

COMBINED PROBIOTIC AND OMEGA-3 SUPPLEMENTATION MITIGATES CHLORPYRIFOS-INDUCED TESTICULAR OXIDATIVE DAMAGE AND ENDOCRINE DISRUPTION

BY

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Abstract

Chlorpyrifos (CPF), a widely used organophosphate pesticide, has been implicated in testicular dysfunction through oxidative and endocrine disruption. Despite growing interest in nutritional interventions, the combined protective effects of probiotic yoghurt and omega-3 fatty acids on CPF-induced testicular injury remain underexplored. Hence, this study aims to investigate the ameliorative potential of probiotic yoghurt and omega-3 fatty acids singly and in combination, against CPF-toxicity in adult male Wistar rats. Sixty adults male Wistar rats were randomly divided into seven groups (n=10 per group): control, CPF (10 mg/kg), Probiotic (10 mg/kg), CPF + Probiotic, Omega-3 fatty acids (2 ml/kg), CPF + Omega-3, and CPF + Probiotic + Omega-3. Treatment with experimental substances was administered concurrently for two weeks (14 days) via oral gavage. 24 hours after the last administration of substances, animals were euthanised and testicular structure, epididymis, and blood samples were collected for post-treatment analysis, i.e., histological processing (H and E and PAS stains), Seminalysis, serum hormone levels, oxidative and inflammatory markers were analysed. Chronic CPF exposure resulted in reduced sperm quality and quantity, accompanied by reduced LH and testosterone levels, suppressed SOD activity, and elevated IL-1 β . Histologically, CPF caused ST degeneration, vacuolations within the germinal epithelium, and disrupted PAS-positive BM. Probiotic yoghurt and omega-3 supplementation, particularly the combined treatment, significantly improved sperm indices, restored hormonal balance, enhanced antioxidant enzyme activity, and reduced inflammatory cytokine concentration. Testicular architecture and BM integrity were preserved in the therapeutic groups, especially in CPF + PRO + OME3 rats. Concurrent administration of probiotic yoghurt and omega-3 fatty acids effectively mitigated CPF-induced testicular toxicity by enhancing antioxidant defence, modulating inflammatory responses, and restoring endocrine and structural integrity. These findings underscore the translational potential of nutritional therapy as a preventive or adjunctive approach against pesticide-related male infertility.

Keywords: chlorpyrifos, male infertility, probiotic yoghurt, omega-3 fatty acids, inflammation, oxidative stress.

Introduction

The testis is an essential reproductive organ responsible for sperm production and steroidogenesis (Virág *et al.*, 2026). Its functional integrity depends on a delicate interplay between germ cells, Sertoli cells, and Leydig cells, all of which are vulnerable to oxidative, endocrine, and inflammatory disruptions (Antinozzi *et al.*, 2025; Virág *et al.*, 2026). Environmental toxicants, particularly organophosphate pesticides such as chlorpyrifos (CPF), are recognised for inducing male reproductive toxicity through multiple biochemical and histological mechanisms (Babazadeh and Najafi, 2017; Mohamed and El-Rahman, 2025). CPF is commonly used in agricultural and domestic pest control but has been associated with testicular degeneration, impaired spermatogenesis, hormonal imbalance, and reduced fertility in experimental and human studies (Rui *et al.*, 2018; Mohamed and El-Rahman, 2025). The compound's lipophilic nature facilitates its accumulation in gonadal tissue, where it generates reactive oxygen species (ROS) and interferes with acetylcholinesterase activity (Montanarí *et al.*, 2024). This oxidative burden triggers lipid

peroxidation, germ cell apoptosis, and breakdown of the seminiferous tubular architecture, ultimately disrupting spermatogenesis and androgen synthesis (Montanari *et al.*, 2024).

