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Histopathological, Pathomorphological and Oxidative Stress Responses of Glyphosate Exposed Probiotics Fed *Clarias gariepinus*

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ABSTRACT: This study assessed changes in tissues and antioxidant enzymatic activities of glyphosate exposed probiotics fed *Clarias gariepinus.* Sublethal exposure of 1/10 and 1/100 LC_{50} at 0.1%, 0.15% and 0.2% probiotics inclusions was carried out for 28days. Histological and oxidative stress biomarkers were determined using standard methods. Histopathological as well as structural changes and antioxidant enzyme activities in studied organs were dose dependent considering the concentration and percentage of glyphosate and probiotics respectively. Exposure at 1/10 and 1/100 LC_{50} erodes and destroys tissue architecture in proportionate fashion expressed as lamella erosion and necrosis in gills and liver respectively with corresponding reduced SOD, CAT and GSH activities in the liver. Regeneration and healing of tissues were more pronounced at *C. gariepinus* fed 0.2% probiotics 1/10 LC_{50} exposure level as against 0.15% probiotics 1/100 LC_{50} exposure level. Antioxidant enzyme activities increased significantly ($p < 0.05$) in glyphosate probiotics fed *C. gariepinus* compared to 0% probiotics fed glyphosate exposed fish. CAT and GSH were higher at 0.2% probiotics compared to control. Level of peroxidation reduced as percentage of probiotics increased with control MDA value the highest compared to those at different inclusion levels. Conclusively, probiotics can help reduce cytotoxic effects of toxicants and stressors in *Clarias gariepinus* at relative inclusion levels.

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The use of chemical substances particularly herbicide for destroying, preventing or mitigating insects and weeds is on the increase worldwide in modern agricultural practices. Water bodies around the world are increasing getting polluted, more than ever in the history as well as the organisms' resident in them. Glyphosate, a typical herbicide have been implicated in causing harm to aquatic lives accessing water bodies via run-offs. It is a broad spectrum systemic herbicide and crop desiccants. It is an organophosphorus compound and a non-selective post emergence herbicide (Ayoola, 2008). Probiotics are non-pathogenic microorganisms that works to benefit the body/system through a number of processes, including reducing intestinal pH, preventing pathogenic organism colonization and invasion, and alter the host immune response (Dimitroglou *et al.*, 2011). Probiotic advantages specific to one species or strain may not apply to another (Williams, 2010). *Saccharomyces cerevisae* (baker's yeast), probiotics

used in this study is a single cell eukaryotic fungus that has the ability to reduce the expression of stress related and inflammatory cytokines in the gut making increase in the weight of fish possible regardless of stressors presence (Ren *et al.,* 2016). These have led to an increased interest in the potential of functional feeds as health promoters. Probiotics improves animal health and well-being (Dawood and Kashio, 2016). *Clarias gariepinus* commonly known as the African sharp tooth catfish is a species of catfish of the family Clariidae, and it's a preferred species for this study due to its several biological characteristics (Skelton, 1993) and its essential economic importance in both fisheries and aquaculture (Teugel *et al.,* 2001). The study therefore assessed the tissue repair and ameliorating effects of probiotics in *Clarias gariepinus* exposed to glyphosate.

MATERIAL AND METHODS

Test Organisms: Clarias gariepinus (fingerlings) was gotten from a commercial fish farm outlet at St. Finbarrs Akoka, Pako bus stop, Lagos state Nigeria. The fishes were transported using plastic containers from the location to the Environmental Biology laboratory, Yaba College of Technology where they were temporarily acclimatized before moving the fish to the experimental set up behind the school college hall.

Preparation of Test Compounds: The commercial formulations of glyphosate were purchased from an outlet in Lagos, Nigeria. Fish meal was obtained from an Agricultural store. The fish meal was grounded to a fine-powder, then incorporated with probiotics - baker's yeast (*Saccharomyces cerevisiae*) at 0.1%, 0.15% and 0.20%. Fish meal without probiotics acts as control. They were all pelletized to 2mm feed size to aid easy digestion.

Toxicity Tests: Range Finding: The concentration of the glyphosate used for the range finding test followed the methods described by Reish and Oshida (1986), Obuotor (2004). The selected fishes were acclimatized for a period of 4 days using dechlorinated water. The weight of the fish was determined every two days. The range finding test was carried out and it lasted for 2 days (48 hours). The test specimens were exposed to the different concentrations, which include 1ml, 10ml, 30ml and control, inside the 4 experimental tanks without feeding them. Observations were made and recorded. Toxicity range value was then estimated from the probit analysis and Spearman Karber method of estimating mortality results.

