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Original article

EFFECTIVENESS OF HORMONE-PROBIOTIC THERAPY IN MITIGATING POST-OVARIOHYSTERECTOMY INFLAMMATION IN FEMALE NEW ZEALAND WHITE RABBITS

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Summary

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Ovariohysterectomy (OVH) causes important hormonal changes, especially lack of estrogen, which can trigger systemic and chronic inflammation. This study expored how OVH affected inflammatory cytokines –interleukin-1β (IL-1β), interleukin-6 (IL-6), tumour necrosis factor-alpha (TNF-α) and C-reactive protein (CRP) in female New Zealand White rabbits. It also assessed the effects of combined estradiol valerate and probiotic therapy as a new post-OVH treatment. Fifty adult females were randomly allocated into five groups: Group 1 – sham-operated control; Group 2 – OVH with combined estradiol-probiotic therapy for 2 months; Group 3 – OVH with estradiol-probiotic therapy for 4 months; Group 4 – OVH without treatment, assessed after 2 months; Group 5 – OVH without treatment, assessed after 4 months. Quantitative analysis revealed that OVH induced substantial elevations in all inflammatory markers in untreated groups. IL-1β increased from 118.9 pg/mL in controls to 138.1 and 162.9 pg/mL after 2 and 4 months without treatment, respectively. IL-6 rose from 122.6 to 143.7 and 174.3 pg/mL, TNF-α from 303.0 to 364.2 and 400.5 pg/mL, and CRP showed the most pronounced rise, from 2.9 mg/L to 16.7 and 47.6 mg/L. In contrast, combined estradiol-probiotic therapy markedly attenuated these elevations, reducing IL-1β to 103.2 and 96.6 pg/mL , IL-6 to 111.4 and 87.1 pg/mL, TNF-α to 280.3 and 176.5 pg/mL, and CRP to 5.1 and 3.7 mg/L, restoring values close to control levels. These findings showed that OVH induced time-dependent immune dysregulation while integrative hormone-probiotic therapy mitigated effectively inflammatory responses. The study presents a new therapeutic approach since synergistic interaction between estrogen and probiotics has not been sufficiently documented in scientific literature. Based on these findings, the combined therapeutic regimen demonstrated a synergistic effect, effectively reducing the inflammatory response and restoring immune homeostasis, and therefore may be a basis for developing optimised post-ovariectomy strategies aimed at restoring immune balance by mitigating chronic inflammation, with potential clinical relevance for patients' management after ovariectomy.

Key words: C-reactive protein, immune modulation, hormone replacement therapy, IL-1β, IL-6, inflammatory cytokines, ovariohysterectomy, probiotics, TNF-α

INTRODUCTION

Estrogen is a major player in the maintenance of immune homeostasis by controlling both pro- and anti-inflammatory pathways that influence immune, metabolic, and neurological functions throughout the female lifespan (Chakraborty *et al*., 2023). Ovariectomy disturbs this hormonal-immune balance since estrogen deficiency results in persistent elevated levels of inflammatory cytokines such as IL-1β, IL-6, and TNF-α indicative of an ongoing systemic inflammatory condition (Collins *et al*., 2017; Wang *et al*., 2025). These chronic changes in immunity have been linked to increased risk for metabolic dysregulation, cardiovascular disease, and impaired tissue repair (Jiang *et al.,* 2021; Le *et al*., 2021). After the ovariohysterectomy (OVH), immune changes become clear through a trend of increased pro-inflammatory cytokines along with reduced anti-inflammatory responses, enabling the activation of several inflammatory signaling pathways. IL-1β promotes prostaglandin production and early inflammatory signaling; IL-6 affects leukocyte differentiation and liver acute-phase responses including CRP production; TNF-α enhances neutrophil recruitment as well as vascular activation (Ng *et al*., 2018; Kumar *et al*., 2025; Li *et al*., 2025). Increased cytokines also activate the hypothalamic-pituitary-adrenal (HPA) axis further changing neurotransmitter balance after OVH while estrogen replacement has been found to reduce these inflammatory and neuroendocrine derangements (Ng *et al*., 2018). The gastrointestinal tract is home to trillions of microbes known as gut microbiota, which form a complex symbiotic system and play crucial roles in host protection, metabolism, and overall health regulation (Vieira *et al*., 2017). Diseases associated with sex hormones are among the top health issues with recent studies indicating a rise in roles played by such microbiota in managing hormone levels through a complex network of microbe-host interactions metabolite synthesis inflammation management, and microbial homeostasis restoration (Dalmolin *et al*., 2024). Various hormone-associated disorders have been linked to gut dysbiosis, highlighting the growing interest in probiotics as modulatory agents capable of reshaping the gut microbiome and influencing hormonal regulation (Jain *et al*., 2024). Probiotics also exert anti-inflammatory effects by lowering acute-phase markers like C-reactive protein (CRP) and improving systemic inflammatory status (Waśkiewicz *et al.* ,2025; Galli,2023), which further validates their potentiality in modulating hormone-related pathways. Besides that, the capacity of probiotics to affect immune signalling pathways plus complement-mediated inflammatory pathways increases their regulatory effect on metabolic as well as hormonal balance (Nikolouzakis *et al*., 2025).

