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Original Article

Ameliorative and protective effects of prebiotic, microbial levan in common carp, (*Cyprinus carpio*) fry under experimental exposure to fipronil

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Abstract: This study investigated the immuno-toxicological effect of the insecticide fipronil at sublethal concentration (10% of LC_{50}) and the potential ameliorative effects of dietary microbial levan in *Cyprinus carpio* fry. Fish were randomly distributed into five treatments in triplicate for 60 days. Five different treatment groups were: levan control L_0F_0 (basal feed + 0% levan without exposure to fipronil), pesticide control L_0F (basal feed + 0% levan with exposure to fipronil), other three dietary supplemented groups exposed to fipronil with different inclusion levels of levan at 0.25% ($L_{0.25}F$), 0.50% ($L_{0.50}F$) and 0.75% ($L_{0.75}F$), respectively. The results revealed that feeding common carp with 0.75% dietary levan significantly reduced (P<0.05) glutathione-S-transferase and glutathione peroxidase levels in various tissues. Lipid peroxidation and heat shock protein level was significantly (P<0.05) reduced with supplementation of levan at 0.75% compared to other groups. Higher glycogen content was observed in high levan fed groups. Although fipronil exposure had no signficiant effect on lipid profile levels, dietary levan supplementation decreased lipid profile level in the fish exposed to fipronil stress. Total immunoglobulin and myeloperoxidase content of common carp showed an increasing trend with the concomitant increase in the level of levan administration in the diet. Overall, results revlealed that microbial levan at 0.75% in the fipronil induced *C. carpio* fry mitigated the stress due to its potent nutraceutical properties, thus presenting a promising immuno-additive for aquaculture.

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Introduction

Insecticides employed in the agricultural practices for the primary aim of eradicating harmful insect's pests and enhancing food production, eventually reach to freshwater bodies through run off (Ghelichpour et al., 2020). These insecticides from agricultural field's overflow pollute the aquatic environment resulting into serious distress to certain physiological and biochemical processes in non-target species even at sub-lethal concentrations (Prusty et al., 2011; Kumar et al., 2016). Fipronil, a broad-spectrum, phenyl-pyrazole insecticides, and its application particularly in the Asian countries is gaining momentum, due to emerging outlaw on the use of organochlorines and organophosphates as agricultural insecticides. Agricultural runoff or drift from aerial or ground based spraying applications of fipronil pose threat to non-target cultured fishes (Fenet et al., 2001; Schlenk et al., 2001; Walse et al., 2004). Once entranced to fish body, marked alterations in many of the key physio-biochemical and haeamatoimmunological processes (Prusty et al., 2011; Akhtar et al., 2012; Gupta et al., 2014a; Kumar et al., 2016) are inevitable. In this context, Gupta et al. (2014b) indicated perturbations of heamato-biochemical responses of *C. carpio* fry as a result of short term exposure of sublethal concentration of fipronil. Further, long term sublethal exposure of fipronil resulted into immune-suppression, delayed adverse health effects, and death of cultivated fishes thus posing heavy economical losses to the fish farmers (Gupta et al., 2013). Hence, there is an utmost need to develop eco-friendly and sustainable methods to control the fish mortality mediated through the sublethal fipronil concentration. Application of herbal products such as extract, phytochemical and prebiotics through dietary

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interventions in aqua-feed seems to be very promising and viable approach to combat pollutant stress (Taheri Mirghaed et al., 2019; Rajabiesterabadi et al., 2020a, b; Fazelan et al., 2020; Ghelichpour et al., 2021).

Prebiotic is nothing but a form of health supplements which selectively stimulate the growth, and/or activity of intestinal microflora associated with health and wellbeing (Gibson and Roberfroid, 1995). Application of fructans as prebiotic has gained attention in aqua feed industry as these are intended to manufacture a mixture of functional prebiotics (Ávila-Fernández et al., 2011). Levan is a unique polymer of carbohydrate containing fructose as monomer in its structure of backbone. Apart from the various useful properties such as bio-compatibility, bio-degradability, renewability, flexibility, and eco friendliness, levan also offers some multi-functional biomedical properties such as anti-oxidant, anti-inflammatory, anti-carcinogenic, anti-AIDS, immune-stimulating, growth augmenting, hyperglycaemic inhibitor etc. (Gupta et al., 2010; Dahech et al., 2011; Huang et al., 2015). Levan is distinctly distributed in plants, yeasts, fungi and bacteria (Jang et al., 2003) and its biosynthesis is usually carried out by an enzyme levansucrase. Several bacterial strains, including *Zymomonas mobilis, Bacillus subtilis, B. polymyxa* and *Acetobacter xylinum* produce extracellular levan of high molecular weight which is serologically active (Clarke et al., 1997).

