

Effect of gut microbiota modulation with probiotics on inflammatory markers in the letrozole-induced polycystic ovary syndrome rat model

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Abstract

Objective: Polycystic ovary syndrome (PCOS) is a common endocrine disorder associated with chronic low-grade inflammation. This study aimed to evaluate whether probiotic supplementation (Lactobacillus and Bifidobacterium) could restore serum interleukin-10 (IL-10) and tumour necrosis factor-alpha (TNF-α) levels in a letrozole-induced PCOS rat model, compared with metformin.

Methods: Twenty-eight female albino Wistar rats were randomly divided into four groups (n=7 each): control (normal diet), PCOS model (letrozole 1 mg/kg plus high-fat diet for 21 days), metformin (300 mg/kg/day for 28 days after induction), and probiotic (1×10⁹ CFU/day of Lactobacillus and Bifidobacterium for 28 days after induction). Serum IL-10 was measured by ELISA, and TNF-α by CLIA. Data were analysed using one-way ANOVA with Bonferroni post-hoc test.

Results: PCOS induction significantly decreased IL-10 by 32.98% (p<0.001) and increased TNF-α by 46.33% (p<0.001) compared with controls. Probiotic treatment significantly raised IL-10 by 27.51% (p<0.001) relative to the model group, but IL-10 remained 14.54% lower than in controls (p=0.003). Probiotics reduced TNF-α by only 4.69% (p=1.000), which was not statistically significant. Metformin increased IL-10 by 36.88% (p<0.001) and reduced TNF-α by 7.71% (p=1.000) compared with the model group.

Conclusion: Probiotic supplementation partially improves the anti-inflammatory IL-10 but does not significantly reduce TNF-α in this PCOS model. Probiotics alone are insufficient to reverse the inflammatory profile of PCOS fully.

Keywords: Polycystic ovary syndrome, Letrozole, Probiotics, Metformin, Inflammation

Plain English Summary

Polycystic ovary syndrome (PCOS) is a common hormone disorder that affects women of childbearing age and often leads to body-wide inflammation. In this study, we tested whether taking probiotics ("good" bacteria) could reduce inflammation in rats with PCOS. We gave rats letrozole and a high-fat diet to create a PCOS-like condition. Then, we treated some rats with probiotics (Lactobacillus and Bifidobacterium) for 28 days, while others received metformin (a standard diabetes medicine) or nothing. We measured two inflammation-related proteins in the blood: IL-10, which fights inflammation, and TNF-α, which promotes it. The PCOS rats had much less IL-10 and much more TNF-α than healthy rats. Probiotics increased IL-10 levels significantly, but not enough to return them to normal. Probiotics did not meaningfully reduce TNF-α. Metformin worked similarly. Our findings suggest that taking

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probiotics alone can help improve some aspects of inflammation in PCOS, but it is not a complete solution. Future research should explore the combination of probiotics with other treatments.

Introduction

Polycystic ovary syndrome (PCOS) is a prevalent endocrine disorder that affects approximately 4–21% of women of reproductive age, depending on the diagnostic criteria used (1). The syndrome is characterised by hyperandrogenism, ovulatory dysfunction, and polycystic ovarian morphology, and it is associated with long-term metabolic complications including type 2 diabetes, cardiovascular disease, and infertility (2). The pathophysiology of PCOS involves complex neuroendocrine, hormonal, and metabolic disturbances. A key feature is an increased luteinising hormone (LH) to follicle-stimulating hormone (FSH) ratio, driven by heightened gonadotrophin-releasing hormone (GnRH) pulsatility, which in turn stimulates excess androgen production and insulin resistance (3). Beyond reproductive and metabolic abnormalities, PCOS is now recognised as a state of chronic low-grade inflammation (4). Women with PCOS have reduced serum levels of the anti-inflammatory cytokine interleukin-10 (IL-10) and elevated levels of pro-inflammatory cytokines such as tumour necrosis factor- α (TNF- α) (5). This inflammatory milieu contributes to insulin resistance, atherogenesis, and the progression of other PCOS-related comorbidities.