Most recent epidemiological data further indicate the hormonal effect of the gut microbiota. As an example, a study of data obtained in the National Health and Nutrition Examination Survey (NHANES 2013-2016) (2699 women were included in the study), 537 of whom declared to consume probiotics in the form of yogurt or supplements, showed that the intake of probiotics was positively correlated with the levels of estradiol (E2) in premenopausal women and had a negative correlation with total testosterone (TT) levels in postmenopausal women. These results indicate that probiotics can be an effective approach to sex hormone balance and possibility to decrease the incidence of hormone-related diseases (Zou *et al*., 2023).

Growing evidence indicates that probiotics exert protective immunomodulatory effects relevant to estrogen-deficiency states. Probiotic strains such as *Lactobacillus plantarum*, *L. reuteri*, *Bifidobacterium lactis* and *B. longum* have demonstrated the ability to enhance macrophage function, promote T-cell activity, support intestinal barrier integrity, and reduce systemic inflammatory markers including IL-6, TNF-α, and CRP (Chung *et al.,* 2017; Mazziotta *et al.,* 2023; Jouriani *et al*., 2025). In estrogen-deficient models, probiotics improve gut microbiota composition, augment estrogen-like metabolites, and mitigate inflammation-related metabolic impairments such as insulin resistance and altered lipid metabolism (You *et al*., 2022; Du and Ying, 2025). These findings highlight the potential of probiotics as an adjuvant therapeutic strategy to counteract immune dysregulation following OVH.

C-reactive protein (CRP) is an acute-phase reactant from the liver induced by IL-6 which is commonly used as a biomarker for systemic inflammation and a predictor of cardiovascular, autoimmune, and infectious conditions (Bedi, 2024; Waśkiewicz *et al*., 2025). Its increase after OVH shows the increased inflammatory load related to estrogen deficiency. Because CRP integrates multiple upstream inflammatory cascades, it is a robust indicator for assessing postoperative immune changes.

The New Zealand White (NZW) rabbits are a suitable experimental model for OVH-related inflammatory studies due to its well-characterised reproductive physiology, sensitivity to estrogen fluctuations (Rahmiati *et al*., 2025), and immune responses that closely resemble those observed in humans undergoing surgical menopause (Brandão & Ozawa, 2024). Moreover, rabbits provide reliable plasma and CSF sampling volumes suitable for serial biomarker quantification (Osborne, 2020).

Our working hypothesis is that OVH causes a prolonged pro-inflammatory response with increases in IL-1b, IL-6, TNF-a, and CRP, and that a combination of estrogen and probiotic treatment will reduce these effects and restore the state of immune homeostasis. The proposed study will determine the systemic inflammatory response to OVH in female New Zealand White rabbits by the measurement of circulating levels of IL-1 b, IL-6, TNF-a, and CRP; as well as the efficacy of estrogen replacement therapy and probiotic supplementation in reversing the inflammatory response to OVH.

