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The ameliorative effects of *Lactobacillus casei* and *Lactobacillus coagulans* probiotics on Spirotetramat induced testicular toxicity based on biochemical and histological studies in male Wistar rats

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Abstract

Probiotics have been found as important elements against reproductive toxicity. So the use of probiotics has been increased day by day to combat with several disorders related to reproductive health. In current study, the potential role of probiotics including *Lactobacillus casei* (L₁) and *Lactobacillus coagulans* (L₂) has been demonstrated against a tetramic acid-derived pesticide Spirotetramat which has been found to cause variable toxicity at different levels in the life cycle of various organisms. For this study, five groups including a control group of male Wistar rats; were studied for toxicological evaluation. A daily dose of Spirotetramat for 28 days (667 mg/kg BW per day) was administered along with probiotics L₁ and L₂ (1x10⁷cfu/ml) in five groups according to protocol. Oxidative stress was estimated by calculating levels of different parameters including Superoxide dismutase (SOD), Lipid peroxidation (LPO), Glutathione peroxidase (GPx) Catalase CAT, and Glutathione (GSH) soon after sacrifice. LPO was found to be increased with GSH, CAT, and GPx and with a significant decrease in SOD ($p < 0.01$) in pesticide-controlled rats as compared to untreated ones. After the treatment by L₁ and L₂, a significant recovery ($p < 0.05$) was observed in toxicity parameters in relation to normal rats. The results also revealed that spirotetramat exposure caused significant reductions in sperm count, motility, and viability in male rats. However, treatment with L₁ and L₂ was found to mitigate these effects. Specifically, co-administration of Spirotetramat with L₁ and L₂ resulted in significant improvements in sperm count, motility, and viability in male Wistar rats.

Keywords: Lactobacillus, probiotics, reproductive toxicity, spirotetramat, testes.

1. Introduction

Probiotics are living microorganisms which are consumed for health benefits. They are found in cultures in various foods like fermented dairy products, probiotic-enriched foods and fermented foods (Mattila *et al.*, 2002) ^[1]. They are known to have diverse functions in living world including immune function, reducing inflammatory processes, treating gastrointestinal and respiratory infections, anti-cancer and anti-allergic effects, lowering of serum cholesterol and blood sugar levels, regulation of the production of inflammatory cytokines and the expression of specific pro-survival or pro-apoptotic genes in hosts' cells (Cong *et al.*, 2003) ^[2] lower plasma glucose, triacylglycerols, as well as decrease in oxidative stress (Barreto *et al.*, 2014; Zhu *et al.*, 2018) ^[3-4] promotes humoral and cell-mediated immunity, stimulates regeneration, corrects metabolism (Yushkova *et al.*, 2019) ^[5] and promotes production of reproductive hormones (Poutahidis *et al.*, 2014) ^[6]. Studies have revealed that there is a correlation between serum cholesterol levels and sperm function. So, the probiotics that improve cholesterol levels may also improve sperm motility and prevent oxidative stress-induced DNA damage in these cells (Dardmeh *et al.*, 2017) ^[7]. Several studies have shown the ability of Lactobacillus to facilitate the excretion of various toxic substances, and thus mitigate their adverse effects on the human health. Some Lactobacillus could efficiently bind to and remove toxic metals such as cadmium (Jama *et al.*, 2012) ^[8]. Therefore, the use of probiotics is becoming popular as an alternative to drugs to control various disorders (Fioramonti *et al.*, 2003) ^[9]. Probiotics have also been found to increase total antioxidant capacity (TAC) and sperm quality including motility, concentration and morphology (Chen *et al.*, 2013) ^[10].

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In diabetic rats. *Lactobacillus casei* has been found to promote sperm maturation (Abasi & Keshtmand, 2020)^[11]. While as *Lactobacillus rhamnosus* have shown promotion in spermatogenesis and increase in the number of Leydig cells by improving seminiferous tubules parameters in mice. (Deabas *et al.*, 2012)^[12]. The aim of the present study was to evaluate the protective effects of *L. Casei* and *L. Coagulans* probiotics on testicular function against spirotetramat-induced reproductive toxicity.

