

# Effect of Probiotics on Allethrin Toxicity: an *In Vivo* Study Using Zebrafish Model

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**Abstract:** Allethrin was widely used in pest management. However, human exposure to the compound shows specific effects. However, few studies have been conducted concerning the effects of allethrin exposure on human health. The time and cost required to conduct these studies in traditional mouse models may partially explain the lack of developmental toxicity research with allethrin. Zebrafish is a cheaper and shorter alternative to rat models, and its use as a model for developmental toxicity research has gradually increased. Considering the above, the goal of this research is to evaluate the protective effects of probiotics in reducing allethrin toxicity by observing different morphological parameters of Zebrafish (*Danio rerio*) after 30 days of treatment of allethrin and probiotics (Individual and combined effects); The protective effect of probiotics in reducing allethrin toxicity by analyzing protein metabolism of treated liver homogenate and blood biochemical parameters of allethrin and probiotic treated (individual and combined effects) Zebrafish. The histopathological findings in the liver of allethrin and treated probiotics (individual and combined effects) are studied in detail; They alternated in the metabolism of proteins and liver-homogeneous antioxidant enzymes allethrin and treated probiotics (individual and combined effects) zebrafish. In conclusion, the zebrafish offers a potent *in vivo* laboratory model for evaluating the side effects of a diverse range of drugs and measuring treatment effectiveness.

**Keywords:** toxicity; allethrin; biochemical parameters; probiotics; histopathology.

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## 1. Introduction

Allethrins are a class of insecticides with structurally similar synthetic chemicals [1]. They are called pyrethroids because they are synthetic versions of pyrethrin, an insecticide that occurs naturally in chrysanthemum flowers [2,3]. Allethrin contains eight stereoisomers in a racemic combination [4]. Some are also sold individually (d-allethrin, bioallethrin, esbiothrin, and S-bioallethrin), showing market value [5–8]. Allethrin is a non-systemic insecticide that is almost primarily used to control flies and mosquitoes in homes and gardens and to control flying and crawling insects in conjunction with other pesticides [9–11]. Allethrin d-trans-isomer is the most toxic structural form of allethrin used in animal systems to control parasites [12–15]. There are mosquito coils, mats, oil formulations, and aerosol sprays to choose from.

The sensible use of allethrin-containing products was considered safe, but as the marketing and demand for these products developed, so did the effective residual level of insecticides, which are significant food and water pollutants [16]. In addition, it can be neurotoxic because it stimulates neurons by changing the membrane's permeability to Na<sup>+</sup> and K<sup>+</sup> ions [17,18]. Overexposure to allethrin is a primary cause of skin and respiratory allergies [19,20]. Because inhalation exposure to allethrin and other pyrethroids in confined and poorly ventilated places is the most common and quickest route of exposure, it is expected to be a significant source of severe poisoning cases in humans [21,22]. After exposure, common symptoms are headache, dizziness, palpitation, chest tightness, and, less frequently, tiredness and poor vision. The epidermis absorbs and accumulates huge quantities of these hazardous materials. This is a slow process, but it is a common hazard to anyone working with chemicals or sprayers.

Ingestion is exceedingly uncommon and inadvertent, but it can cause life-threatening symptoms, including sore throat, nausea, vomiting, stomach pain, oral ulcers, increased secretions and/or dysphagia, coma, and convulsions [23,24]. Several animal species serve as experimental models for biomedical research. Animal models ensure that the results of *in vitro* or rodent studies are consistent and accurate. The zebrafish has become a popular model for scientific study [6,25]. Zebrafishes are a kind of tropical freshwater fish that are linked to minnows. Because it was easier to handle genetically than mice, George Streisinger (University of Oregon) used zebrafish as a biological model for the first time in the 1970s [26].

Despite their relevance as a biological model, zebrafish present many disadvantages, including organ dissimilarity, especially in the respiratory and reproductive systems [27]. Using zebrafish as a model for breathing or human reproduction isn't easy. When administered in sufficient amounts, probiotics are beneficial living bacteria with health benefits to the host [28]. In the past decade, probiotics have become increasingly popular as dietary supplements and treatments for several infectious diseases [29–31]. The most commonly investigated and marketed probiotics are lactic acid bacteria (LAB) and bifidobacteria [32,33].