Augmentation of haemato-immunological and physio-biochemical responses and protection against bacterial challenge have been reported in *C. carpio* (Rairakhwada et al., 2007; Gupta et al., 2014a), *L. rohita* (Gupta et al., 2008, 2015) and *Epinephelus coioides* (Huang et al., 2015) owing to dietary microbial levan supplementation. Of late, present authors reported stress amelioration efficacy of levan by modulation of cytokine expression in the pathogen aggravated rohu, *L. rohita* (Gupta et al., 2018, 2020).

Despite the potential health benefits of prebiotics in fish, very limited information is available about the possible ameliorative effects of prebiotics when fish exposed to pesticides. Thus, the present study was conducted to elucidate possible ameliorating efficacy of dietary microbial levan on the immuno-biochemical responses of *Cyprinus carpio* fry as a result of fipronil exposure.

Materials and Methods

Experimental animals: *Cyprinus carpio* (average weight of 3.31±0.52 g) were procured from Palghar fish farm, Maharashtra, India, transported, stocked in cement tank (1000 L capacity) and left unobstructed for the whole night. On following day, fish were given a mild salt treatment (5%) to ameliorate the handling stress, if any. The stock was acclimatized under aerated condition for 15 days. During acclimation, fish were fed to satiation with control diet having 30% crude protein (CP).

Chemicals: Fipronil ($C_{12}H_4Cl_2F_6N_4OS$) (99.1% pure, Technical grade) was procured from Malti enterprises, Mumbai, India and was kept in an airtight container in the refrigerated condition. A stock solution of fipronil was prepared using analytical grade acetone (solubility of fipronil in acetone is 545.9 $g1^{-1}$ at water pH 9.0). Required quantity of fipronil was drawn from this stock solution for later experimental use. The sublethal fipronil dose of 0.0428 mg l^{-1} ($1/10^{th}$ LC_{50} for 96 h) was calculated as described by Gupta et al. (2013) and selected for this study.

Experimental site, design and feeding: The experimental setup was maintained in the wet laboratory of the Aquaculture division of Central Institute of Fisheries Education (CIFE) Mumbai, and the laboratory analysis was carried out at the Fish Nutrition, Biochemistry and Physiology division. Following a completely randomized design, two hundred and twenty-five fry of *C. carpio* were distributed in five treatment groups in triplicates. The experimental system comprised of 15 uniform size circular fiber reinforced plastic tanks (150 L). Four experimental diets were prepared (Table 1) for feeding the fishes till 60 days and divided into five different treatment groups as: levan control L_0F_0 (basal feed+ 0% levan without exposure to fipronil); pesticide control L_0F (basal feed+0% levan with exposure to fipronil); $L_{0.25}F$ (basal feed+0.25% levan with exposure to fipronil); $L_{0.50}F$ (basal feed+0.50% levan

Table 1. Composition of experimental diets (g %), and its proximate analysis (% dry matter basis).

Ingredients	Experimental diets (% inclusion)			
	$L_0 F_0$	$L_{0.25}F$	$L_{0.50}F$	$L_{0.75}F$
Casein fat free*	35	35	35	35
Gelatin*	9.5	9.5	9.5	9.5
Dextrin*	10	10	10	10
Starch soluble*	25	25	25	25
Cellulose powder*	9.0	8.75	8.5	8.25
Cod liver oil†	4	4	4	4
Sun flower oil‡	4	4	4	4
Vitamin mineral mix§	2.88	2.88	2.88	2.88
Vit-C‼	0.10	0.10	0.10	0.10
Microbial levan	--	0.25	0.5	0.75
CMC*	0.48	0.48	0.48	0.48
Betain Hydrochloride*	0.02	0.02	0.02	0.02
BHT*	0.02	0.02	0.02	0.02
Total	100	100	100	100
Proximate composition of diets				
Organic Matter (OM)	96.63	96.64	96.67	96.59
Crude Protein (CP)	35.10	35.04	35.01	35.33
Ether Extract (EE)	7.56	7.50	7.48	7.61
Ash	3.37	3.20	3.38	3.39
Total Carbohydrate (TC)	53.94	54.25	54.13	53.66
Digestible Energy♯ (DE)	424.31	424.67	423.88	424.51