Spirotetramat is an insecticide derived from tetramic acid and introduced as a new product intended for controlling the insects like whiteflies, aphids, psyllids, mealy-bugs and other sucking insect pests that damage agricultural crops and their yield (Ouyang *et al.*, 2012)^[13] usually by inhibiting acetyl CoA in lipid biosynthesis or through reduction of fecundity or fertility (Wachendorff *et al.*, 2002; Nauen *et al.*, 2005)^[14-15]. These widely application of such sprayed insecticides poses a major hazard for non-target organisms directly or indirectly thereby causing various disorders. Therefore, concern over Spirotetramat use and safety for the environment is a burning issue and its toxicity to the eco-environment and non-target organisms. In rats orally fed with Spirotetramat Liver and genital damage has been observed (Liu *et al.*, 2011)^[16] with a variable metabolite residues among organs and tissues (Wu *et al.*, 2012)^[17]. In aquatic organisms including African catfish, (*Clarias gariepinus*) (Agbohessi *et al.*, 2013)^[18], Chinese toad (*Bufo bufo gargarizans*) tadpoles (Yin *et al.*, 2009)^[19] a significant amount of toxicity has already been reported in various internal organs. Recently, it has been observed that the oxidative damage to testicular cells induced by various xenobiotics, products of abnormal metabolism or ROS can result in testicular dysfunction leading to male infertility (Aggarwal and Said, 2005; Shrilata and Muralidhara, 2007)^[20-21]. These Reactive oxygen species (ROS) interfere in the process of sperm maturation and function, and male fertilization (Wagner *et al.*, 2018)^[22]. In testes unsaturated fatty acids undergo peroxidation and increase the production of free radicals by inducing apoptosis in testicular tissue cells (Alahmar, 2019; Shahrokhi *et al.*, 2014)^[23-24]. The reports of reproductive toxicity of pesticides are a major concern because human spermatogenesis may be vulnerable to chronic exposure to chemicals at very low exposure. Spirotetramat may cause excessive lipid peroxidation (LPO), sterility, sperm aberrations and oxidative injury. Extensive use of broad-spectrum synthetic insecticides results in the destruction of non-target organisms. Since they have a long life period, resulting in bioaccumulation and biomagnification in the environment and in the living organisms (Sahai, 1992)^[25].

2. Materials and Methods

A) Probiotic Strains

The bacterial strains of *L. Casei* and *L. Coagulans* were rejuvenated in MRS (de-Mann Rogosa Sharpe Agar) medium soon after incubation. The strains were got from the Department of Zoology, Saint. Hirdaram College Bhopal.

B) Animals

Male Wistar rats of about 200-250g weight were used for this study and all the procedures were supported and carried out under prescribed ethical committee guidelines.

C) Chemicals

The chemicals for this study were obtained from prescribed companies with purity of grade A. Spirotetramat (insecticide) was obtained from Bayer India Limited. Various types of Kits were used to demonstrate the reproductive toxicity parameters which were purchased from registered stockists.

D) Probiotic Stock Preparation

At 38 °C for 50 hours with anaerobic conditions, *L.casei* and *L.coagulans* cultures were revived separately using MRS broth. A loopful from both cultures was added to 1 ml distilled water and final suspension was prepped up to 10 ml by adding more water. Serial dilutions from N1 to N6 of 1/10 were prepared. On MRS agar medium 100 Microliter of N6 was inoculated to develop the bacterial colonies. A vigorous colony from agar was separated and added to 1ml of distilled water to get a concentration of 1×10^7 cfu/ml.

E) Pesticide Stress Induction

Spirotetramat was administrated (667mg/kg Bw/day) equal to 10000ppm dissolved in water and used as a single dose which was then administrated orally for 4 weeks to achieve sub-acute toxicity, (Young, 2006)^[26].

F) Probiotic Dosage

As per experimental protocol; in rats for 4 weeks, administration of probiotics was done by gavaging orally soon after pesticide dose.

G) Experimental Design

The experimental design was framed by inset of six groups of Male Wistar rats designated as C, T, T₁, T₂ and T₁+T₂ with six rats of near about equal weight in each group as per the design given below.