MATERIALS AND METHODS

Animals, housing, surgical procedures

The Institutional Animal Ethics Committee at the College of Veterinary Medicine, University of Mosul, approved this study (Ref. No. UM.VET.2025.001). Fifty apparently healthy adult female New Zealand White rabbits were purchased from the local market at Kogjali, Mosul for this experimental study. The animals were declared fit for experimentation by a veterinarian and were subjected to ultrasound scanning to ensure that the uteri were free of embryos before starting the experiment.

Rabbits were kept in home-made cages with dimensions 150 cm length × 75 cm width × 65 cm height. The animals were randomly divided into five groups with ten rabbits in each group. The environmental conditions were maintained at ambient temperature between 26 and 29 °C with a light/dark cycle of 14 hours per day. Cages were well-ventilated and coarse sawdust was provided on the floor to absorb moisture. The rabbits were acclimatised to housing conditions and diet for 1 month before the start of experimental procedures. All animals received equal standardised amounts of pelleted feed, while clean water was provided *ad libitum* in non-leaking containers. Fresh green fodder was supplied daily during treatment.

Ovariohysterectomy (removal of both ovaries and uterus) was performed through midline laparotomy under strict aseptic conditions. Animals were fasted for 8 hours before surgery but allowed free access to water up until then. General anaesthesia was induced by intramuscular administration of xylazine (5 mg/kg) and ketamine (35 mg/kg). After achieving an adequate level of anaesthesia, each rabbit was positioned in dorsal recumbency on a warmed surgical table. The abdomen was shaved and disinfected with povidone-iodine followed by alcohol, then opened through a 4–5 cm incision along the linea alba. The uterine horns and ovaries were located and gently brought out. The ovarian blood supply was secured by ligation with absorbable Vicryl sutures, after which the ovaries were removed. Either the body or cervix of the uterus was ligated and cut off, thus completing excision of reproductive organs. Closure of abdominal incision took place in three layers using absorbable sutures; hence there was no need for suture removal thereafter. Post-operative care included subcutaneous administration of enrofloxacin (10 mg/kg) and meloxicam (0.2 mg/kg). All rabbits recovered uneventfully during the follow-up period.

Medications and treatments

- Estradiol valerate (10 mg/mL), produced by JHP Pharmaceuticals, USA, was administered intramuscularly at a dose of 0.1 mg/kg/day to ovariohysterectomised rabbits (Juan *et al.,* 2008).
- Healthy Gut Probiotics (Equa Holistics, LLC, USA) 2.5 mg/mL solution was prepared by dissolving 1 gram of probiotic powder in 400 mL of distilled water (Tian *et al.,* 2025). The solution was administered orally at a dose of 1 mL/kg/day.

The probiotic preparation used in this study contained a mixture of beneficial bacterial strains and yeast, including *Propionibacterium freudenreichii*, *Propionibacterium shermanii*, *Enterococcus thermophilus*, *Lactococcus lactis*, *Lactobacillus casei*, *Bifidobacterium longum*, *Pediococcus acidilactici*, *Pediococcus pentosaceus*, *Bifidobacterium bifidum*, *Bifidobacterium animalis*, *Lactobacillus brevis*, *Lactobacillus fermentum*, *Lactobacillus acidophilus*, *Lactobacillus plantarum*, and active dry yeast, along with rice extract and inulin as prebiotic substrates. The mixed probiotic formulation was standardised to a concentration of 1×10^9 CFU/g.

Experimental design and treatment protocol

Following a 10-day post-surgical recovery period, the 50 female New Zealand White rabbits were randomly assigned into five equal groups (10 animals each) as follows:

- Group 1: Sham surgery (surgical control): experienced incision of the abdomen and without removing the uterus or the ovaries removed. There was no provisional or probiotic therapy.
- Group 2: Ovariohysterectomy + 2 months treatment: total excision of uterus and ovaries and daily treatment with hormone (estradiol valerate 10 mg/mL at a dose of 0.1 mg/kg body

weight, injected intramuscularly (IM) daily for two months) and probiotic: HealthyGut Probiotics (2.5 mg/mL) at 1 mL/kg body weight orally. Length of treatment: 2 months in a row.