Growing evidence supports the role of dietary antioxidants and natural supplements in mitigating such toxic insults (Hadjimbei *et al.*, 2022; Pascariu *et al.*, 2025). Among these, probiotic yoghurt, containing live beneficial bacterial cultures, has been reported to enhance systemic antioxidant status, modulate gut microbiota, and improve reproductive parameters through the gut–testis axis (Hadjimbei *et al.*, 2022; Pascariu *et al.*, 2025). Likewise, omega-3 fatty acids, rich in eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are integral to sperm membrane fluidity, mitochondrial function, and anti-inflammatory regulation (Collodel *et al.*, 2020). However, while both agents individually exhibit protective effects, their combined influence on CPF-induced testicular toxicity has received limited experimental attention. Given the rising incidence of pesticide-related reproductive dysfunction and the global shift toward nutraceutical interventions, it becomes imperative to examine safe, cost-effective strategies capable of protecting testicular integrity. This study, therefore, assessed the ameliorative potential of probiotic yoghurt and omega-3 fatty acids, singly and in combination, against chlorpyrifos-induced testicular injury in mice, integrating hormonal, biochemical, and histological analyses to elucidate their protective mechanisms.

Methodology

Adult male Wistar rats (8-10 weeks old; body weight 180-200 g) were procured from an accredited laboratory animal centre's breeding facility. The animals were transferred in ventilated cages and given seven days to acclimatise before beginning the experiment. During the acclimatisation period, the rats were housed in polypropylene cages with regulated environmental conditions (temperature 22-25°C, relative humidity 50-60%, and 12-hour light / dark cycle). Standard laboratory rodent pellets and clean drinking water were available on a continuous basis. After acclimatisation, the animals were randomly assigned to seven experimental groups (n = 10) using a simple randomisation procedure to reduce allocation bias. The groupings are group A - Control (1ml distilled water), B: - CPF only (10 mg/kg/day, oral), C - Probiotics only (10 ml/kg/day, oral yoghurt), D - Omega-3 only (1 ml/kg/day, oral), E: CPF + probiotics, F: CPF + omega-3, G: CPF + probiotics + omega-3. Technical-grade chlorpyrifos was acquired from Sun Africa Agrochemical Store, Ilorin, Kwara State. Every day, the chemical was freshly produced by dissolving the necessary quantity in distilled water to reach the correct concentration for oral delivery. The probiotic mixture was made from commercial fermented yoghurt that contained live *Lactobacillus* and *Bifidobacterium* cultures. The yoghurt (unsweetened Greek yoghurt) was acquired from FandF healthy drink, Ilorin, and kept at 4°C and warmed to room temperature before use. A pharmaceutical-grade omega-3 fatty acid supplement containing eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) was purchased from One Step Pharmacy, Ilorin. To prevent oxidative deterioration, the capsules were perforated, and the oil content was measured using a calibrated micropipette just before dosage. All substances were administered to the animals via oral gavage, using a separate gavage syringe to ensure accurate dosage, concurrently for 14 days. All administrations were administered approximately at the same time to reduce circadian variability. The animals were starved for 24 hours following the final treatment, and Euthanasia was then performed by cervical dislocation. Blood samples were obtained by heart puncture for hormonal and biochemical analysis. The testes and epididymides were meticulously dissected, free of any connective tissue, and weighed. The cauda epididymis was utilised for seminal analysis, and the testicular tissue was preserved for histological examination. Body weight (weekly) and tissue weight measurements were conducted using a digital analytical balance with a sensitivity of 0.01 g.

Epididymal tissues were carefully dissected out. The epididymides (right and left) were then separated into three segments: caput, corpus, and cauda. The caudal epididymal sperms were collected by slicing the epididymis into tiny pieces in 10 mL of normal saline. The epididymal sperm count was measured using the conventional hemocytometric technique. After diluting epididymal sperm (1:20) in distilled water, 10 μ L of the diluted specimen was transferred to each counting chamber of the hemocytometer (HBG, Hamburg, Germany) and left to stand for 5 minutes in a humid environment to prevent drying. During sedimentation, cells were counted using a light microscope at 400 $\times$ magnification. The sperm count was represented in terms of sperm per millilitre (Babazadeh and Najafi, 2017). A light microscope with a magnification of 400 $\times$ was used to visually assess sperm motility. For this technique, one drop of sperm suspension was deposited on a slide, and a cover slip was placed over it. At least ten fields were examined, and motile sperm percentages were determined. Smears of sperm suspension were made, air-dried, and stained with eosin-nigrosin. Abnormalities in the sperm head, midpiece, and tail were assessed in 200 sperm cells per slide. Viability was determined by eosin exclusion staining, which left living spermatozoa unstained while dead sperm cells absorbed the dye. Percentages were used to calculate the ratio of viable to non-viable spermatozoa (Babazadeh and Najafi, 2017). Determination of Serum sex hormone profile, SOD, and IL-1 β was determined according to the method of Mohamed and El-Rahman (2025) using an Enzyme-linked immunosorbent Assay kit (Diagnostic Products Corporation, USA). All tissue samples were fixed in a Bouin's fluid fixative, then dehydrated in ethyl alcohol and cleaned in xylene for histological analysis. Sections (7 μ m thick) were stained with haematoxylin and eosin, and periodic Acid Schiff (PAS) stain for histopathological analysis. All samples were examined at 400 $\times$ magnification. The results were analysed by Chris Rordens EzAnova (version 1.6.1, Vienna, Austria). The analysis of variance (one-way ANOVA) was used to compare groups, followed by the Tukey post-hoc test. All results were reported as mean $\pm$ SEM. The statistical significance level was set to $p < 0.05$.