*Himedia Laboratories, Mumbai, India, †Densa Pharma, Mumbai, India, ‡Ruchi Soya Industries, Raigad, India,§Composition of vitamin mineral mix (EMIX PLUS) (quantity 2.5kg $^{-1}$), Vitamin A 55,00,000 IU; Vitamin D_3 11,00,000 IU; Vitamin B_2 2,000 mg; Vitamin E 750 mg; Vitamin K 1,000 mg; Vitamin B_6 1,000 mg; Vitamin B_{12} 6 µg; Calcium Pantothenate 2,500 mg; Nicotinamide 10 g; Choline Chloride 150 g; Mn 27,000 mg; I 1,000 mg; Fe 7,500 mg; Zn 5,000 mg; Cu 2,000 mg; Co 450 mg; Ca 500 g; P 300g; L- lysine 10 g; DL-Methionine 10 g; Selenium 50 mgl^{-1}; Selenium 50 mgl^{-1}; Satwari 250 mgl^{-1}; (Lactobacillus 120 million units and Yeast Culture 3000 crore units). ‼Stay C (Hoffman La Roche, Nutley, NJ, USA) 15% ascorbic acid activity, ♯Digestible energy (K cal/100g) = (%CP x 4)+(% EE x 9)+(TC x 4).

with exposure to fipronil) and $L_{0.75}F$ (basal feed+0.75% with exposure to fipronil). Purified microbial levan was procured from Department of Microbiology, Bhavans College, Mumbai, India. Except L_0F_0, all the groups were exposed to sublethal dose of fipronil.

The drinking tap water was used during the experimental trail to make sure that it is not contaminated with any other pollutants. This tap water was used purposefully as the facilities to measure the exact fipronil concentration was unavailable. Aeration was provided through compressed air pump round the clock. Feeding of the fry was done to satiation level (at 4% of their body) and changed accordingly to their biomass gain over a period of 15 days. Daily ration was divided into two parts on the basis of biomass, about 2/3rd of total ration was given at 09:30 h and the rest 1/3rd at 18:30 h. The left over feed and faecal debris were manually siphoned out employing water exchange of approx. 50% of the tank volume on every second days with taking care to provide any discomfort to the animals. To maintain the sublethal effect of the fipronil, test solution was reintroduced with firponil treated water arranged from the stock solution. We followed the methodology as previously described by Das and Mukherjee (2003) and Prusty et al. (2011) to maintain sublethal concentration. Water quality parameters from each replicate tank monitored on daily basis, were dissolved oxygen (DO), temperature, and pH, while alkanlity and hardness

were monitored on weekly basis. All water quality parameters (dissolved oxygen: 6.48–7.2 mg l^{-1}; pH: 7.35–7.60; temperature: 26.8-27.2°C; alkalinity 51-59 mg l^{-1} and hardness 56-66 mg l^{-1}) were found to be within the normal range for rearing of *C. carpio* (Gupta et al., 2013).

Proximate analysis of feed: As per the standard methods of AOAC (1990), proximate composition of the experimental diets was determined (Table 1). The moisture content was determined by drying the samples at 105°C to a constant weight. Nitrogen content was estimated by automated Kjeldahl apparatus (2200 Kjeltec Auto distillation, Foss Tecator, Sweden) and CP was estimated by multiplying nitrogen percentage by 6.25. Ether extract (EE) was measured using a Soxtec system (1045 Soxtec extraction unit, Tecator, Sweden) using diethyl ether (boiling point, 40-60°C) as a solvent and ash content was determined by incinerating the samples in a muffle furnace at 600°C for 6 h. Total carbohydrate was calculated by difference i.e., total carbohydrate % = 100−(CP%+EE%+Ash%). The digestible energy (DE) values of experimental diets and tissues were calculated as described by Halver (1976).

