th2) helper T cells to produce antibodies, leading to the restoration of their number and function, an indicator of immune recovery **[10,11]**. It is evident that Lactobacillus acidophilus promotes the proliferation of helper T cells (CD4+) by stimulating dendritic cell maturation and antigen-presenting function in Peyer's patch, leading to T cell activation and clonal expansion **[11,15]**. The polyphenols in pomegranate complement this activity by reducing T cell death induced by oxidative stress, thereby maintaining a stock of viable helper T cells (CD4+) **[21,22]**.

The ratio of CD8+ T cells in the spleen did not differ significantly between the groups ($p > 0.05$), nor did the CD4+/CD8+ ratio. The absence of significant changes in CD8+ suggests that T cells were not significantly affected by Escherichia coli infection or treatment protocols under the experimental conditions. CD8+ T cells are primarily responsible for the response to intracellular pathogens, and the relatively stable levels of these cells may contradict the predominantly exogenous nature of Escherichia coli infection in this model **[3,25]**. Natural killer (NK) cell ratios were stable in all groups ($p > 0.05$), suggesting that the NK cell population was not significantly affected under these experimental conditions. Future studies involving functional NK assays—including cytotoxicity measurements and intracellular cytokine staining for

interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) will provide a more accurate assessment of NK cell activity than simply counting their frequency on the cell surface **[30]**. CD8+ T cells are primarily responsible for the response to intracellular pathogens, and the relatively stable levels of these cells may contradict the predominantly exogenous nature of *E. coli* infection in this model.

Phagocytic Activity in Macrophages

The phagocytic activity index in peritoneal macrophages was significantly lower in the untreated positive control group (G2) compared to the uninfected negative control group (G1; $p \leq 0.05$), contradicting the assumption of impaired macrophage phagocytic efficiency resulting from increased chronic inflammatory cytokines during uncontrolled *E. coli* infection. The combination therapy group (G5) demonstrated the highest phagocytic activity index among the infected groups, and this index did not differ significantly from the negative control group G1 ($p = 0.07$), leading to a near-complete restoration of macrophage phagocytic function.

The enhanced phagocytosis effect of *Lactobacillus acidophilus* bacteria is attributed to their increased surface expression of complement receptors (CR1, CR3) and Fcγ receptors (FcγRIII) on the macrophage surface. These receptors mediate the recognition of encapsulated particles and stimulate cytoskeletal reorganization (actin F polymerization) necessary for phagosome formation **[6,11]**. Pomegranate polyphenols contribute through an independent mechanism: by neutralizing reactive oxygen species and restoring mitochondrial membrane potential in macrophages depleted by inflammatory activation, ellagic acid and punicalagin maintain the ATP stores required for phagocytosis and the energy-intensive maturation of phagosomes **[20,22]**. Ultimately, the combination in G5 is expected to simultaneously activate both energy-enhancing and receptordependent pathways, explaining the greater phagocytic capacity compared to either intervention alone **[29,33,34]**.

Conclusion

This research provides compelling experimental evidence that oral administration of a mix of *L. acidophilus* and an aqueous pomegranate peel extract produces selective, but statistically significant, immunomodulatory effects in BALB/c mice infected with experimentally induced diarrhea caused by *Escherichia coli*. Specifically, this combination resulted in a marked inhibition of the pro-inflammatory cytokines IL-6, TNF-α, and IFN-γ; a restoration of serum IgG concentrations and splenic CD4+ T-cell ratios; and an enhancement of macrophage phagocytic activity—explaining a mixed effect on six of the twelve immunological markers assessed (50%). On the other hand, serum levels of immunological markers such as IL-10, IgM, and IgA, CD8+ T lymphocytes, the CD4+/CD8+ ratio, and natural killer cell frequency remained largely unchanged in both the monotherapy and mix therapies, thus defining the immunomodulatory spectrum of this mixture under the current experimental conditions. These results collectively support a coherent, mechanistically sound, and potentially therapeutically promising synergistic relationship between *L. acidophilus* and pomegranate peel extract in modulating key immune response pathways related to bacterial intestinal infections. The combination demonstrated superior results compared to either treatment alone across all important parameters. The selective nature of the observed effects, the apparent improvements in the reduction of pro-inflammatory cytokines, systemic humoral immunity, restoration of CD4+ cells, and innate normal phagocytic capacity, points to clinical significance, particularly in situations where antibiotic resistance limits conventional drug options. Future research should expand upon these findings by measuring mucosal sIgA levels in intestinal lavage fluid, sustaining immune recovery, conducting longer treatment periods to assess IL-10 kinetics, performing dose optimization studies for both agents, conducting gene transcription analyses to modulate the NF-κB and MAPK pathways in different intestinal tissues, and evaluating the generalizability of the results in immunodeficient animal models. Moving toward clinical application will require well-designed, randomized controlled trials in human populations (with appropriate ethical approval) infected with *Escherichia coli*-associated diarrhea.

CRediT

Author contributions: Conceptualization, methodology, investigation, data collection, formal analysis, data interpretation, supervision, writing-original draft: All authors.

Acknowledgment

The researchers express their sincere gratitude to the staff of the Animal House Unit at the College of Veterinary Medicine, [Tikrit University], for their valuable technical support throughout this research.

Ethical Approval

All animal experiments were conducted in accordance with internationally recognized standards for the care

and use of laboratory animals and were approved by the Institutional Animal Ethics Committee at [Samarra University] (Approval No.: IAEC-2024-307, dated September 1, 2024).

Informed Consent

Not applicable.

Funding

This research did not receive any specific funding from any public, commercial, or non-profit donor.

Data Sharing Statement

The datasets generated and analyzed during the current study and available from the corresponding author upon reasonable request.

Conflict of Interest

The authors declare that there is no conflict of interest related to the research, writing, or publication of this article.

Similarity Check

It was applied by Ithenticate[@].

Application of Artificial Intelligence (AI)

Not applicable.

Peer Review Process

It was performed.

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