Results

Figure 1 shows the effects of probiotic (yoghurt) and omega-3 fatty acids on the testicular weight of mice exposed to chlorpyrifos. Between and within experimental groups, no statistically significant differences ($p > 0.05$) in the testicular weight of animals.

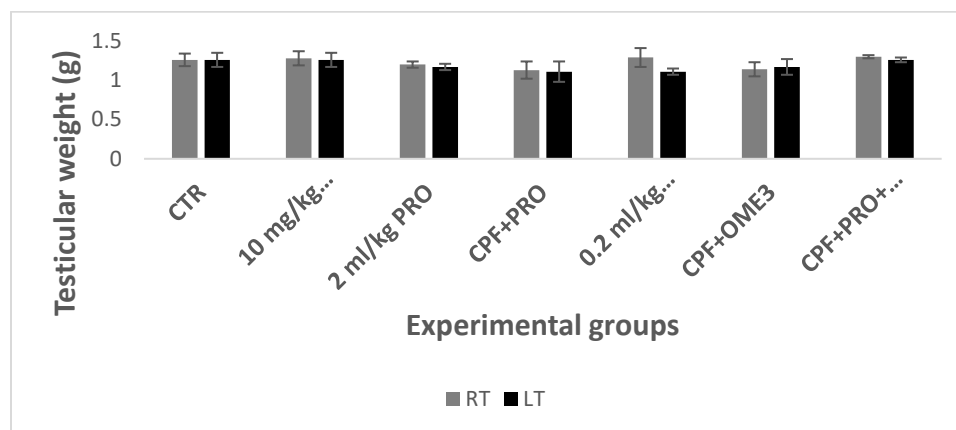


Figure 1: showing the effects of probiotic (yoghurt) and omega-3 fatty acid on animals' testicular weight. Each bar represented as Mean $\pm$ SEM. Number of mice/groups = 8. CPF – chlorpyrifos, PRO – probiotic yoghurt, OME3 – omega-3 fatty acid.

Table 1: Effects of probiotic (Yoghurt) and omega-3 fatty acid on the semen quality in chlorpyrifos-treated animals

Groups	Semen Conc. (*10 ⁶)	Motility (%)	Live and Death Ratio (%)	Morphology (%)
Control	494±20.7	89±1.86	89±3.34	94±1.07
10 mg/kg CPF	274±34.3*	55±7.23*	57±7.39*	57±5.30*
2 ml/kg PRO	402±34.4 α	86±4.56 α	83±3.51 α	89±2.96 α
CPF+PRO	264±19.8*	67±3.16*	69±3.23*	72±3.31* α
0.2 ml/kg OME3	276±45.0*	80±9.33	78±6.56	81±6.30 α
CPF+OME3	426±22.5 α	90±2.34 α	92±2.30 α	91±2.29 α
CPF+PRO+OME3	432±14.4 α	86±3.44 α	88±3.22 α	85±4.19 α

Table 1: Effects of probiotic (yoghurt) and omega-3 fatty acid on the seminal quality of experimental animals. Data represented as Mean $\pm$ SEM, *p<0.05 compared to the control group, α p<0.05 compared to the CPF group. Number of mice/groups = 10. CPF – chlorpyrifos, PRO – probiotic yoghurt, OME3 – omega-3 fatty acid.

Table 2 shows the effects of probiotic (Yoghurt) and omega-3 fatty acid on the hormone profile in chlorpyrifos-treated mice.