Group	Remarks
C	Control
T	Toxicity Control
T ₁	L ₁ Treated
T ₂	L ₂ Treated
T ₁ +T ₂	L ₁ +L ₂ Treated

L₁= *L.casei* L₂= *L.coagulans*

The rats were sacrificed after 4 weeks after mild ether anaesthesia. From the punctured heart using disposable syringes blood was collected from each rat and transferred immediately in to tubes already containing EDTA (Ethylene diamine tetra acetic acid) as blood anticoagulant. Tubes carrying samples were centrifuged at 3500rpm for 15 minutes to separate the plasma for biochemical valuations. Tubes with plasma samples were kept in a deep freezer until examined for biochemical estimations. Before homogenization testes were removed and then washed with cold saline. Testes and epididymis were then weighed for calculation of relative organ weights using the ratio of organ weight/ body weight x100 formula. 10% buffered formalin was used to fix one test for histopathological study. Second testes were homogenized using ice-cold KCl₄ (150mM) with a ratio of tissue weight to homogenate equal to 1:10. Homogenates were centrifuged at 20,000 g for 10 min at 4° for determination of enzyme activities from the supernatant. From this homogenate, serial dilutions were obtained for the calculation of the concentration of LPO, GSH, and Total Protein.

H) Sperm Parameters

For determination of sperm count, sperm motility and abnormalities in sperms, the epididymis was crushed in 5ml of saline and incubated for half an hour at 37 °C for smooth flow of sperms from tubes of epididymis. A warm microscope slide was loaded with one drop of the mixture. Using a phase contrast microscope, the percentage of motility was determined at a magnification of 400X. Sperms were allowed to dry after removal of the coverslip and stained with eosin (1%) to observe the morphological abnormalities along different fields. Neubauer's hemocytometer was used to calculate total sperm count (Yokoi *et al.*, 2003)^[27].

I) Estimation of oxidative stress and testosterone level

Oxidative stress was confirmed by determining lipid peroxidation (LPO) (Ohkawa *et al.*, 1979)^[28], Superoxide dismutase (SOD) (Kakkar *et al.*, 1984)^[29], Catalase CAT (Sinha (1972)^[30], Glutathione peroxidase (GPx) (Rotruck *et al.*, (1973)^[31] and Glutathione (GSH) (Habig *et al.*, (1974)^[32]. Overnight kept serum and interstitial fluid obtained from tunica albuginea centrifuged at the rate of 54xG for 20min was used for determining Testosterone levels by automated Erbachem analyzer by chemiluminescence method.

J) Statistical Methods

For statistical calculation, Graph Pad InStat software was used. Results were calculated by determining mean of standard error and in relation to mean of observed values. One-way analysis of variance to compare and calculation of variance using Dunnet's test.

3. Results

I) Testicular toxicity

A) Changes in Lipid peroxidation (LPO)

LPO significantly increased (305.5% in testes tissue) ($p < 0.01$) in rats administrated with pesticide as compared to control. On the other hand, the treated groups with L₁ and L₂ showed a significant ($p < 0.01$) fall in LPO (57.5%). The group treated

with L₁ revealed a significant ($p < 0.05$) reduction in LPO (32.5%) and that treated with L₂ reduced LPO significantly ($p < 0.01$) to 41.2% in liver tissue.

B) Changes in Glutathione (GSH)

GSH was found to be declined significantly ($p < 0.01$) in concentration (73.2% in testes tissue) in pesticide-administrated rats as compared to untreated ones. Dosage with L₁ and L₂, separately, and both revealed a significant ($p < 0.01$) rise in the level of GSH (95.5%, 132.4%, and 160.4%, respectively) in spirotetramat-induced toxicity in rats as compared to normal control.

C) Changes in Superoxide dismutase (SOD)

In toxicity-induced rats with spirotetramat, SOD levels significantly ($p < 0.01$) reduced (41.5% in testes tissue) in comparison with untreated rats. Administration of L₁ and L₂, separately and both revealed a significant ($p < 0.01$) rise in the level of SOD (47.5%, 44.5%, and 55.5%, respectively) in spirotetramat-induced toxicity in rats as compared to normal control.

D) Changes in Catalase (CAT)

CAT was found to be reduced significantly ($p < 0.01$) (32.09% in testes tissue) in comparison to normal rats. After the treatment with L₁, it resulted in a significant ($p < 0.05$, 41.05%) rise in CAT whereas in a combination of L₁ and L₂, and L₂ separately it showed a significant ($p < 0.01$) increase in CAT in spirotetramat induced rats in their tissue (55%) when compared to control.