- Group 3: Ovariohysterectomy + 4 months treatment: identical surgical incision and postoperative regimen as for Group 2, with duration of 4 months.
- Group 4: Ovariohysterectomy untreated (follow-up 2 months): only excision of the uterus and ovaries and nothing more. A period of 2 months was allowed to animals.
- Group 5: Ovariohysterectomy untreated (follow-up 4 months): identical to Group 4, with period of observation 4 months.

Blood plasma sampling and assay of cytokines and CRP

Blood samples from the control group were collected after the sixth day of postoperative recovery, whereas blood samples from the other groups were collected after two and four months, respectively, for the hormone-treated, probiotic-treated, and untreated groups. Blood samples were obtained from the cephalic vein of the forelimb using a sterile cannula. The animals were manually and gently restrained to minimise stress and ensure stability during sampling. A tourniquet was not used, as the vein could be easily visualised through gentle warming or light massage of the area. The cannula was directly inserted into the vein, and an appropriate volume of blood was drawn using a sterile syringe. Samples were immediately transferred into tubes containing the anticoagulant EDTA to prevent clotting. Following collection, the tubes were kept on ice for a short period, then centrifuged at 3000 rpm for 15 minutes at 4°C to separate the plasma layer. The supernatant plasma was carefully transferred to sterile polypropylene tubes and stored at −80°C until biochemical analyses were performed.

Circulating serum concentrations of pro-inflammatory cytokines – interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α), C-reactive protein (CRP) were determined using a sandwich enzyme-linked immunosorbent assay (ELISA) designed for the quantitative measurement of these biomarkers in rabbit serum according to the manufacturer's instructions (Elabscience, USA). ELISA assays were performed using kits from SunLong Biotech company, specifically manufactured for rabbits, with the following catalogue numbers: CRP (SL0061Rb), IL-1β (SL0112Rb), IL-6 (SL0117Rb), and TNF-α (SL0217Rb). Blood samples were collected and centrifuged at 3000 rpm for 15 min to obtain serum, which was then stored at −80 °C until analysis. All measurements were performed in duplicate to ensure accuracy. The concentrations of IL-1β, IL-6, and TNF-α were expressed in pg/mL , while CRP levels were expressed in mg/L.

Statistical analysis

Statistical analysis was conducted using one-way ANOVA followed by Duncan's multiple range test for group mean comparison. Data are presented as mean ± SD, with statistical significance at $P \leq 0.05$.

RESULTS

The results demonstrated a clear effect of ovariohysterectomy on the serum concentrations of inflammatory cytokines (IL-1β, IL-6, TNF-α) and C-reactive protein (CRP) in female New Zealand White rabbits, with significant differences observed between the different groups ($P \leq 0.05$).

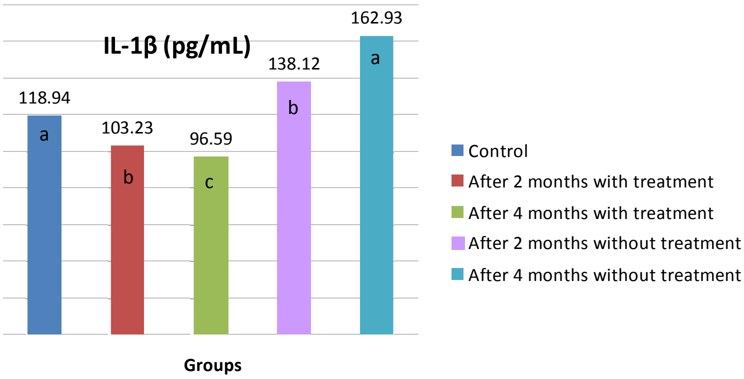

Fig. 1. Effects of ovariohysterectomy and estradiol–probiotic therapy on IL-1β levels (mean ±SD, n=10) across experimental groups. Different letters in the columns indicate significant differences among means according to Duncan's test.