Acute Toxicity Test: 2 litres of water was measured into six experimental tanks and duplicated, for the concentration of 1ml, 3ml, 6ml, 12ml, 24ml and control for glyphosate. Seven (7) fishes were added to each tank. The five concentrations of each herbicide were administered to the fish tanks once and the response of the fishes was monitored for 96hrs. From the acute test, a probit analysis table was obtained and the LC_{50} (6.251ml/l) was determined from the table and also 1/10th (0.625ml/l), 1/100th (0.0625ml/L) of LC_{50} was obtained.

Sublethal Toxicity Test: 1/10 LC_{50} and 1/100 LC_{50} glyphosate concentration were used for chronic toxicity test probiotics, 0.1%, 0.15%, and 0.2% were equally used for the experiment. Seven fish of similar average weight were placed in each tank and 1/10 and 1/100 LC_{50} glyphosate dropped in respective tanks. Probiotics of different concentrations were added in experimental set-up and another set-up without probiotics (0%) stood as the control. Tanks were duplicated as A and B. Exposure lasted for 28days.

Histological Analysis: Cut-up: The samples were gross-examined for any observable lesion, cut-up/dissected into smaller portions if very big, and sampling areas with remarkable observations taken. They were then placed in a well labelled embedding tissue cassette before they were processed using the automatic tissue processor.

Tissue Processing (Microm STP 125. Thermo-fisher - USA): Tissues were then processed with a a 24hr-Automatic tissue processor.
The tissue processor held 12 beakers containing various grades of reagents, beaker 1 (formalin for further fixation, beakers 2,3,4,5,6,7 different grades of alcohol 70%, 90%, 95% and 3 x IPA, used for proper dehydration and two changes of absolute. Beakers 8 and 9 contains 2 changes of xylene, a clearing agent that clears and replaces the alcohol off the tissue sample. Beakers 10,11 and 12 contains 3 changes of molten paraffin wax at 60^0C ,an infiltrating/ impregnating agent.

Embedding: The samples after processing were embedded into paraffin blocks; using an automatic tissue embedding center.

Microtomy, rotatory microtome (Microm HM 325. Thermo-fisher - USA): Each block was placed on the rotary microtome and trimmed to expose their surfaces. The sections were cut at 2-3microns for lymph nodes and other tissues were cut at 4microns, they were gently placed on each well labelled slide and with the aid of a curved floating forceps, the sections were floated out on a hot water bath already maintained at 45^{o}c each, the labelled slides were then

used to pick the section that were free of creases, whilst ensuring that each of the sections adheres to the centre of the slide. Slides were subsequently dried on a hot air oven already maintained at 60^{o}c to ensure proper attachment (Bancroft *et al*., 2014).

Antioxidant Enzymes Assay: Homogenizing Sample: The organs were dissected and the liver and gills were removed, the fleshes of the animal were also cut. The post mitochondria fraction of the organs of the animal was prepared as fellow; the organs of the animal were washed in an ice cold 1.15% KCL solution, blotted and weighed. They were then homogenized with 0.1M phosphate buffer (pH 7.2), putting the organs each into the mortar; laboratory sand was added to it (acid washed sand) and it was blended in the mortar with pestle together. The resulting homogenate was centrifuge at 2500rmp speed for 15mins then it was removed from the centrifuge and the supernatant was decanted and stored -20oC until analysis.

Determination of Superoxide Dismutase (SOD) activity: Superoxide Dismutase activity was determined by its ability to inhibit the auto-oxidation of epinephrine determined by the increase in absorbance at 480nm as described by Sun and Zigma (1978). The reaction mixture (3 ml) contained 2.95 ml 0.05 M sodium carbonate buffer pH 10.2, 0.02 ml of liver homogenate and 0.03 ml of epinephrine in 0.005 N HCL was used to initiate the reaction. The reference cuvette contained 2.95 ml buffer, 0.03 ml of substrate (epinephrine) and 0.02 ml of water. Enzyme activity was calculated by measuring the change in absorbance at 480 nm for 5 min. $\sum = 4020M^{-1}\ cm^{-1}$.