E) Changes in Glutathione peroxidase (GPx)

A significant fall in GPx levels ($p < 0.01$) (71.20%) was reported in toxicity-induced rats as compared to normal rats. The oral gavage of L₁ and L₂, separately and in combination of both showed a significant ($p < 0.01$) rise in the testicular tissue (56.00%, 68.50%, and 90.50) as compared to the control.

Table 1: Probiotic (L₁ and L₂) control over oxidative stress in testicular tissue in Wistar rats.

Parameters	Control Group	Toxicity Group	L ₁ Group	L ₂ Group	L ₁ +L ₂ Group
LPO [#] Level	1.07±0.06	2.80±0.05 ^z	2.95±0.10 ^{*,z}	2.50±0.10 ^{**y}	1.90±0.10 ^{**x}
GSH ^{##} Level	5.50±0.05	2.30±0.25 ^z	4.90±0.20 ^{**z}	4.50±0.10 ^{**y}	5.90±0.50 ^{**x}
SOD ^{###} Level	11.50±0.05	6.70±0.15 ^z	10.90±0.10 ^{**z}	9.90±0.10 ^{**y}	11.90±0.15 ^{**z}
CAT ^{###} Level	28.5±1.10	19.9±0.45 ^z	24.90±2.00 ^{*,z}	25.50±2.5 ^{**z}	28.10±0.10 ^{**y}
GPx ^{###} Level	1.05±0.05	0.40±0.10 ^z	0.62±0.10 ^{**z}	0.50±0.10 ^{**z}	0.69±0.05 ^{**z}

#), (nmol MDA/h/g) tissue.

##), (µmol/g) tissue

###), (µmol/min/mg) Protein

L₁ Group = Induced with *L. Casei*, L₂ Group = Induced with *L. Coagulans*, L₁ and L₂ Group = Mixture of both probiotics.

Mean (\bar{X}) ±SEM (N=no of rats=6 rats/ group).

^x $p > 0.05$, ^y $p < 0.05$, ^z $p < 0.01$ compared to control, * $p < 0.05$,

** $p < 0.01$ compared to Toxicity control.

SEM=Standard error of mean,

LPO-Lipid peroxidation

MDA-Malondialdehyde

GPx-Glutathione peroxidase

GSH-Glutathione

SOD-Superoxide dismutase

CAT-Catalase

II) Effect of Spirotetramat on the total testes weights

A significant decrease in the relative weight of testes and epididymis on treatment with Spirotetramat was found ($p < 0.05$). However, no change in weight was witnessed in rats treated with L₁ and L₂ (Fig. 1).