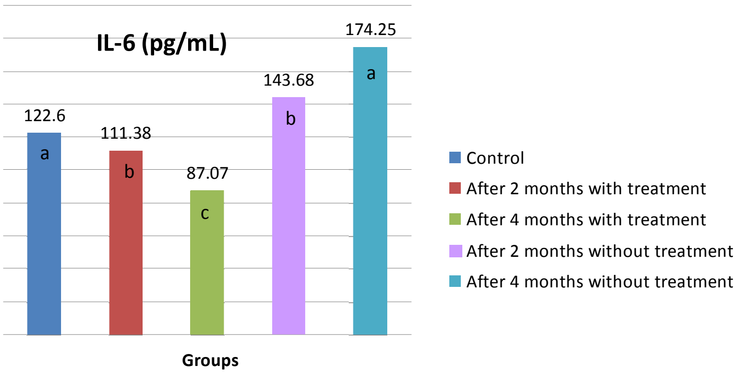

Fig. 2. Effects of ovariohysterectomy and estradiol–probiotic therapy on IL-6 levels (mean ±SD, n=10) across experimental groups. Different letters in the columns indicate significant differences among means according to Duncan's test.

IL-1β (Fig. 1) decreased significantly in the treated groups after two and four months (103.23±22.91 and 96.59±2.65 pg/mL, respectively) compared to the control group – 118.94±37.03 pg/mL. In contrast, it increased markedly in the untreated groups, reaching 138.12±0.03 and 162.93±58.40 pg/mL after two and four months. A similar pattern was observed for IL-6 (Fig. 2) – an average level of 122.60±11.23 pg/mL in the control group, reduced concentrations in the treated groups to 111.38±46.06 and 87.07±6.54 pg/mL and elevated values in the untreated groups to 143.68±35.81 and 174.25±88.12 pg/mL after two and four months, respectively.

The concentration of TNF-α was 303.00±60.17 pg/mL in the control group (Fig. 3). In the treated groups, TNF-α decreased to 280.31±38.20 and 176.49±26.51 pg/mL after two and four months, respectively. In untreated groups, it was significantly higher with levels of 364.23±66.12 and 400.50±82.23 pg/mL after similar periods of time. As for CRP, it slightly increased after two months 5.1±0.08 mg/L and then decreased after four months 3.7±1.2 mg/L vs control group concentrations (2.9±0.24 mg/L) (Fig. 4). In contrast, CRP was dramatically higher in the untreated groups, reaching 16.7±6.02 after two and 47.6± 4.64 mg/L after four months.

These results confirm that ovariohysterectomy induces a pronounced immune-inflammatory response, reflected by elevated inflammatory cytokines and CRP levels, while subsequent hormone therapy combined with probiotics mitigates these effects and maintains values at lower levels compared to the untreated groups.

Fig. 5 illustrates the temporal changes in IL-1β, IL-6, TNF-α, and CRP and the relationship between inflammatory markers, time, and treatment. Visual analysis reveals a synchronised trend among these markers; while levels progressively increased in untreated groups from two to four months post-ovariohysterectomy, the combined estrogen-probiotic therapy effectively suppressed this rise, maintaining levels near baseline. This parallel progression indicates a coordinated systemic in-

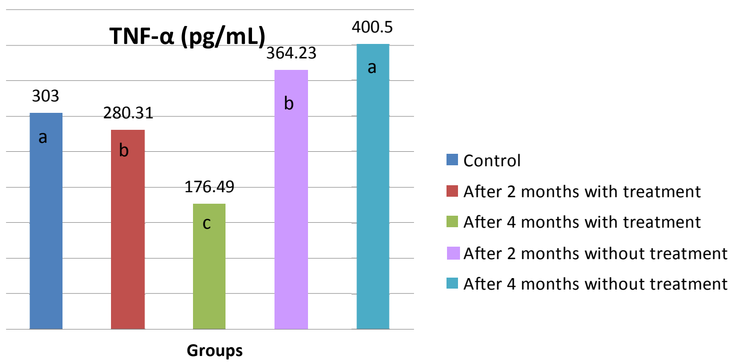

Fig. 3. Effects of ovariohysterectomy and estradiol–probiotic therapy on TNF-α levels (mean ±SD, n=10) across experimental groups. Different letters in the columns indicate significant differences among means according to Duncan's test.