Catalase activity Determination: Catalase activity was determined according to Sinha *et al*., (1972). It was assayed colorimetrically at 620nm and expressed as µmoles of H_2O_2 consumed/min/mg protein at 25^{0}C. The reaction mixture (1.5ml) contained 1.0ml of 0.01M phosphate buffer (pH 7.0), 0.1ml of tissue homogenate and 0.4ml of 2M H_2O_2. The reaction was stopped by the addition of 2.0ml of dichromate-acetic acid reagent (5% potassium dichromate and glacial acetic acid were mixed in 1:3 ratio).
$\sum = 40M^{-1}\ cm^{-1}$.

Reduced Glutathione Determination: The reduced glutathione (GSH) content of liver tissue as non-protein sulphydryls was estimated according to the method described by Sedlak and Lindsay (1968). To the homogenate 10% TCA was added, centrifuged. 1.0ml of supernatant was treated with 0.5ml of Ellmans reagent (19.8mg of 5,5-dithiobisnitro benzoic acid (DTNB) in 100ml of 0.1% sodium nitrate) and 3.0ml of phosphate buffer (0.2M, pH 8.0). The absorbance was read at 412nm.
$\sum = 1.34 \times 10^4\ M^{-1}\ cm^{-1}$.

Lipid Peroxidation: Malondialdehyde (MDA), an index of lipid peroxidation was determined using the method of Buege and Aust (1978). 1.0 ml of the supernatant was added to 2 ml of (1:1:1 ratio) TCA-TBA-HCl reagent (thiobarbituric acid 0.37%, 0.24N HCl and 15% TCA) tricarboxylic acid- thiobarbituric acid-hydrochloric acid reagent boiled at 100^{o}C for 15 min, and allowed to cool. Flocculent materials were removed by centrifuging at 3000 rpm for 10 min. The supernatant was removed and the absorbance read at 532 nm against a blank. MDA was calculated using the molar extinction coefficient for MDATBA- complex of $1.56 \times 10^5\ M^{-1}CM^{-1}$.

Determination of Glutathione –S- Transferase Activity: Glutathione –S- transferase activity was determined by the method according to Habig *et al.,* (1974).

Statistical Analysis: Data were analysed using SPSS version 2.0. The mean values were compared using Analysis of Variance. Means were presented as mean $\pm$ standard deviation while p-value was set at 0.05 level of significance.

RESULTS AND DISCUSSION

Histopathology of Gills: Gills Of Clarias gariepinus Exposed To 1/10 LC_{50} Glyphosate: Figure 1 shows the histopathological slides of the gills of *Clarias gariepinus* exposed to 1/10 LC_{50} of glyphosate contamination and fed with probiotics supplemented feed. Necrosis and destruction of both primary and secondary lamellae (severe lamellar necrosis) in the control *C. gariepinus*. Those fed with 0.1% and 0.15% probiotics supplemented feed showed mild lamellar necrosis of the gills while those fed with 0.2% probiotics supplemented feed showed relative clear gill architecture.

Gills of Clarias gariepinus exposed to 1/100 LC50 glyphosate: The results of histopathological evaluation of the gills of *Clarias gariepinus* exposed to 1/100 LC_{50} of glyphosate contamination and fed with probiotics supplemented feed are shown in Figure 2. Result showed mild lamellar necrosis in the gills of *Clarias gariepinus* fed with 0.1% and 0.2% probiotics supplemented feed. On the other hand, the gills of *Clarias gariepinus* fed with the control feed (0% probiotics) and those fed with 0.15% probiotics supplemented feed showed clear tissue morphology.

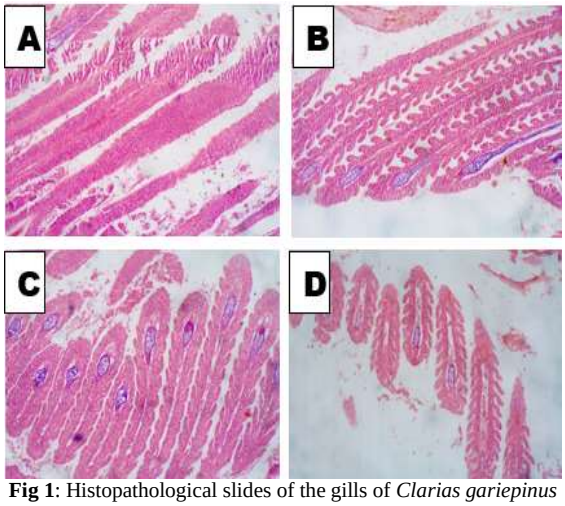

Fig 1: Histopathological slides of the gills of *Clarias gariepinus* exposed to 1/10 LC_{50} glyphosate contamination and fed with probiotics supplemented feed [A = 0% P, B = 0.1% P, C = 0.15% P, D = 0.2% P]