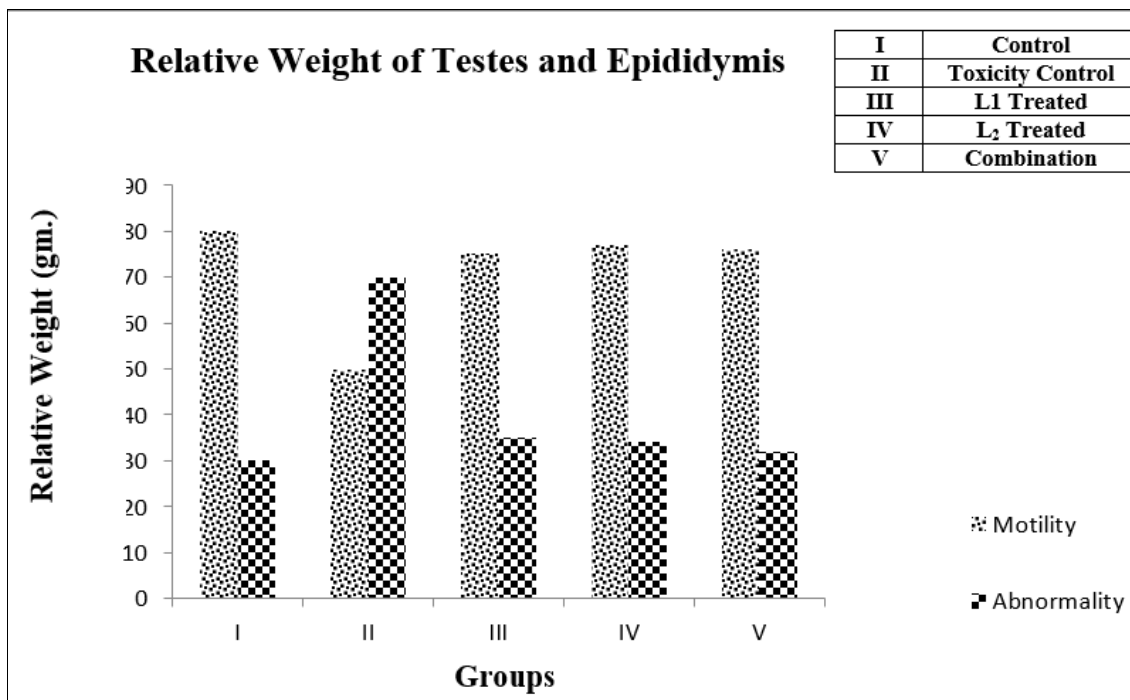


Fig 1: Effect of L₁ and L₂ on relative weights of testes in Wistar rats induced with Spirotetramat toxicity (mean ± SE **p*<0.05 with respect to control).

III) Defensive role of L₁ and L₂

In group II treated with Spirotetramat, there was a significant (*p*<0.01) reduction in number of sperms/gram of epididymus (Fig. 2) with increased abnormality (*p*<0.05) and decreased motility (*p*<0.001). However, a significant increase in number

of sperm cells was reported up to the mark of normal with enhanced motility and decreased abnormality with the treatment of L₁ and L₂ both in combination and individually (Fig 3). The treatment with L₁ and L₂ has reduced the changes brought about by Spirotetramat-induced parameters.

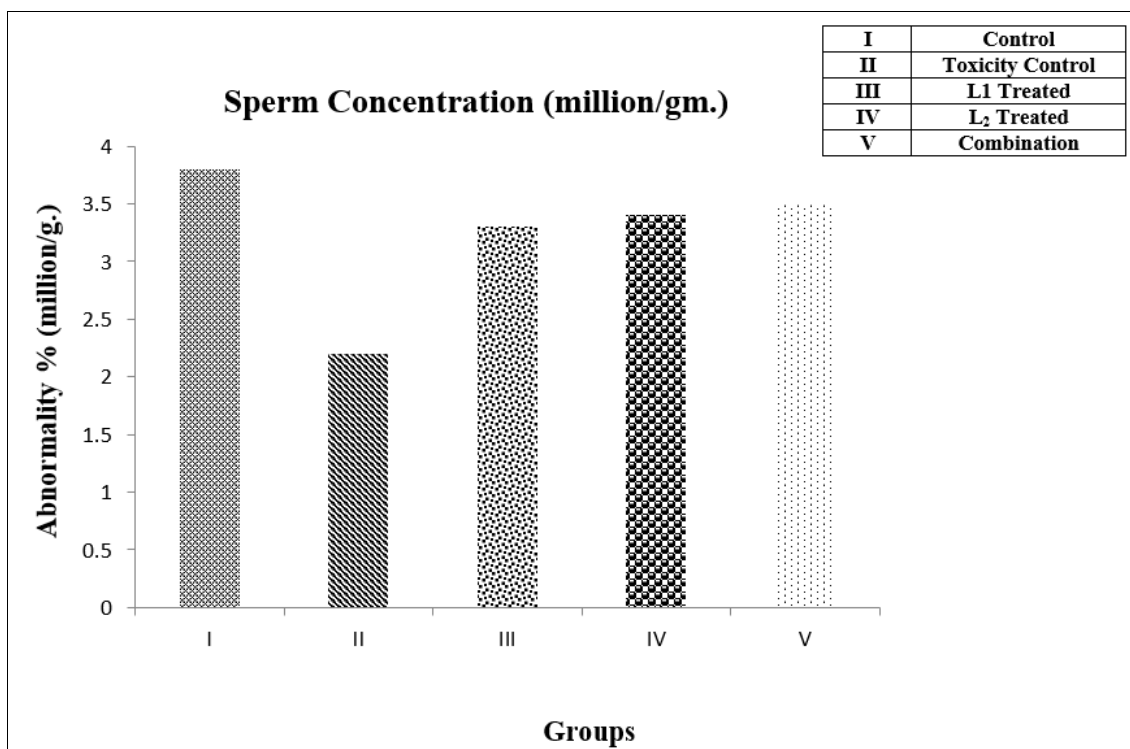


Fig 2: Change in epididymal Sperm concentration by L₁ and L₂ in Wistar rats induced with Spirotetramat toxicity (mean ± SE **p*<0.01) with respect to control.