Sample preparation: At the end of the experiment, two fish from each replicate with a total of six from each treatment were anaesthetized with CIFECALM (CIFE, Mumbai, India) at 50 µl l^{-1}. Whole intestine was dissected out aseptically and collected for enzyme assay after removing the intestinal contents and immediately homogenized (5%) in chilled sucrose solution 0.25 M using a teflon coated mechanical tissue homogenizer. During homogenization, care was taken by keeping the sample over ice to avoid heating. In a cooling centrifuge, homogenized samples were centrifuged (6000×g for 10 min) and supernatants were collected in 2 ml Eppendorf tube and stored at –20ºC for subsequent enzyme assays.

Enzyme assays: Protease activity in the intestinal tissue was determined by the casein digestion method of Drapeau (1974). Lipase activity in the intestinal tissue was determined based on Cherry and Crandell (1932). ALP activity in the intestinal tissue was determined by the method of Garen and Levinthal (1960). Phosphate liberated was estimated at OD of 660 nm (Fiske et al., 1925). ACP activity was estimated using the same method as ALP, except that acetate buffer (0.2 M, pH 5) was used in place of bicarbonate buffer. Protein estimation in the tissue was carried out using method of Lowry et al. (1951).

Antioxidant assay: Gluthathione-S-transferase (GST) activity was measured in the liver, gill, brain and kidney tissues following the method of Habing et al. (1974) using S-2, 4-dinitrophenyl glutathione (CDNB) as substrate. The method was based on the principle of formation of adduct of CDNB, S-2, 4-dinitrophenyl glutathione and was monitored by measuring the increase in absorbance at 340 nm against blank. Glutathion peroxidase (GPx) was measured in the liver, gill and kidney tissues following the method of Paglia and Valentine (1967). GPx activity was analysed based on conversion of glutathione into glutathione disulphide, and decomposition of 1 µM of the substrate to the product. GSH levels were measured based on the formation of 2-nitro 5-thiobenzoic acid.

Tissue Glycogen: Liver and muscle glycogen content (mg g^{-1}) was estimated colorimetrically by the method described by Hassid and Abraham (1957). The tissue was placed in a pre-weighed centrifuge tube containing 3 ml of 30% KOH. After the weight of the tissue had been recorded, the tubes were placed in a boiling water bath for 20 min. After cooling, 5 ml of 95% ethanol was added to precipitate the glycogen. The precipitate obtained was dissolved in 1 ml of distilled water and again precipitated with 95% ethanol and centrifuged. The glycogen precipitate was then dissolved in distilled water, and this solution was used to estimate the quantity of glycogen. In 0.1 ml of aliquot, 5 ml of anthrone reagent was added and mixed by swirling the tube. The tubes were covered with glass marble and heated for 10 min in boiling water, followed by cooling, and the absorbance was recorded at 590 nm. The reading was compared with that of standard glycogen.

Collection of blood serum: For serum, two fish from each replicate and a total of 6 from each treatment were anesthetized as above; the blood was collected

without anticoagulant and allowed to clot for 2 h, centrifuged (3000 g for 5 min) and then kept at –80°C until use.

Serum cholesterol, triglyceride, LDL (low density lipoprotein) and (VLDL) (very low density lipoprotein) cholesterol: Serum cholesterol was measured by method of (Palkonen et al., 1957) by using commercially available kit from Sigma Aldrich, Saint Louis, USA. In brief, 20 µl of serum was mixed with 2 ml of reaction solution (enzyme solution with colour reagent). The absorbance of samples was measured at 540 nm against the reagent blank value. Serum triglycerides were measured by the method described (Kaplan 1985) using commercially available kit from Sigma Aldrich, Saint Louis, USA. In brief, 10 µl of serum was mixed with 1 ml of reaction solution. The absorbance of sample was measured against the reaction solution. The increase in absorbance, measured at 540 nm, due to the formation of the quinoneimine dye. The increase in absorbance was directly proportional to the glycerol concentration in the sample. True serum triglycerides were calculated by subtracting the free glycerol concentration in the sample from total triglycerides. Serum LDL and HDL contents were measured using the kit of Daiichi Pure Chemicals Co., LTD. Chuo-ku, Tokyo, Japan. Initially, 3 µL of sample was mixed with 300 µL of reagent 1 and kept for 5 min and then $A_{700/600\ nm}$ was measured. This was followed by the addition of 100 µL of reagent 2 in the same microtitre plate. Again the $A_{700/600nm}$ was measured for the same sample. The absorbance was measured by using the instrument Chem Well of Awareness Technology Inc. Palm City, Florida, USA. Estimation of VLDL was calculated by (Sattyanarayanan 2001). The value of VLDL was calculated by density gradient centrifugation method based on the following formula:

VLDL = Total cholesterol– (HDL+LDL)

The estimated values of VLDL were expressed in mg dl^{-1}

Lipid peroxidation (LPO) and HSP70: LPO was determined by the procedure of Uchiyama and Mihara (1978) in the liver, gill, kidney and brain tissues. Briefly, 0.25 ml of homogenate was mixed with 25 ml of 10 mM butylated hydroxytoluene (BHT). 3 ml phosphoric acid (1%) and 1 ml of 0.67% thiobarbituric acid (TBA) were added and mixture was incubated at 90°C for 45 min. The absorbance was measured at 535 nm. The rate of LPO was expressed as nanomoles of thiobarbituric acid reactive substance (TBARS) formed/h/mg of protein using a molar extinction coefficient of $1.56 \times 105\ M^{-1}\ cm^{-1}$.

HSP70 levels (EIA kit, catalog no. EKS-700B) in the liver, muscle and gill was determined following the manufacturer's instructions (Bioguenix/Enzo Life Science, Mumbai, India). The absorbance was read on the ELISA plate reader (Biotek India Pvt. Ltd.).

Myeloperoxidase content and total immunoglobulin: Total myeloperoxidase content present in serum was measured according to Quade and Roth (1997) with some modifications. About 15 µL of the serum was diluted with 135 µL of hank's balanced salt solution (HBSS) without Ca^{2+} or Mg^{2+} in 96-well plates. The wells were added with 25 µL of 20 mM 3,3′ 5, 5′- tetramethyl benzidine hydrochloride (TMB) (Hi-media) and 25 µL of 5 mM H_2O_2 (Qualigens) (Both substrates of MPO prepared freshly). The reaction was terminated after 2 min by adding 50 µL of 4 M sulphuric acid (H_2SO_4). The plate was centrifuged (400 g) for 10 min, and 150 µL of the supernatant from each well was transferred into new 96-well plates. The absorbance was measured at 450 nm in a microplate reader (Biotek India Pvt. Ltd.).

Total immunoglobulin level was measured and calculated as per the protocol of Siwicki et al. (1994) with minor modification. OD measurement was performed at 595 nm. The serum total immunoglobulin concentration was calculated by subtracting the concentration of proteins in the supernatant from the total protein concentration in the serum before precipitation with PEG. The total immunoglobulin level was expressed as unit ng dL^{-1}.

Statistical analysis: Mean value of all parameters were subjected to one-way analysis of variance (ANOVA) to study the treatment effect and Duncan's Multiple Range Tests (DMRT) were used to determine the significant differences between the

Table 2. Effect of dietary microbial levan on specific enzyme activities in the digestive tract of *Cyprinus carpio* fry exposed to sublethal fipronil stress for 60 days.

Treatments	Protease	Lipase	ALP	ACP
L_0F_0	12.78^{a}±0.73	1.44^{a}±0.06	27.33^{c}±1.22	45.44^{c}±1.95
L_0F	7.16^{b}±0.36	0.66^{b}±0.03	40.09^{b}±1.92	87.85^{a}±2. 31
$L_{0.25}F$	8.73^{b}±0.55	0.80^{b}±0.09	45.72^{b}±1.89	83.94^{a}±3. 07
$L_{0.50}F$	8.91^{b}±0.70	0.87^{b}±0.12	39.18^{b}±1.13	67.22^{b}±1.89
$L_{0.75}F$	12.48^{a}±0.41	1.33^{a}±0.12	28.79^{c}±2.58	40.61^{c}±2.41

Activities are expressed as follows: Protease as micromole of tyrosine released per min per mg protein; Lipase activity as units per hour per mg protein; ALP and ACP as nanomoles p-nitrophenol released per min per mg protein at 37°C; Values in the same column with different superscript (a, b, c) differ significantly (P<0.05). Data are expressed as mean ±SE (n=6)

Table 3. Effect of dietary microbial levan on glycogen content in liver and muscle tissues of *Cyprinus carpio* fry exposed to sublethal fipronil stress for 60 days.