Only a few spore-forming species of the genus *Bacillus* are used, despite the spore-forming nature of *Bacillus* spp. offer significant advantages over LAB as probiotics. *Bacillus licheniformis* is a bacterial species that may be found in the soil [34]. It's a gram-positive mesophilic bacteria. The probiotic *B. licheniformis* is utilized in animal feed. Isolates have been proven to prevent illness and promote growth and are commercially available. Although many clinical investigations have yet to be completed, certain isolates are probiotic in humans (and are also commercially available) [35]. In light of the foregoing, the major goal of this study is to determine if probiotics have any protective benefits in lowering allethrin toxicity with zebrafish as a model system.

## 2. Materials and Methods

### 2.1. Maintenance of test species and exposure to a toxicant.

Before being employed in the testing, adult zebrafish were introduced to soft water in a laboratory environment for two weeks after being purchased from a local commercial dealer. The fish were then kept in aquaria at a temperature of 26°C in well-oxygenated water (pH 7.0) with a 14:10 h light: dark cycle. Respective probiotic bacteria were grown in 5ml broth overnight at 37°C using aerobic conditions at 200rpm. The following day turbidity was determined, 100ml of suspension culture was centrifuged, and the OD value was recorded at

600nm. 100 ml suspension was centrifuged at 5000 rpm for 10 minutes. Pellet was dissolved in saline water (pH 7.5). The fish were fed a commercial diet (TetraBits, Melle, Germany) and live blood worms regularly. Water temperature was kept constant at 22–24°C; pH was kept at 6.8– 7.2; dissolved oxygen was kept at 5–7 mg/L, and conductivity was kept at 650 IS/cm.  $1 \times 10^8$  CFU of probiotic bacteria, which were dissolved in the saline water (pH 7.5), has been added directly into the water. Heavy metal concentrations were near nil or not detectable, and the water composition met the fishery water quality requirement. Every day, the water in each group was entirely replaced, and the aquaria were properly cleaned. The chemicals and solvents were purchased from commercial vendors and are analytical grade. Water that contains probiotics has been changed every day, thus reducing the excess feed of probiotics which may result in contamination. In general, if contamination is detected, based on the type of contamination, antibiotics such as ampicillin or streptomycin can be used [36].

### 2.2. Experimental groups and sample preparation.

Zebra fishes were randomly divided into four groups, as given below, with 07 fish in each group and seven fish livers used to compose one sample (n=7 pools).

Group 1: Control - (Untreated): Not exposed to allethrin or probiotic

Group 2: Allethrin exposed (10 mg/per liter daily for 30 days)

Group 3: Treated with Probiotic (*Bacillus licheniformis* -  $1 \times 10^8$  CFU/L for 30 days) – Gram-positive

Group 4: Treated with both probiotic *B. licheniformis* (containing  $1 \times 10^8$  CFU/L) and allethrin exposed (10 mg/per liter).

### 2.3. Estimation of growth and survival rate.

The early embryonic and adult phases of fish differ significantly in appearance and physiology. As a result, those phases may be impacted differently and, therefore, more vulnerable to hazardous substances. We investigate how changes in body mass-related metrics reflect the reorganization process throughout development, given that the fish embryo's growth occurs without external resources and relies solely on endogenous supply. After 30 days of therapy, I measured the body length (from mouth to caudal peduncle), body weight, and survival rate of fish in all four experimental groups [37].

### 2.4. Estimation of protein content (Total- structural & soluble proteins).

Proteins are high-molecular-weight chemical molecules that are highly complicated. They are composed of carbon (C), hydrogen (H), and oxygen (O), as well as around 16% nitrogen (N: range 12–19%), phosphorus (P), and sulfur (S). Proteins have a different fundamental structure than other physiologically essential macromolecules like carbohydrates and lipids. Soluble proteins have more than 70% solubility, while insoluble proteins have less than 30% solubility. A distinctive amino acid pattern or motif in structural proteins forms a skeleton to the functional properties of a multicellular organism, cell, or substance [38]. The shapes and sizes of soluble proteins vary, and each has a unique set of functions [39]. The total protein test measures the quantity of two different types of proteins in the fluid portion of your blood. They are albumin and globulin. Lowry *et al.* (1951) estimated total protein in liver homogenate using BSA as a standard [40].