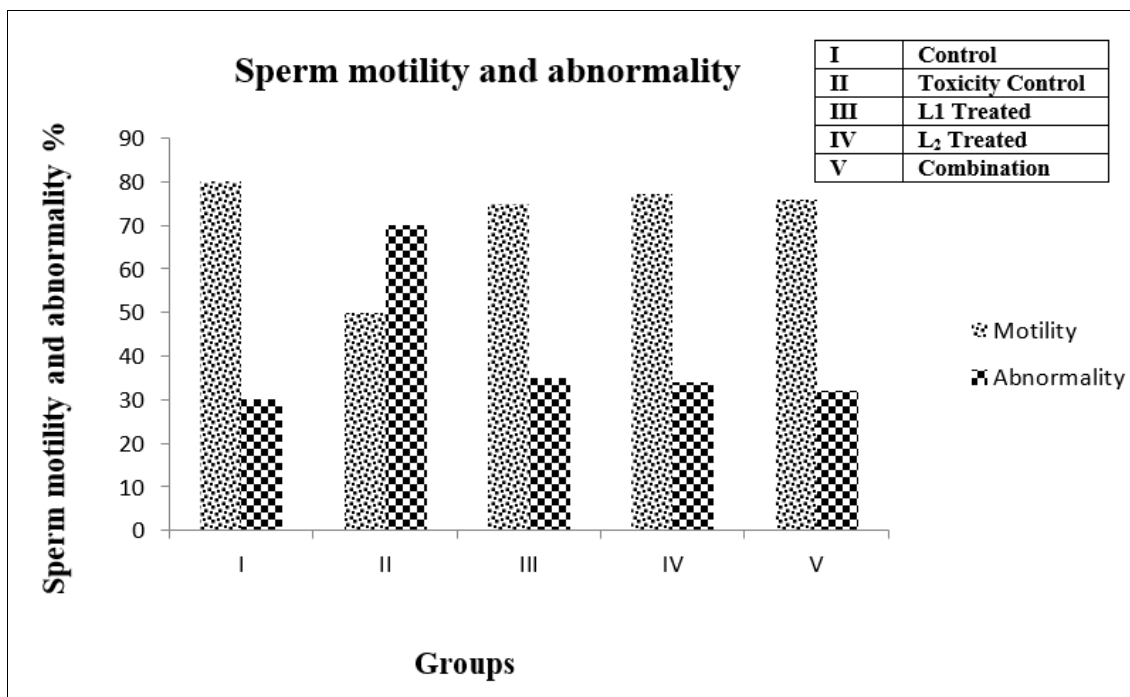


Fig 3: Change in epididymal Sperm motility and abnormality by L₁ and L₂ in Wistar rats induced with Spirotetramat toxicity (mean ± SE **p*<0.001) with respect to control.

IV) Change in Testosterone level

Testosterone level was found to be declined significantly (*p*<0.001) in the serum of rats induced with Spirotetramat as compared to the control group. However, a slight increase in

the levels of testosterone was observed in combination and individually treated groups with L₁ and L₂, possibly due to the toxicity of Spirotetramat when compared to the control (Fig.4).