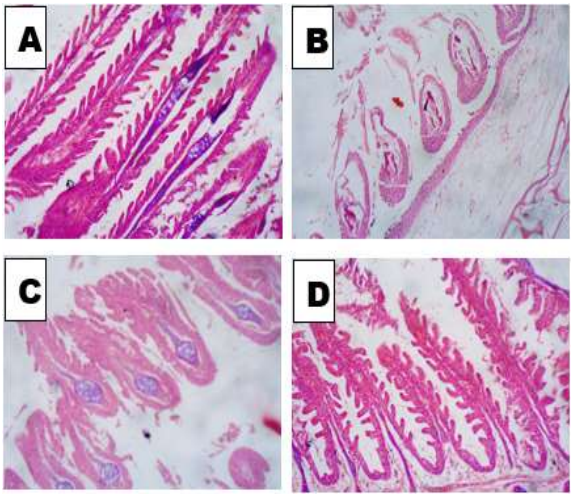

Fig 2: Histopathological slides of the gills of *Clarias gariepinus* exposed to 1/100 LC50 glyphosate contamination and fed with probiotics supplemented feed [A = 0% P, B = 0.1% P, C = 0.15% P, D = 0.2% P]. Comparatively, degree of necrosis was more severe in fig, 1 than fig. 2.

*Liver of Clarias gariepinus exposed to 1/10 LC_{50} glyphosate***:** Figure 3 represents the histopathological slides of the liver of *Clarias gariepinus* exposed to 1/10 LC_{50} glyphosate contamination and fed with probiotics supplemented feed. Normal liver tissues were observed in the *Clarias gariepinus* fed with 0.1%, and 0.2% probiotics supplemented feed. On the other hand, liver of *Clarias gariepinus* fed with the control feed (0% probiotics) showed vascular congestion with edema. Parallel radially arranged plates of hepatocytes, with the portal space and periportal zone filled with a smooth to slightly floccular pink fluid material common with edema admixed with congested aggregates of red blood cells were also observed.

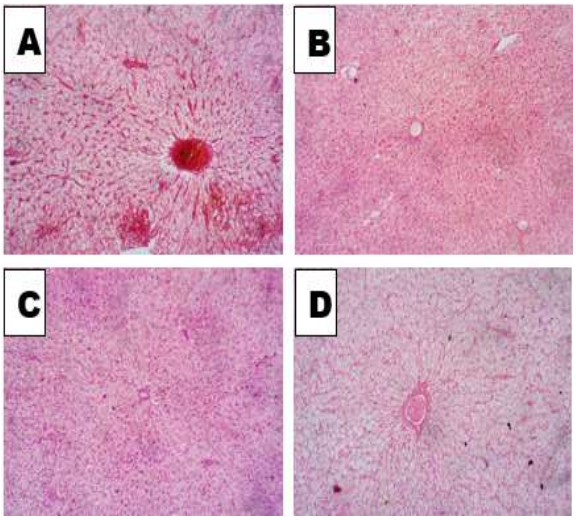

Fig 3: Histopathological slides of the liver of *Clarias gariepinus* exposed to 1/10 LC_{50} glyphosate contamination and fed with probiotics supplemented feed [A = 0% P, B = 0.1% P, C = 0.15% P, D = 0.2 P]

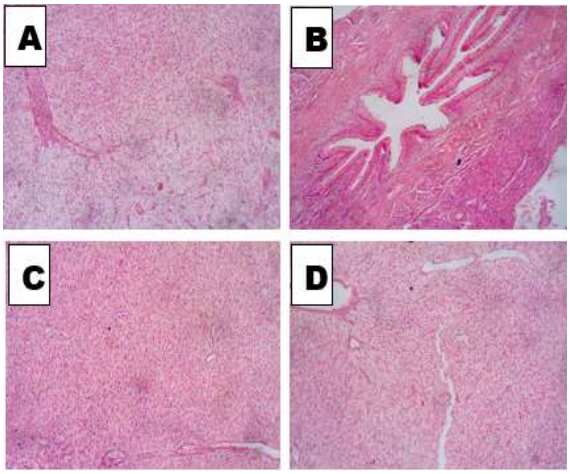

Fig 4: Histopathological slides of the liver of *Clarias gariepinus* exposed to 1/100 LC_{50} glyphosate contamination and fed with probiotics supplemented feed [A = 0% P, B = 0.1% P, C = 0.15% P, D = 0.2% P].