Treatments	Glycogen content (mg g wet tissue^{-1})	
	Muscle	Liver
L_0F_0	1.39^{ab}±0.06	21.31^{c}±0.79
L_0F	1.21^{c}±0.01	18.36^{d}±0.99
$L_{0.25}F$	1.28^{b}±0.03	19.68^{bc}±0.98
$L_{0.50}F$	1.34^{ab}±0.04	19.83^{ab}±0.84
$L_{0.75}F$	1.37^{a}±0.02	21.09^{a}±0.86

Values in the same column with different superscript (a, b, c, d) differ significantly (P<0.05). Data are expressed as mean ±SE (n=6).

mean value. Comparisons were made at 5% probability level. All the data were analyzed using statistical package SPSS (Version 16) (SPSS Inc., Chicago, IL, USA).

Results

Enzyme assays: Dietary levan supplementation significantly (P<0.05) affected protease, lipase, ALP and ACP activity in the intestine of *C. carpio* fry in different experimental groups (Table 2). Highest (P<0.05) protease activity among levan fed group was observed in the ($L_{0.75}F$) group fed with 0.75% levan supplementation and exposed to fipronil which was comparable to L_0F_0 group fed without levan supplementation and reared without exposure of fipronil stress. Lipase activity was recorded highest (P<0.05) with highest level of dietary levan fed $L_{0.75}F$ group and varied significantly from all other groups except L_0F_0. Lowest value of both protease and lipase activity was observed in the L_0F group fed without levan supplemented diet and exposed to fipronil. Fish fed 0.75% levan and exposed to fipronil had significantly (P<0.05) lowest intestinal ALP activity than the other treatment group except L_0F_0 group. A decreasing trend in ACP activity of intestine was observed with increasing concentration of levan and lowest (P<0.05) activity was observed in the group exposed to fipronil and fed with 0.75% dietary levan which was similar to the group fed without levan and reared without fipronil stress. Higher value of ALP and ACP activities (P<0.05) were observed in the order of $L_0F > L_{0.25F} > L_{0.50F}$ groups (Table 2).

Antioxidant assay: The oxidative stress in terms of GST and GPx in the liver, gill, brain and kidney of *C. carpio* exposed to fipronil stress for 60 days are presented in Table 5. The cellular stress indicators such as GST and GPx were noticeably elevated (P<0.05) in fipronil exposed group with compared to all other treatment groups. We found that supplementation of levan at 0.75% diet significantly amended the oxidative stress levels in all the tissues which were reflected in terms of reduced GST and GPx levels.

Tissue glycogen content: Dietary levan supplementation had significant (P<0.05) impact on the glycogen content in both liver and muscle of experimental groups (Table 3). Glycogen content was recorded higher in the liver than the muscle. Lowest

Table 4. Impact of dietary levan on serum lipid profile (mg%) of *Cyprinus carpio* fry exposed to sublethal fipronil stress for 60 days.

Treatments	Cholesterol	Triglyceride	LDL[1]	VLDL[2]
L_0F_0	154.67^{bc}±6.38	246.33^{c}±8.81	54.64^{b}±0.90	32.76^{b}±5.48
L_0F	162.33^{c}±2.40	253.33^{c}±3.17	55.30^{b}±3.56	31.10^{b}±5.38
$L_{0.25}F$	149.00^{abc}±4.16	230.33^{b}±2.90	43.56^{a}±4.24	22.08^{ab}±0.18
$L_{0.50}F$	144.00^{ab}±4.16	226.33^{ab}±1.45	43.33^{a}±2.69	18.38^{a}±3.25
$L_{0.75}F$	35.67^{a}±5.36	214.33^{a}±2.40	38.91^{a}±2.06	15.27^{a}±0.92

[1]LDL- Low-density lipoprotein, [2]VLDL-Very low-density lipoprotein. Values in the same column with different superscript (a,b,c,d) differ significantly (P<0.05). Data are expressed as mean ±SE (n=6).

Table 5. Impact of dietary levan on on glutathione-s-transferase (GST) and glutathione peroxidase (GPx) in liver, gill, brain and kidney tissues of *Cyprinus carpio* fry reared under fipronil stress for 60 days.