Groups	LH (mIU/ml)	FSH (mIU/ml)	TT (ng/ml)
CTR	0.28±0.03	1.48±0.12	0.53±0.05
10 mg/kg CPF	0.15±0.02*	2.70±0.34*	0.31±0.03*
2 ml/kg PRO	0.34±0.02 α	2.51±0.34*	0.54±0.02 α
CPF+PRO	0.30±0.02 α	1.96±0.29	0.53±0.05 α
0.2 ml/kg OME3	0.29±0.01 α	1.99±0.30	0.50±0.04 α
CPF+OME3	0.18±0.02*	1.96±0.27	0.53±0.04 α
CPF+PRO+OME3	0.19±0.02	1.62±0.18 α	0.63±0.05 α

Table 2: Effects of probiotic (yoghurt) and omega-3 fatty acid on the sex hormone levels in experimental animals. Data represented as Mean $\pm$ SEM, *p<0.05 compared to the control group, α p<0.05 compared to the CPF group. Number of mice/groups = 10. CPF – chlorpyrifos, PRO – probiotic yoghurt, OME3 – omega-3 fatty acid.

Figure 2 shows the effects of probiotic (yoghurt) and omega-3 fatty acid on superoxide dismutase (SOD) levels in chlorpyrifos-induced oxidative stress. CPF+PRO, 0.2 ml/kg OME3, and CPF+OME3 groups increased SOD levels significantly (*P>0.05) compared to the control group, while all experimental groups significantly increased SOD compared to the 10 mg/kg CPF group.

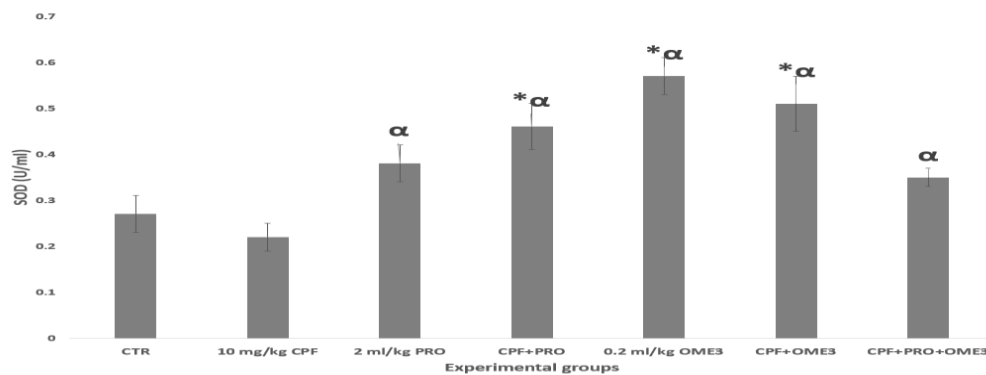


Figure 2: effects of probiotic (yoghurt) and omega-3 fatty acid on superoxide dismutase (SOD) concentration. Each bar is represented as the Mean $\pm$ SEM. * $p < 0.05$ compared to the control group, $\alpha p < 0.05$ compared to the CPF group. Number of mice/groups = 10. CPF – chlorpyrifos, PRO – probiotic yoghurt, OME3 – omega-3 fatty acid.

Figure 3 shows the effects of probiotic (yoghurt) and omega-3 fatty acid on interleukin-1 β (IL-1 β) levels in chlorpyrifos-induced testicular injury. All experimental groups, compared to the control and CPF groups, showed significantly reduced (* $p < 0.05$, $\alpha p < 0.05$) IL-1 β levels.