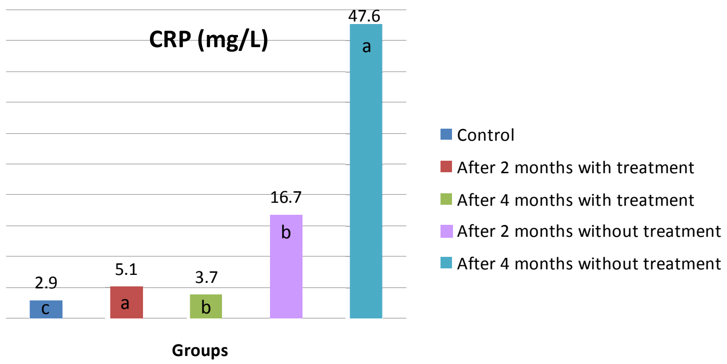

Fig. 4. Effects of ovariohysterectomy and estradiol–probiotic therapy on CRP levels (mean ±SD, n=10) across experimental groups. Different letters in the columns indicate significant differences among means according to Duncan's test.

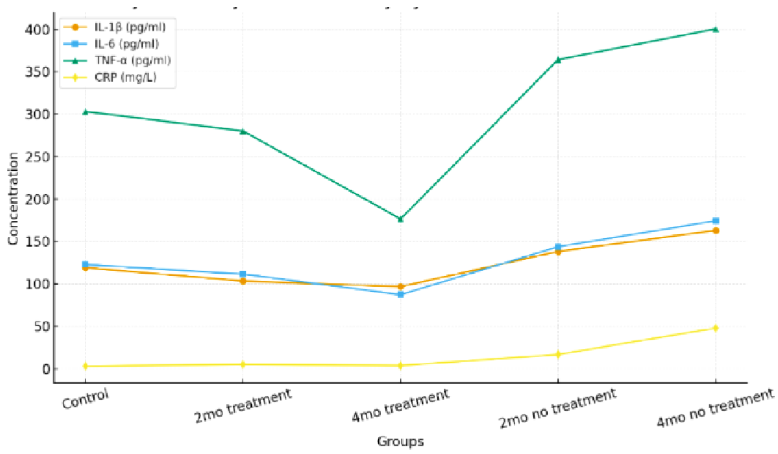

Fig. 5. Effects of ovariohysterectomy and subsequent hormone and probiotic treatment on inflammatory markers and their correlation with time in female New Zealand White rabbits.

flammatory response to both hormonal loss and therapeutic intervention. The integrated inflammatory response was significantly influenced by the duration of therapeutic intervention and whether or not treatment was applied. Concentrations for both IL-1β and IL-6 had significant decreases after two months of treatment which continued after four months while TNF-α showed more pronounced reduction at four months of treatment. On the other hand, all measured cytokines – IL-1β, IL-6 as well as TNF-α in untreated rabbits increased progressively. CRP reached the highest levels after four months without treatment highlighting critically time and absence of therapeutic intervention in escalation of the inflammatory response.

DISCUSSION

Inflammation is a basic physiological response to protect and repair tissues after an injury, usually resolving when the damaging factor is removed (Jia *et al.*, 2021). However, if inflammation persists or becomes chronic, it can be pathological. It has been implicated in the pathogenesis of cardiovascular diseases, neurodegenerative diseases, and autoimmune diseases. Physiological inflammation induced by ovariohysterectomy (OVH) is necessary for wound healing. The sudden estrogen deficiency that accompanies OVH may exacerbate inflammatory reactions and delay recovery (Moore *et al.*, 2024).

Neutrophils, monocytes, and macrophages produce pro-inflammatory cytokines such as IL-1β and IL-6, TNF-α, acute-phase proteins like CRP in order to coordinate the immune response and manage tissue repair. Estrogen through ERα and ERβ receptors plays a critical role in regulating these responses by inhibiting phagocyte-induced inflammation, controlling cytokine and chemokine production, as well as promoting the resolution phase of inflammation (Li *et al.*, 2025; Lin *et al.*, 2025). Following OVH, the loss of estrogen-mediated control deregulates these pathways resulting in elevated inflammatory markers and increased vulnerability to immune dysregulation (Suszczyk *et al*., 2024; Tian *et al*., 2024). This also applies to mucosal immunity where estrogen downregulates Toll-like receptor signaling along with immune mediators such as S100A8 allowing maintenance of tissue homeostasis within dynamic microbial environments.