Metformin, an oral biguanide primarily used for type 2 diabetes, is commonly prescribed off-label for PCOS. It improves insulin sensitivity, reduces hepatic gluconeogenesis, and enhances peripheral glucose uptake. Beyond its metabolic effects, metformin has been shown to modulate the gut microbiota by increasing beneficial bacteria such as *Lactobacillus* and *Bifidobacterium*, thereby strengthening the intestinal barrier and reducing systemic inflammation (6).

The gut microbiota comprises trillions of microorganisms that play essential roles in maintaining host health. These microbes produce short-chain fatty acids (SCFAs) such as acetate and propionate, which nourish colonocytes, reinforce the intestinal barrier, and exert anti-inflammatory effects (7). Dysbiosis – an imbalance in gut microbial composition – leads to a loss of SCFA-producing bacteria (including *Lactobacillus* and *Bifidobacterium*) and an overgrowth of pathogenic species such as *Clostridium difficile* and *Klebsiella*. This dysbiosis increases intestinal permeability, allowing bacterial toxins and antigens to enter the bloodstream and perpetuate systemic inflammation (8). In PCOS, gut dysbiosis has

been implicated as both a consequence and a driver of chronic inflammation (9).

Given the emerging role of the gut–ovary axis, probiotic supplementation has been proposed as a potential adjunctive therapy for PCOS. However, evidence from animal models remains limited. We hypothesised that restoring gut microbial balance with a probiotic mixture of *Lactobacillus* and *Bifidobacterium* would reduce systemic inflammation in a letrozole-induced PCOS rat model. The objective of this study was to evaluate the effects of this probiotic intervention on serum IL-10 and TNF- α levels, using metformin as a comparator.

Materials and Methods

Animals and housing

Twenty-eight adult female albino Wistar rats weighing 150–200 g were obtained from the animal facility of the University of Babylon. The rats were housed in standard polypropylene cages (four per cage) under controlled environmental conditions: temperature $22\pm 2^{\circ}\text{C}$, relative humidity $55\pm 5\%$, and a 12-hour light/dark cycle. They had free access to standard chow and tap water. All animals were acclimatised for seven days before the start of the experiment.

Study design and groups

After acclimatisation, the rats were randomly assigned to four experimental groups ($n=7$ per group) using a computer-generated random number sequence. Group allocation was concealed from the investigators responsible for outcome assessment (blinding).

Control group

Rats received a normal diet throughout the experiment and were given distilled water orally as a vehicle.

PCOS model group: PCOS was induced by daily oral administration of letrozole (1 mg/kg body weight) dissolved in 2% dimethyl sulfoxide (DMSO) via a gavage tube, combined with a high-fat diet (45% energy as fat, 20% as protein, 35% as carbohydrate; 4.73 kcal/g, Research Diets, New Brunswick, USA) for 21 days (10). After induction, they were switched back to a normal diet for the remaining 28 days but received no further treatment.

Metformin group

PCOS was induced as described above. After the 21-day induction period, rats received metformin (300 mg/kg/day, dissolved in distilled water) orally by gavage for 28 days while being maintained on a normal diet.

Probiotic group

PCOS was induced as described above. After induction, rats received a daily oral dose of a probiotic mixture containing 1×10^9 colony-forming units (CFU) of *Lactobacillus* and *Bifidobacterium* strains (Medwise, United Kingdom) suspended in distilled water for 28 days.

All treatments were administered between 9:00 and 10:00 AM.

Confirmation of PCOS induction

To confirm the success of PCOS induction, vaginal smears were collected daily from all rats during the induction period. A sterile cotton swab moistened with normal saline was gently inserted into the vagina, and the smear was spread onto a clean glass slide. The smear was stained with methylene blue and examined under a light microscope ($\times 400$ magnification) to determine the stage of the oestrous cycle (11). The presence of persistent dioestrus (predominance of leukocytes) or a prolonged, irregular cycle was considered indicative of PCOS-like features.

Blood collection and serum preparation

At the end of the 28-day treatment period, all rats were anaesthetised with diethyl ether. Blood samples (approximately 3–5 mL per rat) were collected via cardiac puncture using a 21-gauge needle. The blood was allowed to clot at room temperature for 30 minutes, then centrifuged at 3,000 rpm for 10 minutes. The serum supernatant was carefully aspirated, aliquoted into Eppendorf tubes, and stored at -20°C until biochemical analysis.