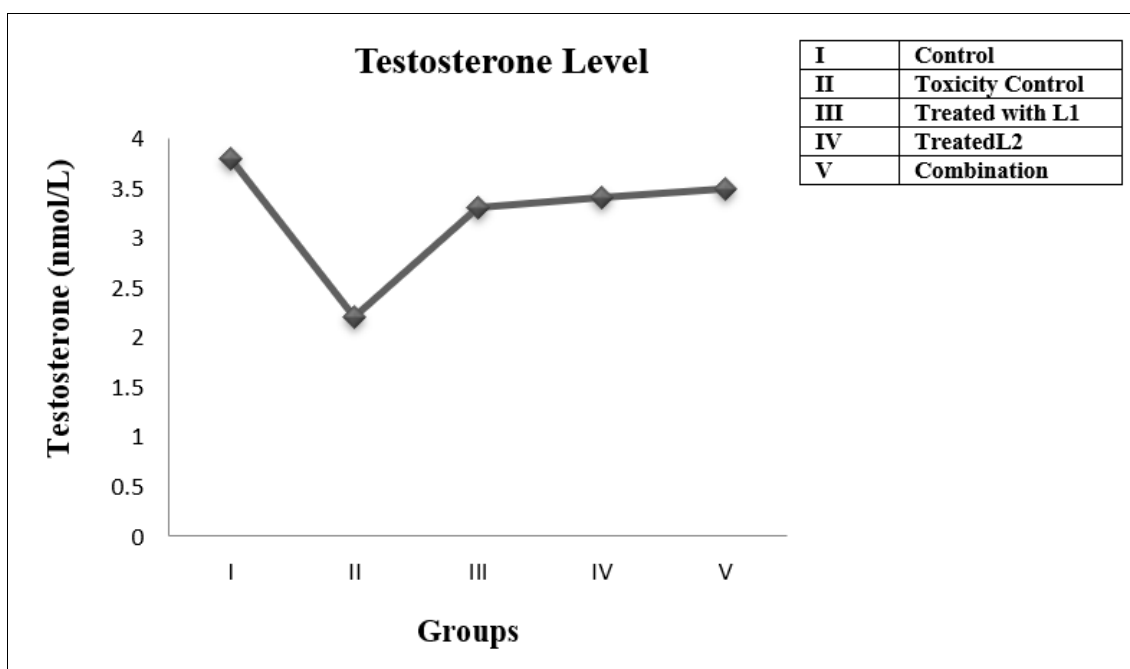


Fig 4: Change in Testosterone levels by L₁ and L₂ in Wistar rats induced with Spirotetramat toxicity (mean ± SE **p*<0.05) with respect to control.

V) Histological impact of L₁ and L₂

In toxicity control group, a scathing deterioration of seminiferous tubules with diminution and depletion in germinal epithelial cells and Leydig cells was reported as clearly visible; possibly due to action of Spirotetramat.

Among tubules, blood vessels have been found to be in a cramming position with cellular debris in the lumen. The groups treated with L₁ and L₂ displayed a standard testicular morphology and spermatogenesis with a trivial relapse of spermatozoa and spermatids (Fig 5).