### 2.5. *Blood biochemical assay.*

Blood was taken from treated zebrafish into separate tubes without anticoagulant and centrifuged at 2200-2500 RPM for 15 minutes, with the serum obtained being utilized for biochemical analysis. All blood parameters were calculated using diagnostic kits provided by SD fine, kanbaxy, span diagnostics ltd., India, according to the kit's instructions.

### 2.6. *Measurement in blood.*

The plasma and leukocyte layers are separated from venous blood containing heparin or citrate. Three times with isotonic NaCl, the erythrocyte sediment is rinsed. Adding four parts by volume of distilled water to a stock hemolysate containing -5 g Hb/100 ml creates a stock hemolysate containing -5 g Hb/100 ml. Before the test, a 1:500 dilution of this concentrated hemolysate is produced with phosphate buffer, and the Hb (hemoglobin) concentration is measured in duplicate (e.g., by the method of Drabkin). 0.1 or 0.02 ml capillary blood is hemolyzed in 250 or 50 ml distilled water. The hemoglobin content of the blood must be measured in a separate blood sample if it is to be used as a reference point.

### 2.7. *Measurement in tissues.*

Catalase in tissues with relatively high activity, such as the liver and kidney, can be measured spectrophotometrically if complete lysis of all organelles and clear (or very faintly colored) solutions or extracts can be produced. A detergent (e.g., 1 percent Triton X-100) must be used in the production of the stock homogenate (1 + 9 or 1 + 19); otherwise, too low values will occur. Further dilutions can be produced with phosphate buffer, pH 7.0 (1: 100 to 1:500, depending on tissue and species) (1: 100 to 1:500, depending on tissue and species). However, if the material after organelle lysis cannot be diluted to this level, the significant UV absorption of Triton X-100 must be considered. Alternatives include digitonin (0.01 percent) and sodium cholate (0.25 percent). Catalase activity in tissue samples is often reported in milligram wet weight or milligram total N [41].

### 2.8. *Histopathology studies of the liver.*

Hematoxylin must be dissolved in both hot water and pure alcohol. Combine the two solutions, and then bring them to a boil. Remove from the heat, mix in mercuric oxide, and quickly cool. If glacial acetic acid is added, rapid nuclear staining results, but the solution's shelf life is shortened. Therefore, if any, acetic acid should be added to the working solution. The counterstain that turns the cytoplasm rose-colored is eosin. Individuals can choose the eosin's intensity. "Eosin Y" is the most often utilized eosin. Yellowish is denoted by the letter "Y." It can be found in forms that are either alcohol- or water-soluble. The water-soluble version of eosin Y is often used in the alcohol-water solution that is described here. Eosin should be dissolved in water and added to 95% alcohol (one part eosin solution with four parts alcohol). Add a few drops of acetic acid to the finished mixture (0.4ml). The acetic acid makes eosin's staining more intense. The stain should be hazy when ready to use; if it is clear, add a few drops of acetic acid. Staining the control slides will standardize the solution.

2.9. Method of staining.

Sections are deparaffinized in xylene for 10 to 20 minutes. Clean the hematoxylin. Sections for hydration: For one to two minutes, consume 100% or 95% alcohol. Rinse first with distilled water, then with tap water. Hematoxylin stain for three to five minutes. Use tap water to wash. Check under a microscope after differentiating the segment with 1% HCl in 70% alcohol 1-2 dips. Return slides to HCl if more distinction is required. Slides should be washed for 15 minutes under running water. Slides should be stained with eosin for 1-4 minutes. Differentiation and dehydration alcohol, 95% 5-6 dips Absolute alcohol 5-6 dips. Two times in xylene, clear slides. Slides should be mounted using a mounting medium (Permount or DPX), and 40X magnification should be used for observation. The means of triplicates were used to calculate all experimental data in the results.

3. Results and Discussion

3.1. Growth & Survival Rate

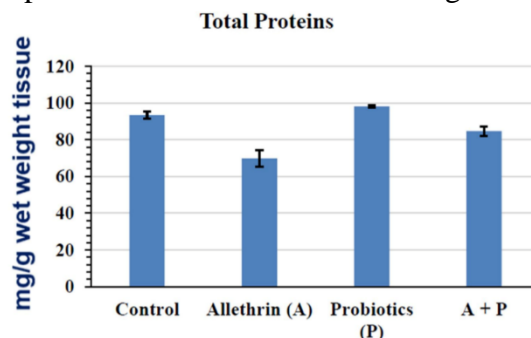
Before zebra fish treatment, the body weight (g) and the body length (cm) in Group-1, Group-2, Group-3, and Group-4 are within normal limits. The findings of 30 days of therapy revealed an increase in body weight (g) and body length (cm) in all four experimental groups. The survival rate (%) of zebra fishes after 30 days of treatment was found to be normal in Groups 1 & 3, liberal in Group 4, and shortened in Group 2, as shown in Table 1.