Measurement of IL-10 and TNF- α

Serum IL-10 levels were quantified using a commercially available rat IL-10 enzyme-linked immunosorbent assay (ELISA) kit (Elabscience, China) following the manufacturer's instructions. The assay sensitivity was 1.0 pg/mL, and the intra-assay and inter-assay coefficients of variation were $<8\%$ and $<10\%$, respectively. Serum TNF- α levels were measured using a rat TNF- α chemiluminescent immunoassay (CLIA) kit (Elabscience, China) according to the manufacturer's protocol. The detection limit was 0.5 pg/mL, with intra-assay and inter-assay CVs of $<6\%$ and $<9\%$, respectively.

All samples were analysed in duplicate, and the mean values were used for statistical analysis.

Statistical analysis

Data were expressed as mean $\pm$ standard deviation (SD). Normality of distribution was assessed using the Shapiro–Wilk test, and homogeneity of variances was confirmed with

Levene's test. Between-group comparisons were performed using one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test for multiple comparisons. A p-value of less than 0.05 was considered statistically significant. All statistical analyses were conducted using SPSS version 26.0 (IBM Corp., Armonk, NY, USA).

Results

Confirmation of the PCOS model

Vaginal smear cytology during the induction period revealed that all rats in the letrozole-treated groups (model, metformin, and probiotic groups) exhibited persistent dioestrus, characterised by a predominance of leukocytes and the absence of cornified epithelial cells. In contrast, control rats showed regular 4- to 5-day oestrous cycles. This confirmed the successful induction of PCOS-like reproductive abnormalities.

Effect on serum interleukin-10 (IL-10)

One-way ANOVA revealed a statistically significant difference in serum IL-10 levels among the four groups ($F(3,24)=38.14$, $p<0.001$). As shown in Table 1, rats in the PCOS model group had a marked reduction in IL-10 compared with the control group (54.287 ± 5.645 vs. 81.001 ± 4.053 pg/mL; a decrease of 32.98%; $p<0.001$). This finding confirms that letrozole-induced PCOS is associated with a suppressed anti-inflammatory state.

Treatment with metformin significantly increased IL-10 levels to 74.306 ± 3.962 pg/mL, which was 36.88% higher than the model group ($p<0.001$). Importantly, the IL-10 level in the metformin group did not differ significantly from that of the control group ($p=0.217$), indicating that metformin fully normalised this anti-inflammatory cytokine.

Probiotic treatment also raised IL-10 significantly compared with the model group, reaching 69.224 ± 7.481 pg/mL (a 27.51% increase; $p<0.001$). However, IL-10 in the probiotic group remained 14.54% lower than in the control group ($p=0.003$), indicating incomplete restoration. There was no statistically significant difference between the probiotic and metformin groups ($p=0.317$), suggesting that both interventions had comparable effects on IL-10 in this model.

Effect on serum tumour necrosis factor-alpha (TNF- α)

Serum TNF- α levels also differed significantly among the groups ($F(3,24)=15.92$, $p<0.001$). The PCOS model group exhibited a substantial elevation of TNF- α (58.640 ± 8.138 pg/mL) compared with the control group (40.075 ± 2.647 pg/mL), representing a 46.33% increase

($p < 0.001$; Table 1). This confirms the pro-inflammatory state induced by letrozole. Metformin treatment produced a modest reduction in TNF- α to 54.121 ± 4.909 pg/mL, which was 7.71% lower than the model group, but this difference was not statistically significant ($p = 1.000$). Similarly, probiotic treatment lowered

TNF- α to 55.889 ± 7.832 pg/mL (a 4.69% reduction relative to the model group; $p = 1.000$). Both metformin and probiotic groups had TNF- α levels that remained significantly higher than the control group (35.05% and 39.46% higher, respectively; $p = 0.002$ and $p = 0.001$). Neither treatment was able to normalise TNF- α .