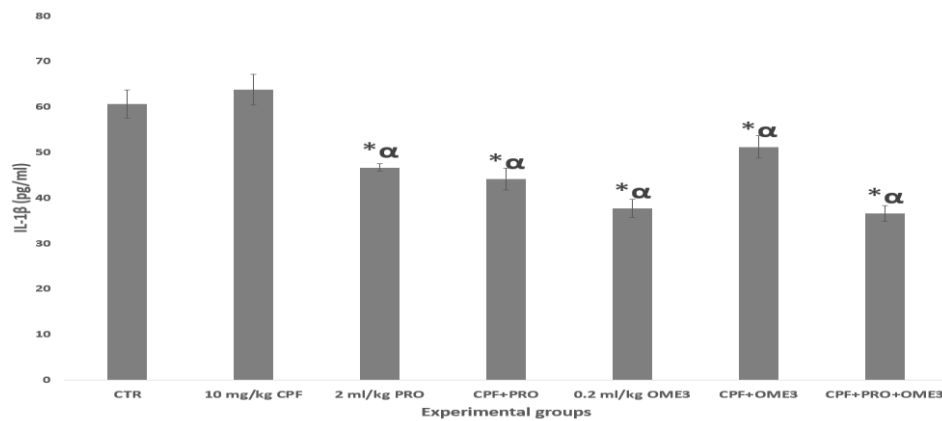


Figure 3: effects of probiotic (yoghurt) and omega-3 fatty acid on interleukin-1 β . Each bar is represented as the Mean $\pm$ SEM. * $p < 0.05$ compared to the control group, $\alpha p < 0.05$ compared to the CPF group. Number of mice/groups = 10. CPF – chlorpyrifos, PRO – probiotic yoghurt, OME3 – omega-3 fatty acid.

Figure 4 (A – G) shows representative testicular sections stained with Hematoxylin and Eosin from rats in various experimental groups at a magnification of x400, showing well-preserved cytoarchitecture of the seminiferous tubule (ST), intact germinal epithelium and abundant spermatozoa in the lumen (L) in the CTR, PRO, and OME3 groups (fig. 4A, 4C, 4E). In the CPF-only group (fig. 4B), shrunken (atrophied) ST, germinal cell disorganisation, marked vacuolation within the germinal epithelium, and sparsely populated lumina (L) were noted. In the CPF + PRO and CPF + OME3 groups (Fig. 4D, 4F), improvement in the morphology of the ST and spermatogenic organisation, the lumen (L) contains visible spermatozoa. However, focal vacuolation persists, whereas the combined therapy group CPF + PRO + OME3 showed near-normal testicular histoarchitecture, with distinct epithelial layering and dense luminal spermatozoa.

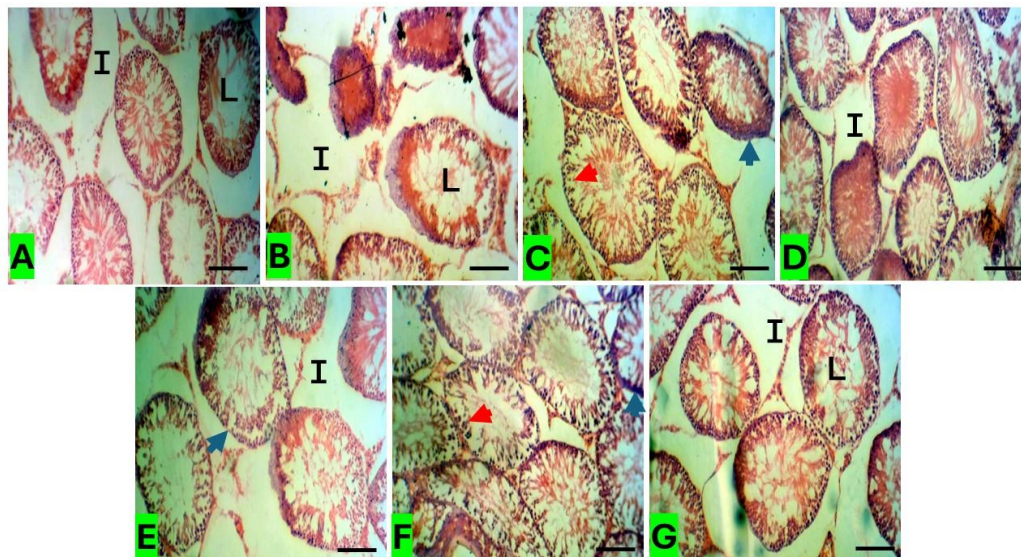


Figure 4 (A – G): Representative photomicrographs showing the effects of probiotic (yoghurt) and omega-3 fatty acid on CPF-induced testicular injury in experimental animals. Stain – Haematoxylin and Eosin stain. Magnification – 400x. Scale bar - 50µm. **I** – interstitial space, **L** – lumen, **blue arrow** – Basement membrane, **red arrow** – germinal epithelium (BM). (A) – Control, (B) – CPF (10 mg/kg), (C) – PRO (10 mg/kg), (D) – CPF + PRO, (E) – OME3 (2 ml/kg), (F) – CPF + OME3, (G) – CPF + PRO + OME3.