Our results align with these mechanistic insights. OVH significantly raised levels of IL-1β, IL-6, TNF-α, and CRP at both two- and four-months post-surgery in untreated rabbits with maximum levels seen at four months. This increase over time indicates a cumulative effect of prolonged estrogen deficiency on systemic inflammation. On the other hand, though estrogen replacement therapy plus probiotics normalised cytokine levels completely reductions started already at two months further improving by four months more pronounced for TNF-α at four months of combined therapy indicating an enhancement over time in anti-inflammatory effects.

Restoration of inflammatory homeostasis can be attributed to multiple estrogen-mediated mechanisms: modulation of both innate and adaptive immune responses, balancing Th1/Th2 activity, and suppression of Toll-like receptor signaling (Hoffmann *et al*., 2023; Bartkowiak-Wieczorek *et al.,* 2024). Probiotics possibly enhanced these effects via gut microbiota regulation, intestinal barrier strengthening, and interaction with immune receptors (TLRs and NLRs) modulating systemic inflammation (Cross *et al*., 2023; Silva *et al*., 2024). Therefore, the synergistic action of estrogen plus probiotics may provide a robust approach to mitigating OVH-induced immune dysregulation.

The presented results have wider physiological implications and translational significance. Increased post-OVH cytokines may indicate not only acute inflammation related to surgical trauma but also the possibility of chronic inflammatory diseases if hormonal deficits persist. The restoration of cytokine balance via combined therapy stresses the significance of early intervention after estrogen loss to avoid long-term immune and metabolic dysregulation, as seen in postmenopausal women and animal models (Umur *et al*., 2024; Vicariotto *et al*., 2024).

The observed data further reveal a synchronised relationship among IL-1β, IL-6, TNF-α, and CRP, indicating an integrated inflammatory network that was significantly influenced by the treatment and the time elapsed. This emphasises that both the duration of therapeutic interventions and their presence are critical in determining the trajectory of inflammatory responses. The pronounced suppression of all measured cytokines by the combined estrogen–probiotic therapy supports its potential to restore immune homeostasis more effectively than either intervention alone.

This study has shown the effect of OVH on the rise in immune-inflammatory markers and the combined estradiol–probiotic therapy as a mitigating agent, however, has some limitations. The study did not include a probiotic-only group, which makes it difficult to determine the independent effect of probiotics compared to their synergistic interaction with estradiol. The sample size was limited to five groups of ten rabbits each; hence, statistical power is reduced and generalisability becomes an issue. Therapy protocols were not tested at different doses or timings and measurements were only done at specific time points (2 and 4 months) with no long-term continuous monitoring of changes in inflammatory markers.

Future studies should be larger randomised controlled trials with standardised therapy protocols and extended follow-up periods for long-term changes to be assessed. Studies on interactions between the gut–brain axis and hormones would help in clarifying biological mechanisms that underlie synergistic effects between estradiol and probiotics. Safety and cost-effectiveness should also be evaluated for clinical application so that better management of chronic inflammation after OVH in rabbits can be opened up to other species.

CONCLUSIONS

The current study results confirm that ovariohysterectomy induces a strong immunoinflammatory reaction in female New Zealand White rabbits, as indicated by increased serum levels of IL-1β, IL-6, TNF-α, and CRP. OVH-induced estrogen deficiency tilts the balance between pro- and anti-inflammatory signals toward a progressive increase in systemic cytokines. These results highlight the need to target both hormonal and microbial regulatory pathways in postoperative strategies for inflammation control after surgical menopause with potential applications in optimising post-surgical care not only in animal models but possibly human clinical settings too.

Combined estradiol valerate and probiotic therapy effectively opposes this trend by restoring cytokine levels toward baseline, exhibiting a synergistic immunomodulatory effect. The synergistic action of estrogen and probiotics presents an interesting strategy for treating post-operative immune dysregulation that can be translated into practice for postmenopausal women receiving ovariohysterectomy.

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