Table 1: Serum inflammatory marker levels in the experimental groups

Group	IL-10 (pg/mL)	TNF- α (pg/mL)
Control	81.001 ± 4.053	40.075 ± 2.647
PCOS model	$54.287 \pm 5.645^*$	$58.640 \pm 8.138^*$
Metformin	$74.306 \pm 3.962^\#$	$54.121 \pm 4.909^\dagger$
Probiotic	$69.224 \pm 7.481^{*\#^\dagger}$	$55.889 \pm 7.832^\dagger$

*Data are mean $\pm$ SD. $p < 0.05$ vs. control; $^\#p < 0.05$ vs. PCOS model; $^\dagger p < 0.05$ vs. control (Bonferroni post-hoc test)

Discussion

This study investigated whether probiotic supplementation with *Lactobacillus* and *Bifidobacterium* could improve the inflammatory profile in a letrozole-induced PCOS rat model. The key findings are: (i) letrozole plus a high-fat diet successfully induced a PCOS-like phenotype with persistent dioestrus, reduced IL-10, and elevated TNF- α ; (ii) probiotic treatment significantly increased IL-10 but did not fully restore it to control levels; (iii) neither probiotics nor metformin produced a statistically significant reduction in TNF- α ; (iv) the effects of probiotics on IL-10 were comparable to those of metformin.

The PCOS model reproduces chronic inflammation

The significant decrease in IL-10 and increase in TNF- α in the model group align with the well-documented low-grade inflammatory state in PCOS (4, 5). Letrozole, an aromatase inhibitor, reduces oestrogen synthesis, leading to compensatory luteinising hormone hypersecretion and ovarian androgen excess. Hyperandrogenism promotes adipose tissue dysfunction and macrophage infiltration, which in turn drives TNF- α secretion while suppressing IL-10 production (12). Our findings are consistent with those of Xu and Dai, who reported reduced anti-inflammatory markers and elevated pro-inflammatory cytokines in PCOS (13). The high-fat diet used in this study likely exacerbated metabolic inflammation, as dietary lipids can trigger endotoxaemia and toll-like receptor 4 (TLR4) activation (14).

Probiotics partially restore IL-10 but not TNF- α

Probiotic supplementation significantly increased IL-10 levels by 27.51% compared with the model group. This is an encouraging finding, as IL-10 is a master regulator of intestinal and systemic immune homeostasis. The proposed mechanisms include: (i) direct interaction of

probiotic strains with dendritic cells and macrophages, promoting a regulatory T-cell (Treg) phenotype that secretes IL-10 (15); (ii) production of short-chain fatty acids (SCFAs) that activate G-protein-coupled receptors (e.g., GPR43) on immune cells, enhancing IL-10 expression (7); and (iii) reinforcement of the intestinal barrier, reducing lipopolysaccharide (LPS) translocation and subsequent TLR4-mediated inflammation (8, 16).

However, the lack of a significant effect on TNF- α is noteworthy. Several explanations are plausible. First, TNF- α is a highly potent and rapidly induced cytokine, and its regulation may require more sustained or higher-dose probiotic interventions. Second, the 28-day treatment period may have been insufficient to reverse established inflammatory changes, particularly those driven by androgenic and metabolic pathways independent of gut microbiota. Third, the probiotic dose (1×10^9 CFU/day) might be suboptimal for TNF- α suppression; higher doses or multi-strain formulations with known anti-TNF- α properties (e.g., certain *Lactobacillus rhamnosus* strains) could be more effective (17). Fourth, systemic TNF- α in PCOS may also be produced by ovarian theca cells and adipose tissue, which are less directly influenced by gut microbiota than intestinal immune cells (12).

Interestingly, metformin also failed to significantly reduce TNF- α , despite its known anti-inflammatory properties via AMPK activation and NF- κ B inhibition (18). The observed 7.71% reduction, though non-significant, suggests a trend that might reach statistical significance with a larger sample size. A post-hoc power analysis indicates that to detect a 10% difference in TNF- α with 80% power and $\alpha = 0.05$, approximately 25 rats per group would be required. Thus, the current study was underpowered for TNF- α as a primary outcome.

Comparison with human studies

Ghanei and colleagues conducted a randomised, double-blind, placebo-controlled trial in women with PCOS and found that probiotic supplementation for 12 weeks significantly reduced high-sensitivity C-reactive protein (hs-CRP) and improved IL-10 levels (19). However, that study did not measure TNF- α directly. Another human trial reported that multi-strain probiotics reduced serum TNF- α in PCOS patients after 12 weeks (20). The discrepancy between those human findings and our animal model may be due to differences in intervention duration (12 weeks vs. 4 weeks), probiotic strains, and species-specific gut microbiota composition.