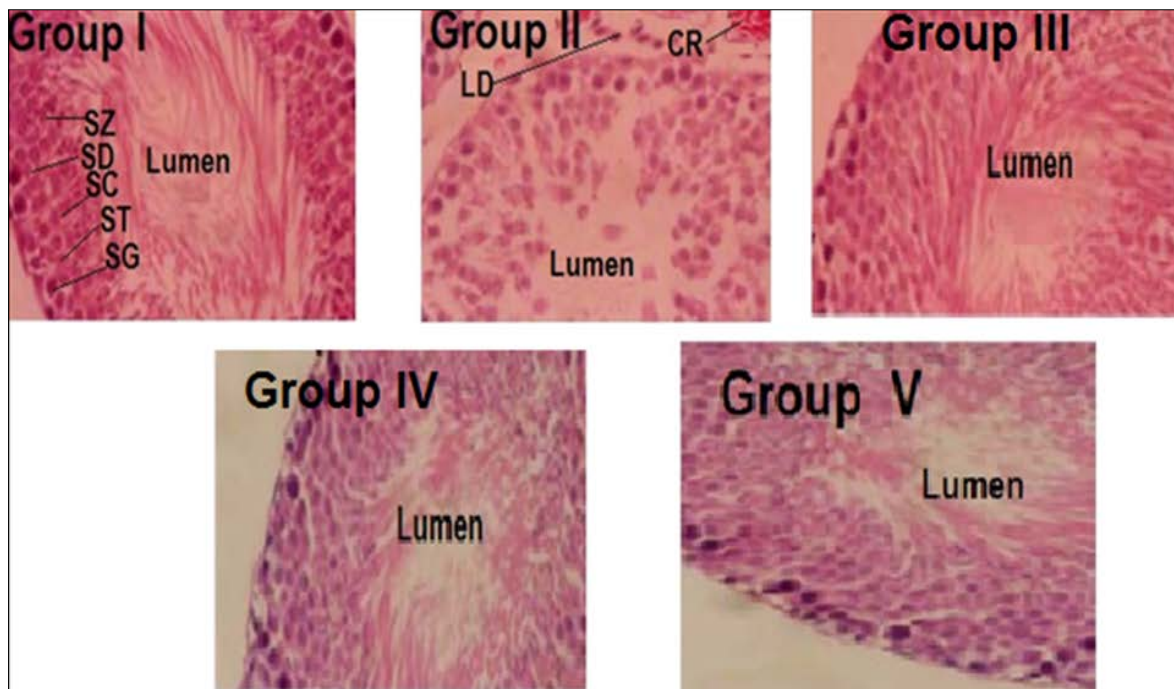


Fig 5: Exposure of the seminiferous tubules among different groups displaying normal to disrupted organization of germinal epithelium cells at different stages of spermatogenesis. Spermatogonia (SG), Sertoli cell (ST), spermatocytes (SC), spermatids (SD) and spermatozoa (SZ) Leydig cells (LD) and cramping of blood vessels (CR) between the tubules. (H and E, X400).

4. Discussion

Pesticides are chemicals that not only affect the target organisms but also impact non-target organisms. Spirotetramat, an insecticide is usually meant to deal with a group of agricultural insects like aphids, whiteflies, bugs etc. to minimize their damage to crops (Oyang *et al.*, 2012)^[13]. It causes several defects in organisms like the liver and genitals in rats (Liu *et al.*, 2011)^[16]; accumulated as metabolites (Wu *et al.*, 2012)^[17] and activated acid phosphatases (Liu, 2011)^[16] and oxidative injury by producing free radicals behaviour (Zepeda *et al.*, 2017; Mostafalou *et al.*, 2013)^[33-34]. These free radicals cause oxidative deterioration *viz.* LPO (Oberley, 1988)^[35]. Therefore, concern about the use of Spirotetramat for reproductive toxicity is increasing day by day. So there is an immediate need to find out alternative therapy to combat with the issue. Probiotics have performed a significant role in contribution to the control of reproductive toxicity that has been demonstrated clinically. (Mengheri, 2008)^[36]. In rats, probiotics have been reported to act against various intolerances like dyslipidemia (Yadav *et al.*, 2007)^[37]. Along this study, the LPO was found to be increased in tissue after exposure to Spirotetramat which reflects that generation of free radicals might be responsible for this elevation. After administration of probiotics, the level of LPO was found to be significantly decreased in treated rats. This reflects that probiotics have an ameliorative role in decreasing LPO. Reduced Glutathione (GSH) performs in contradiction of reactive oxygen species (ROS) (Yadav *et al.*, 2008)^[38] and its levels in present study have been found to be reduced due to Spirotetramat. However, probiotics reversed the said change by decreasing the stress and biosynthesis of GSH. Decrease in GPx by free radical production (Anuradha and Selvam, 1993)^[39] was also found to be elevated by the administration of probiotics. Like-wise CAT and SOD levels have also been reported to be increased significantly using probiotics. The results also reveal that a significant change in morphology, motility, count of sperm cells and a decrease in testosterone

levels after administration of spirotetramat. Co-administration of probiotics in Spirotetramat-induced testicular toxicity shows a decrease in histopathological changes with protective cover against Spirotetramat in bringing down the cell count. To conclusion, it may be expected that oxidative stress contributes to the testicular toxicity induced by Spirotetramat in male wistar rats. Probiotics have shown a protective role to fight against Spirotetramat-induced testicular toxicity and oxidative stress in rats.

5. Future Scope

Spirotetramat is a recently announced insecticide in the fields of agriculture that has severely affected non-target organisms, so, the substitute use of probiotics can lessen the ill properties of non-target organisms hence deprivation of biodiversity and conservation of ecological balance can be reinstated to some amount.

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7. Conflict of Interest: None.

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