Figure 5 (A – G) shows testicular sections stained with Periodic Acid-Schiff (PAS), highlighting the carbohydrate-rich basement membrane (BM) of the STs. The BM (red arrow) of the control, PRO, and OME3 groups (fig. 4A, 4C, 4E) showed strong PAS positivity demarcating the ST and intact spermatogenic series compared to the disrupted and weakly PAS-reactive BM and defective spermiogenesis observed in the CPF-only group (fig. 4B), while moderate PAS reactivity around the BM was observable in the CPF + PRO, CPF + OME3, and CPF + PRO + OME3 (fig 4D, 4E, 4G) groups.

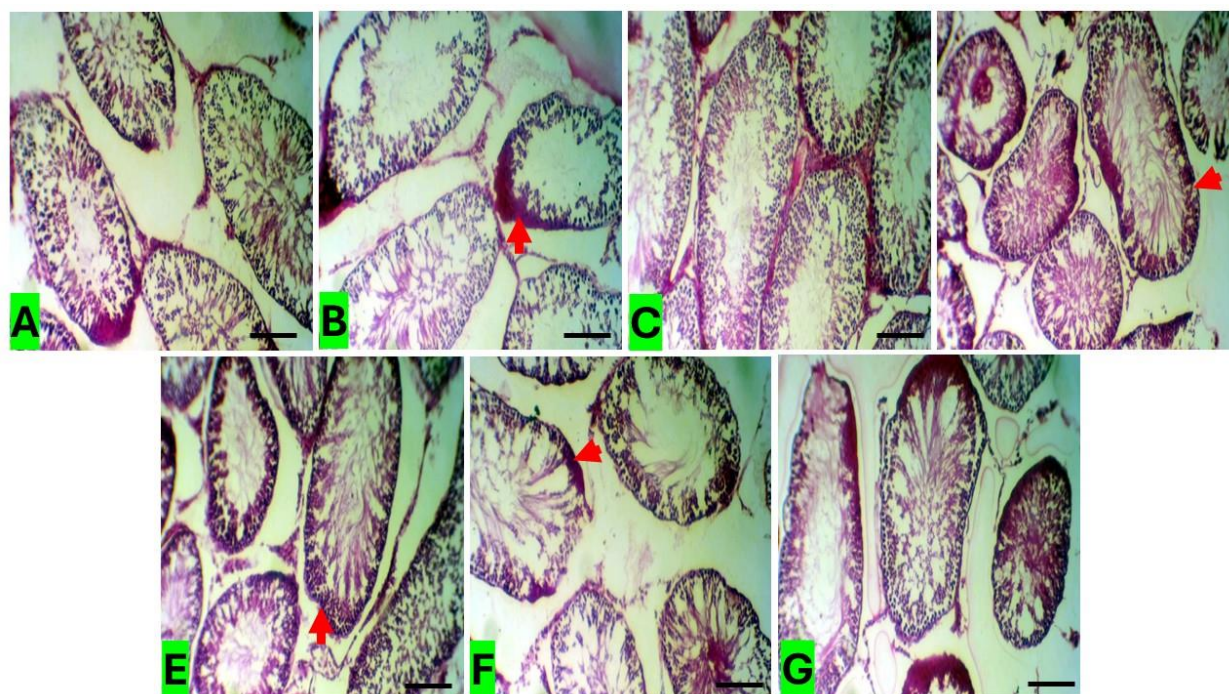


Figure 5 (A – G): Representative photomicrographs showing the effects of probiotic (yoghurt) and omega-3 fatty acid on CPF-induced testicular injury in experimental animals. Stain – Periodic Acid-Schiff (PAS) stain. Magnification – 400x. Scale bar - 50µm. Red arrow – Basement membrane (BM). (A) – Control, (B) – CPF (10 mg/kg), (C) – PRO (10 mg/kg), (D) – CPF + PRO, (E) – OME3 (2 ml/kg), (F) – CPF + OME3, (G) – CPF + PRO + OME3.