**Table 1.** Growth and survival rate of zebrafish before and after 30 days of treatment.

Parameter	Group-1 (control)	Group-2 (A)	Group-3 (P)	Group-4 (A+P)
Body weight (g)	Before treatment			
	0.18 ± 0.01	0.20 ± 0.01	0.19 ± 0.02	0.20 ± 0.01
	After treatment			
	0.45 ± 0.03	0.36 ± 0.10	0.51 ± 0.05	0.41 ± 0.03
Body length (cm)	Before treatment			
	0.75 ± 0.03	0.82 ± 0.02	1.04 ± 0.01	0.89 ± 0.05
	After treatment			
	1.23 ± 0.04	0.95 ± 0.05	1.18 ± 0.12	1.08 ± 0.05
Survival (%) After treatment	100	80	100	95

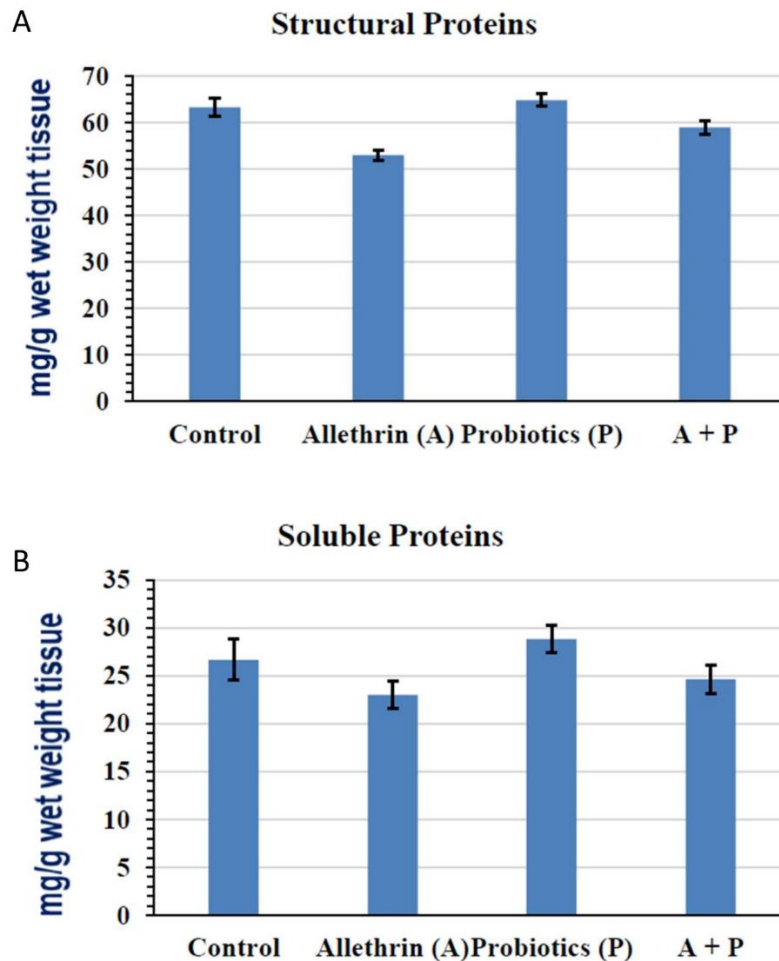
3.2. Total protein content.

In biochemistry research and development laboratories, measuring the protein concentration in an aqueous sample is crucial for uses ranging from enzymatic investigations to giving information for biopharmaceutical lot release. Hence, the total protein content was estimated, including both the structural and soluble proteins, after 30 days of treatment with probiotic and allethrin compounds. The values obtained are given in Figure 1.