Liver of Clarias gariepinus exposed to 1/100 LC_{50} glyphosate: The liver of *Clarias gariepinus* fed with the control feed (0% probiotics) showed vascular congestion with edema (Figure 4). The liver also showed parallel radially arranged plates of hepatocytes, with the portal space and periportal zone filled with a smooth to slightly floccular pink fluid material common with edema admixed with congested aggregates of red blood cells. However, no abnormalities were observed in the liver tissues of the *Clarias gariepinus* fed with 0.1%, 0.15% and 0.2% probiotics supplemented feed.

Antioxidant Enzymes Activities of Liver and Gills: Clarias gariepinus exposed to 1/10 LC_{50} of

glyphosate: Activities of antioxidant enzymes in the liver and gills of *Clarias gariepinus* exposed to 1/10 LC_{50} of glyphosate and fed with probiotics supplemented feed are presented in Table 1.

Liver: Activities of GSH and SOD was significantly higher in the liver of the *C. gariepinus* fed with 0.2% probiotics supplemented fed compared to those fed on the 0.1% and 0.15% of probiotics supplemented feeds, and the control. Activities of GSH and SOD recorded in the *C. gariepinus* fed with probiotics supplemented feed were however not significantly different. Also, catalase activity was highest in the *C. gariepinus* fed with 0.2% probiotics supplemented feed and this was not significantly different from those fed with 0.15% probiotics supplemented feed. On the other hand, level of MDA was significantly highest in the control *C. gariepinus* and lowest in the *C. gariepinus* fed with 0.2% probiotics supplemented feed. Aside the control, levels of MDA recorded in the other experimental groups were not significantly different.

Gills: Activity of GSH was observed to significantly increase in the gills of *C. gariepinus* fed with probiotics supplemented feed with increase in the proportion of supplementation from 0.1% to 0.2%. Hence, GSH activity was lowest in the control *C. gariepinus* (fed 0% probiotic supplemented feed) and highest in those fed with 0.2% probiotic supplemented feed. On the other hand, activities of SOD and MDA were highest in the control *C. gariepinus* (fed 0% probiotics supplemented feed) while activities of Catalase were highest in *C. gariepinus* fed with 0.2% probiotics supplemented feed.

Table 1: Activities of antioxidant enzymes in the liver and gills of *Clarias gariepinus* exposed to 1/10 LC_{50} of glyphosate and fed with probiotics supplemented feed

		GSH (nm/mg pro)	SOD (nm/mg pro)	CAT (nm/mg pro)	MDA (nm/mg pro)
Liver	0% P	61.52 ± 0.74^{b}	4.47 ± 0.57^{b}	15.19 ± 0.27^{c}	2.54 ± 0.06^{a}
	0.1% P	75.22 ± 0.31^{b}	3.47 ± 0.10^{b}	19.23 ± 0.33^{b}	1.92 ± 0.03^{b}
	0.15% P	77.91 ± 1.29^{b}	5.74 ± 0.06^{b}	28.00 ± 0.71^{a}	1.31 ± 0.01^{b}
	0.2% P	247.16 ± 2.87^{a}	11.43 ± 0.04^{a}	28.63 ± 0.89^{a}	1.27 ± 0.03^{b}
Gills	0% P	24.83 ± 1.06^{d}	2.13 ± 0.04^{a}	10.28 ± 0.40^{c}	3.27 ± 0.10^{a}
	0.1% P	98.46 ± 0.65^{c}	1.43 ± 0.01^{b}	21.33 ± 0.47^{b}	2.69 ± 0.13^{b}
	0.15% P	105.72 ± 2.86^{b}	1.47 ± 0.03^{b}	24.88 ± 1.13^{a}	2.42 ± 0.03^{b}
	0.2% P	207.54 ± 2.89^{a}	1.57 ± 0.03^{b}	25.53 ± 0.75^{a}	1.77 ± 0.03^{b}

^{abcd}Mean (±Standard deviation) in the same column for liver and gills respectively having similar superscripts are not significantly different at $p < 0.05$; %P = Percentage probiotics supplements in feed

Table 2: Activities of antioxidant enzymes in the liver and gills of *Clarias gariepinus* exposed to 1/100 LC_{50} of glyphosate and fed with probiotics supplemented feed