Discussion

The present study evaluated the effects of probiotic yoghurt and omega-3 fatty acids on chlorpyrifos (CPF)-induced testicular injury, focusing on the spermatogenic, hormonal, oxidative, and inflammatory responses. CPF exposure (10 mg/kg) for two weeks produced characteristic features of testicular toxicity, whereas concurrent administration of probiotic and omega-3 fatty acids demonstrated substantial amelioration across functional and histological indices. CPF exposure, at 10 mg/kg, caused marked degeneration of the seminiferous epithelium, tubular atrophy, and vacuolation, with luminal depletion of spermatozoa. These findings align with previous reports describing organophosphate-mediated impairment of germ cell maturation and basement membrane integrity via lipid peroxidation and enzyme inhibition of acetylcholinesterase (Mohamed and El-Rahman, 2025). Histochemical disruption of PAS-reactive BM further reflects CPF-induced compromise of the Sertoli cells interface, critical for the maintenance of the blood-testis barrier and nutrient exchange. Contrastingly, co-treatment with probiotics and omega-3 restored tubular architecture and re-established spermatogenic layering. The CPF + PRO + OME3 group, in particular, displayed nearly normal spermatogenesis, confirming that combined nutritional therapy supports germ cell regeneration and reconstitution of basement membrane glycoproteins. These findings emphasise the therapeutic synergy of dietary antioxidants in maintaining testicular microstructure under toxic stress.

Significantly reduced sperm concentration, motility, viability, and morphology were noted in the CPF-only group, alongside decreases in luteinizing hormone (LH) and testosterone levels (Kamalesh, 2024; Mohamed & El-Rahman, 2025). The elevated follicle-stimulating hormone (FSH) levels may represent a compensatory pituitary response to Sertoli cell dysfunction. Such hormonal and spermatogenic alterations are consistent with endocrine disruption resulting from testicular oxidative injury. Probiotics and omega-3 supplementation significantly improved sperm parameters and normalised hormonal profiles, with testosterone restored to near-control levels (Afeiche *et al.*, 2013; Falsig *et al.*, 2019). Omega-3 fatty acids, rich in docosahexaenoic and eicosapentaenoic acids, are integral to sperm membrane fluidity and steroidogenesis, whereas probiotics may enhance cholesterol metabolism and modulate the gut-testis axis. The observed recovery supports a dual mechanism involving restoration of Leydig cell function and enhanced hormonal signalling along the hypothalamo-pituitary-gonadal axis (Hosseini *et al.*, 2019). Probiotic and omega-3 treatment significantly enhanced superoxide dismutase (SOD) activity and suppressed interleukin-1 β (IL-1 β), indicating effective redox stabilisation and anti-inflammatory modulation compared to elevated IL-1 β and reduced SOD levels, suggesting oxidative and inflammatory stress as key mediators of CPF testicular cytotoxicity (Montanari *et al.*, 2024). These findings agree with the established role of reactive oxygen species (ROS) in lipid peroxidation, germ cell apoptosis, and mitochondrial dysfunction. Probiotics promote systemic antioxidant defence through the production of short-chain fatty acids and upregulation of the host antioxidant enzymes (Pascariu *et al.*, 2025), while omega-3 fatty acids downregulate nuclear factor-kappa B (NF-KB) signalling, thereby limiting cytokine release (Collodel *et al.*, 2020). The combined intervention thus restored the biochemical balance necessary for testicular homeostasis and spermatogenic continuity. Chronic exposure to organophosphates such as CPF has been associated with male subfertility, endocrine disturbance, and altered sperm quality and function (Rui *et al.*, 2018). The present findings suggest that concurrent intake of probiotic-rich foods and omega-3 supplementation may offer a cost-effective adjunctive strategy to mitigate environmental or occupational toxicant exposure, particularly in agricultural populations at risk of reproductive impairment. Furthermore, the structural and functional restoration observed in the combined treatment group underscores a translational opportunity to explore nutritional therapeutics as non-pharmacological interventions in idiopathic male infertility linked to oxidative and inflammatory aetiologies.

Conclusion

The study provides compelling evidence that combined probiotic and omega-3 supplementation holds therapeutic promise for the prevention and management of toxicant-induced male reproductive dysfunction, with potential implications for occupational health and clinical reproductive medicine. Chronic chlorpyrifos exposure to adult male rats induced significant testicular degeneration, hormonal imbalance, oxidative stress and inflammatory activation in adult male Wistar rats. Concurrent administration of probiotic yoghurt and omega-3 fatty acids markedly alleviated these alterations, restoring spermatogenic activity, endocrine balance, and testicular histoarchitecture. These highlight the complementary protective mechanisms of these agents, acting through antioxidative, anti-inflammatory, and endocrine modulating pathways.

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