**Figure 1.** Total protein content in liver homogenate after treatment.

The graph showing the total protein content in control and probiotic-treated groups was found to be normal. The total protein content of Group-4 was high compared to the total protein content of Group-2, which had been allethrin-treated. When compared to Groups 1 and 3, it was also low. Structural & soluble protein content in liver homogenate after 30 days of treatment is shown in Figures 2A & 2B. Figure 2A shows the level of structural protein was normal in the control and probiotic-treated groups, liberal in the combined A+P group and low in the allethrin-exposed group. Figure 2B shows the amount of soluble protein in control and probiotic-treated groups was normal; it was liberal in the combined A+P group and low in the allethrin-exposed group.



**Figure 2.** (A) Structural & (B) soluble protein content in liver homogenate after 30 days of treatment.

### 3.3. Blood Biochemical Assay

A blood biochemical assay was performed after 30 days of treatment to fish. Table 2 shows probiotics' effect on zebrafish's allethrin toxicity, as measured by hematological parameters.

**Table 2.** Effect of probiotics in reducing allethrin toxicity with reference to hematological parameters in zebrafish.

Blood parameter	Group -1	Group -2	Group -3	Group -4	Range (%)
	(control)	(A)	(P)	(A+P)	
<b>White blood cell differential counts</b>					
<b>Lymphocytes (%)</b>	78.23 ± 2.12	61.21 ± 2.31	79.28 ± 2.75	70.91 ± 2.34	71-92
<b>Monocytes (%)</b>	09.15 ± 0.91	4.74 ± 0.74	12.14 ± 1.21	8.91 ± 1.42	44696
<b>Neutrophils (%)</b>	12.31 ± 0.71	6.45 ± 1.14	13.18 ± 1.21	09.12 ± 0.48	44610
<b>Eosinophils</b>	0.91 ± 0.02	0.69 ± 0.02	1.07 ± 0.17	0.85 ± 0.0	0-2
<b>Basophils</b>	0.86 ± 0.03	0.28 ± 0.03	1.21 ± 0.12	0.54 ± 0.04	0-2

Serum Biochemical analytes					
Albumin	1.8 ± 0.06	0.95 ± 0.08	1.8 ± 0.45	1.31 ± 0.14	3.3 g/dl
ALP	6.32 ± 0.41	3.31 ± 0.31	6.45 ± 0.32	4.14 ± 0.09	2.0 – 10.0 U/L <sup>3</sup>
ALT	351 ± 7.21	241 ± 4.21	365 ± 6.36	318 ± 5.23	343.0-410.0 U/L
Total bilirubin	0.42 ± 0.03	0.18 ± 0.02	0.45 ± 0.01	0.31 ± 0.01	0.2 – 0.6 mg/dl
BUN	3.1 ± 0.21	2.14 ± 0.03	3.95 ± 0.7	2.82 ± 0.31	3.0 – 4.0 mg/dl
Calcium	12.5 ± 1.64	08.31 ± 1.31	13.81 ± 1.12	10.32 ± 2.41	10.3-18.6 mg/dl
Phosphorus	21.4 ± 1.18	18.32 ± 1.45	23.21 ± 1.81	20.14 ± 1.15	20.3-24.3 mg/dl
Glucose	90.21 ± 5.14	71.12 ± 1.48	92.27 ± 1.45	89.12 ± 1.23	91.0 g/dl
Potassium	5.23 ± 0.12	4.42 ± 0.23	6.45 ± 0.23	5.01 ± 0.01	5.2 – 7.7 mEq/L
Total Protein	4.2 ± 0.14	3.91 ± 0.21	4.61 ± 0.05	0.42 ± 2.14	4.0 – 5.8 g/dl

The kind of component and its associated range are represented in the first and last columns. The WBC differential count for all groups is in the normal range after 30 days of therapy, although the count in Group-2 fishes has decreased. The same is true for serum components. Group 4 has a lower range of components than Groups 1 and 3 but a higher range than Group 2.

### 3.4. Antioxidant enzyme levels.

These enzymes' levels rise only when the body is exposed to a harmful substance. When undesirable free radicals are present in the body, these enzymes become active and remove them. When compared to other groups, the levels of lipid peroxidation, glutathione peroxidase (GPx), glutathione reductase (GR), catalase, and superoxide dismutase (SOD) were considerably higher in Group-2 fishes (Table 3). The enzyme levels in Group-1 fishes that were not treated (control) were normal, whereas the liver enzymes in Group-4 (A+P) were somewhat higher.