Study limitations

The following limitations should be considered when interpreting our results.

Limitations:

Small sample size (n=7 per group). This limited statistical power, particularly for TNF- α , where modest differences did not reach significance. A post hoc power calculation suggests that 20–25 rats per group would be needed to detect a 10% difference in TNF- α .

No direct measurement of gut microbiota composition. Although we hypothesised that probiotics modulate the gut microbiome, we did not perform faecal 16S rRNA sequencing or quantitative PCR to confirm changes in *Lactobacillus* and *Bifidobacterium* abundance or to assess overall microbial diversity.

Lack of a combined metformin–probiotic group. This would have allowed evaluation of potential synergistic effects. Metformin itself alters gut microbiota, and adding probiotics might produce additive or supra-additive anti-inflammatory effects.

No histological confirmation of PCOS. While vaginal smears confirmed persistent dioestrus, we did not perform ovarian histology to document cystic follicles, theca hyperplasia, or reduced corpora lutea – the gold standard for PCOS confirmation in animal models.

Single time-point measurements. Cytokine levels were measured only at the end of the treatment period. Longitudinal sampling would help determine the time course of improvement and whether effects are sustained.

Limited generalisability. The findings are specific to the letrozole-induced model, which does not fully recapitulate all aspects of human PCOS, particularly the metabolic and psychological dimensions.

Future directions

Studies should include a combined metformin plus probiotic arm to test for synergy.

Longer treatment durations (8–12 weeks) and dose-ranging studies are needed.

Mechanistic investigations should measure SCFA levels, intestinal permeability markers (e.g., zonulin, LPS), and regulatory T-cell populations.

Translation to human PCOS requires well-designed randomised controlled trials with adequate sample sizes and clinically relevant outcomes (e.g., menstrual cyclicity, hirsutism, metabolic parameters).

Conclusion

Polycystic ovary syndrome is a complex endocrine disorder accompanied by chronic low-grade inflammation, reflected in reduced IL-10 and elevated TNF- α . In a letrozole-induced rat model, probiotic supplementation with *Lactobacillus* and *Bifidobacterium* for 28 days significantly improved IL-10 levels but did not normalise them and had no significant effect on TNF- α . Metformin produced a similar pattern. Therefore, probiotic use alone represents a supportive, rather than definitive, strategy for alleviating inflammation in PCOS. Combination therapies targeting multiple inflammatory pathways, including probiotics with metformin or anti-androgen agents, warrant further investigation.

List of Abbreviations

AMPK: Adenosine monophosphate-activated protein kinase
ANOVA: Analysis of variance
CFU: Colony-forming unit
CLIA: Chemiluminescent immunoassay
DMSO: Dimethyl sulfoxide
ELISA: Enzyme-linked immunosorbent assay
FSH: Follicle-stimulating hormone
GnRH: Gonadotropin-releasing hormone
GPR43: G protein-coupled receptor 43
hs CRP: High-sensitivity C-reactive protein
IL 10: Interleukin 10
LH: Luteinising hormone
LPS: Lipopolysaccharide
NF κ B: Nuclear factor kappa B
PCOS: Polycystic ovary syndrome
SCFA: Short-chain fatty acid
SD: Standard deviation
SPSS: Statistical Package for the Social Sciences
TLR4: Toll-like receptor 4
TNF α : Tumour necrosis factor alpha
Treg: Regulatory T cell

Declarations

Ethics Approval and Consent to Participate

This study was approved by the Research Ethics Committee of the University of Babylon (Approval No. A-0059, 19 October 2025). All animal

procedures were conducted in accordance with the ARRIVE guidelines and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (8th edition, 2011). Consent to participate is not applicable as this study did not involve human subjects.

Consent for Publication

All authors have given their explicit consent for the publication of this work under the Creative Commons Attribution Non-Commercial 4.0 International license.

Data Availability

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

Competing Interests

The authors declare that they have no competing interests, financial or otherwise, that could have influenced the work reported in this manuscript.

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Author Contributions

HB worked on the conceptualisation, investigation, writing the original draft and project administration.

BAA worked on the methodology, formal analysis, supervision, writing the review and editing.

HLF worked on the validation, resources, data curation, writing the review and editing.

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