		GSH (nm/mg pro)	SOD (nm/mg pro)	CAT (nm/mg pro)	MDA (nm/mg pro)
Liver	0% P	62.87 ± 1.13^{d}	4.26 ± 0.37^{a}	20.18 ± 0.25^{b}	3.00 ± 0.42^{a}
	0.1% P	92.01 ± 2.84^{c}	3.66 ± 0.08^{a}	23.03 ± 1.46^{a}	2.35 ± 0.06^{b}
	0.15% P	166.43 ± 3.39^{b}	1.46 ± 0.04^{b}	23.94 ± 1.33^{a}	1.63 ± 0.04^{c}
	0.2% P	178.39 ± 1.84^{a}	0.67 ± 0.01^{c}	24.38 ± 0.54^{a}	1.52 ± 0.03^{c}
Gills	0% P	55.61 ± 0.86^{d}	1.29 ± 0.01^{c}	17.24 ± 0.34^{c}	2.08 ± 0.11^{a}
	0.1% P	110.42 ± 3.42^{c}	3.25 ± 0.28^{b}	20.35 ± 0.49^{b}	1.40 ± 0.14^{b}
	0.15% P	187.93 ± 2.87^{b}	5.54 ± 0.06^{a}	20.45 ± 0.64^{b}	1.29 ± 0.01^{b}
	0.2% P	204.45 ± 4.88^{a}	5.15 ± 0.07^{a}	24.73 ± 1.03^{a}	1.12 ± 0.03^{b}

^{abcd}Mean (±Standard deviation) in the same column for liver and gills respectively having similar superscripts are not significantly different at $p < 0.05$; % P = Percentage probiotics supplements in feed.

Clarias gariepinus exposed to 1/100 LC_{50} of glyphosate: Table 2 shows the activities of antioxidant enzymes in the liver and gills of *Clarias gariepinus* exposed to 1/100 LC_{50} of glyphosate and fed with probiotics supplemented feed.

Liver: Activity of GSH was significantly higher in the *C. gariepinus* fed with 0.2% probiotic supplemented feed. In contrast, SOD activity was highest in the control *C. gariepinus* (fed with 0% probiotics supplemented feed) and lowest in *C. gariepinus* fed with 0.2% probiotics supplemented feed. There was a significant difference among the different groups. Activity of Catalase was however significantly lowest in the control *C. gariepinus* and highest in *C. gariepinus* fed with 0.2% probiotics supplemented feed. There was no significant difference among the groups fed with 0.1% to 0.2% probiotics supplemented feed. Level of MDA was also significantly highest in the control *C. gariepinus* fed with 0% probiotics supplemented feed and lowest in the group fed with 0.2% probiotics supplemented feed.

Gills: Activities of GSH, SOD and Catalase were significantly higher in the *C. gariepinus* fed with 0.2% probiotics supplemented feed when compared to those

fed with the different proportions of probiotics supplemented feed, and the control. On the other hand, activity of MDA was significantly highest in the control *C. gariepinus* and lowest in the *C. gariepinus* fed with 0.2% probiotics supplemented feed. Probiotics were observed to be more proficient rebuilding tissues and inhibiting degeneration of organs. Glyphosate has strong disrupting tendencies as found in this study, particularly at $1/10^{th}$ LC_{50} concentration. Degree of tissue destruction is dose dependent as corroborated by Muhammed, 2021. The ability to repair tissues by probiotics is attributed to inherent immune-modulatory factors (Seyram *et al.,* 2021). Healing of tissues in this study was profound at 0.15% and 0.2% inclusion levels. Amin Shavandi *et al.,* (2020) and Jovanka *et al.,* (2017) further agreed with the tissue repair mechanism of probiotics as observed in this study. Oxidative stress biomarkers are tools to assess the stress level of an organism. Level of activity of these enzymes increased significantly in probiotics fed *C. gariepinus* 0.2% inclusion level compared to control (0% probiotics at 1/10 and 1/100 LC_{50} concentration) Superoxide dismutase (SOD), Catalase (CAT) and GSH activities were inhibited at both 1/10 and 1/100 exposure levels. However, reduced activity of MDA was observed in probiotics fed *C. gariepinus* across board, with highest activation at 0.2% probiotics inclusion level (Sayed *et al.,* 2021). Miguel *et al.,* (2021) agrees with the antioxidant action of probiotics in this study.

In conclusion, probiotics incorporation in feeds at relative inclusion levels can positively affect the overall fish physiology.

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