**Table 3.** Antioxidant enzyme levels in liver homogenate.

Liver	Lipid peroxidation nmol MDA/g wet wt	Glutathione peroxidase (GPx) nmol min-1 mg protein-1	Glutathione reductase (GR) nmol min-1 mg protein-1	Catalase nmol min-1 mg protein-1	Superoxide Dismutase (SOD) Unit inhibition/min/mg (tissue)
Group -1 (control)	245.63 ± 5.26	15.82 ± 1.23	10.21 ± 1.28	160.43 ± 7.28	0.42 ± 0.01
Group -2 (A)	278.45 ± 3.42	19.43 ± 1.42	14.61 ± 0.81	231.61 ± 5.74	1.13 ± 0.21
Group -3 (P)	231.23 ± 3.54	14.84 ± 2.64	10.72 ± 1.45	171.31 ± 2.93	0.38 ± 0.01
Group -4 (A+P)	253.78 ± 5.24	17.27 ± 1.22	12.23 ± 1.24	210.42 ± 5.21	0.810.04

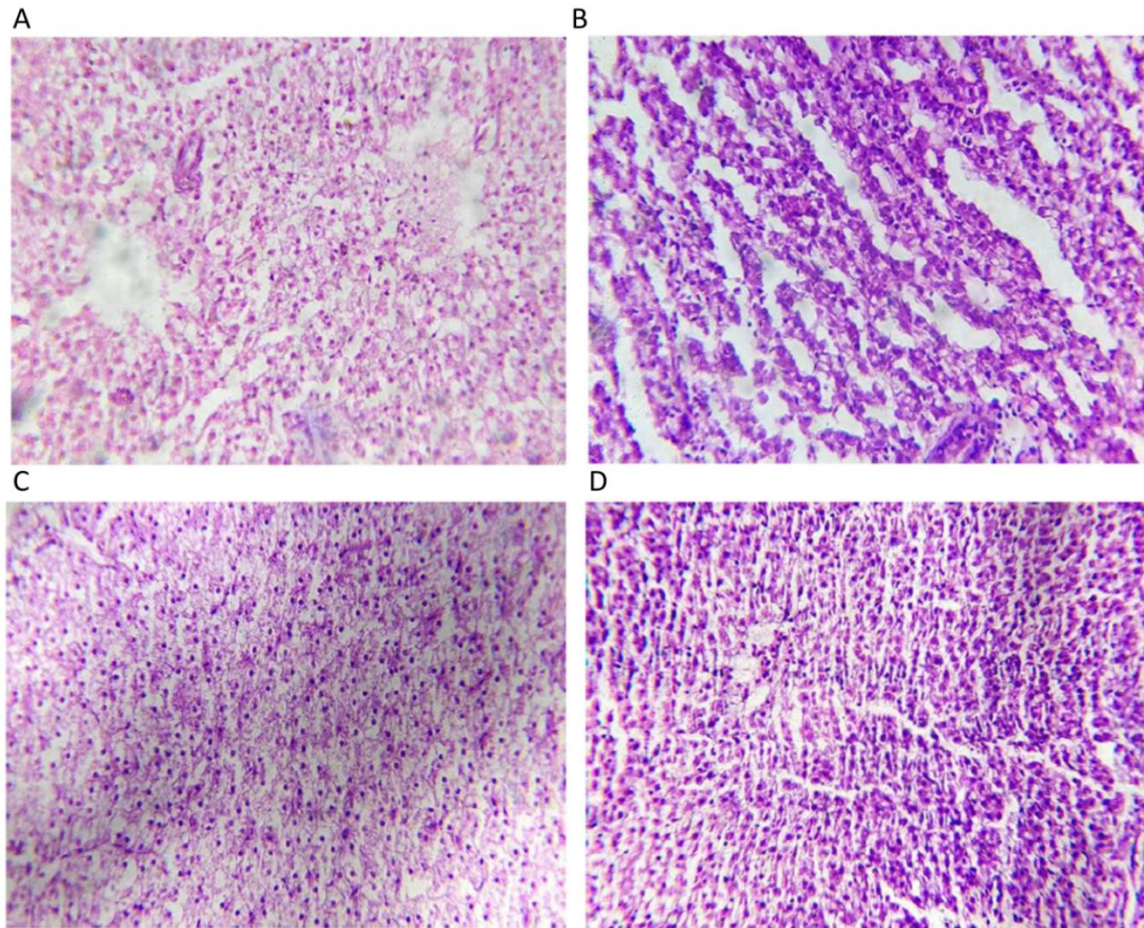
### 3.5. Histopathological studies of liver tissue.

The hepatocyte cells in zebrafish liver tissue after 30 days of therapy are shown in the figures below. The images were captured at a magnification of 40X. Untreated (control) fish's liver tissue cells showed normal, healthy cells in Figure 3A. The liver tissue cells were destroyed in Figure 3B as a result of exposure to the allethrin compound. Because of the probiotic therapy, the cells in Figure 3C were healthy and compact. The liver tissue cells in Figure 3D were injured and then regenerated.

## 4. Discussion

Several pyrethroids are harmful. However, little study has been done on the effects of developing pyrethroid exposure. The time and cost required to conduct these investigations in conventional mouse models may explain the shortage of developmental toxicity research with pyrethroids. Zebrafish are a less expensive and time-consuming alternative to rat models, and

their usage as a model for developmental toxicity research has been gradually growing. Zebrafish were used as an animal model to mimic the changes inside the human body as their genome is much more similar to humans. Most fish toxicity studies have been conducted on an adult or adolescent fish. However, utilizing live fish has been criticized for being unethical. The most promising alternative method to the traditional acute fish toxicity assessment using live fish has been identified as the zebrafish toxicity model. A greater fecundity and quick growth have made it possible to evaluate more samples in a shorter amount of time.



**Figure 3.** Liver tissue changes after treatment. (A) Group – 1 (control); (B) Group – 2 (Allelethrin exposed); (C) Group – 3 (Probiotic treated) and (D) Group – 4 (Allelethrin + Probiotic treated).

The compound allelethrin was commonly used to control pests. However, exposure of humans to this compound shows some effects. So the present study helps to investigate the allelethrin toxicity when exposed to lower concentrations (10 mg/per liter) of zebrafish for 30 days. Also, it helps to analyze the probiotic ability to reduce the adverse effects of allelethrin. In this study, after treatment for 30 days at all the optimal conditions, the allelethrin has adversely affected the morphological characteristics of zebrafish, showing a decrease in length and weight of the body; however, treatment with probiotics has reduced these adverse effects of allelethrin. Each component of protein metabolism is also reduced when treated with allelethrin, but when treated with probiotics, it shows an increase to the normal range. Levels of antioxidant enzymes and blood biochemical properties also confirm the synergistic properties of probiotics in reducing allelethrin toxicity. This says that the allelethrin causes toxicity on exposure. Throughout this study, it was confirmed that treatment with probiotics reduces the adverse

effects of allethrin. Further in-depth animal model studies are required to confirm the probiotic ability to reduce allethrin toxicity with exact mechanisms.

Due to its tiny size, genetic makeup, superior breeding skills, and, most significantly, its molecular pathways and physiological resemblance to humans, the zebrafish is an excellent candidate for biosafety investigations. Zebrafish has emerged as a model for evaluating the cardio-, neuro-, or genotoxicity of medications, which is indicative of its benefits over other animal models in terms of the 3Rs (replacement, reduction, and refinement). We stress the zebrafish model as a superior vertebrate toxicological model with the potential to considerably enhance drug discovery in toxicology in light of these benefits. As consumer demand for probiotic meals grows, numerous new foods are anticipated to incorporate probiotics in the near future. Before commercializing new probiotic cultures for human and animal consumption must be thoroughly and accurately tested for safety and effectiveness. According to several studies, some *Bacillus* strains with a history of safe usage in the food industry are increasingly being integrated with health-promoting "functional foods" to give digestive and immunological health advantages. *Bacillus* can also be used as a medicinal, preventive, and growth supplement for animals and humans as an alternative to frequently used LAB.

## 5. Conclusions

Finally, the zebrafish is excellent *in vivo* preclinical model for testing the harmful effects of various medications and determining treatment effectiveness. The probiotic *Bacillus licheniformis* helps in reducing the toxic effects. But more research needs to be done to determine the lethal concentration of the allethrin compound.

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## Conflicts of Interest

The authors declare no conflict of interest.

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