	Treatments				
	L_0F_0	L_0F	$L_{0.25}F$	$L_{0.50}F$	$L_{0.75}F$
GST-Liver	0.18^{b}±0.07	0.27^{c}±0.02	0.17^{b}±0.01	0.18^{b}±0.03	0.11^{a}±0.08
GST-Gill	0.19^{b}±0.01	0.37^{d}±0.02	0.28^{c}±0.05	0.23^{c}±0.02	0.14^{a}±0.02
GST-Brain	0.32^{b}±0.04	0.51^{d}±0.04	0.43^{c}±0.03	0.33^{b}±0.06	0.24^{a}±0.03
GST-Kidney	0.26^{c}±0.02	0.49^{d}±0.06	0.23^{bc}±0.01	0.25^{c}±0.04	0.14^{a}±0.01
GPx-Liver	4.54^{b}±0.60	8.56^{c}±0.42	2.21^{a}±0.18	2.11^{a}±0.27	2.23^{a}±0.06
GPx-Gill	4.66^{b}±0.35	10.43^{e}±1.07	7.87^{d}±1.04	5.67^{c}±0.77	3.01^{a}±0.22
GPx-Brain	3.98^{b}±0.32	11.02^{d}±0.70	4.20^{b}±0.53	7.11^{c}±0.41	1.69^{a}±0.14
GPx-Kidney	3.97^{b}±0.41	7.50^{e}±0.65	5.48^{c}±0.39	6.25^{d}±0.42	2.01^{a}±0.24

Values in the same row with different superscript (a, b, c, d) differ significantly (P<0.05). Data expressed as Mean ±SE (n=6). GST and GPx: Units mg protein^{-1}.

(P<0.05) value of glycogen level in both liver and muscle was observed in the L_0F group fed without levan supplemented diet and exposed to fipronil. Higher value among levan fed groups was observed in the $L_{0.75}F > L_{0.50}F > L_{0.25}F$ groups (Table 3).

Lipid profile of serum: The lipid profile of serum of the experimental groups at the end of the feeding trial is represented in Table 4. Supplementation of dietary levan significantly (P<0.05) affected cholesterol, triglycerides, LDL and VLDL of the experimental groups. As the incorporation of levan increased in the diet, the value of serum cholesterol decreased consequently in the experimental groups. Similar trend was observed for triglyceride and VLDL contents and the lower value was observed in the $L_{0.75F}$ group fed with 0.75% levan and exposed to fipronil stress. The lower value of LDL cholesterol was observed in the group fed with higher levan and exposed to fipronil stress. Higher value of serum cholesterol, triglycerides and LDL were observed in the L_{0F} group fed without levan supplementation and exposed to fipronil.

LPO and HSP-70: The LPO levels were significantly higher (P<0.05) in the liver, gill, kidney and brain tissues of fipronil exposed group compared to all other groups. Further, the level of LPO was significantly (P<0.05) reduced with supplementation at 0.75% diet compared to other treatment groups in all the tissues except LPO level in the brain which was similar (P>0.05) to the control L_0F_0 and $L_{0.50}F$ levan fed groups. The stress biomarkers HSP-70 of *C. carpio* exposed to fipronil and fed with different doses of levan for 60 days are presented in Table 6. The HSP 70 in gill, liver and muscle were noticeably (P<0.05) elevated in fipronil exposed group (L_0F), whereas higher supplementation of levan led to consequential reduction in the levels of HSP-70 and hence lowest (P<0.05) was found in the group fed at at 0.75% diet compared to other treatment groups in all the tissues.

Myeloperoxidase content and Total immunoglobulin (Ig): Total immunoglobulin content of *C. carpio* showed a significant increasing trend with the concomitant increase in the level of dietary levan concentration. The content of total immune-globulin was observed highest (P<0.05) in the group fed at 0.75% levan compared to other treatment

Table 6. Impact of dietary levan on lipid peroxidation (LPO) in liver, gill, brain and kidney tissues and HSP-70 in the gill, liver and muscle tissues of *Cyprinus carpio* fry reared under fipronil stress for 60 days.

	Treatments				
	L_0F_0	L_0F	$L_{0.25}F$	$L_{0.50}F$	$L_{0.75}F$
LPO-Liver	$16.41^{b}\pm0.25$	$31.23^{d}\pm3.12$	$16.98^{b}\pm1.54$	$18.98^{c}\pm1.51$	$12.92^{a}\pm1.44$
LPO-Gill	$12.86^{b}\pm0.52$	$27.29^{d}\pm0.83$	$19.23^{c}\pm1.90$	$19.40^{c}\pm1.54$	$10.49^{a}\pm0.89$
LPO-Kidney	$17.62^{b}\pm0.43$	$46.51^{e}\pm0.78$	$34.34^{d}\pm0.85$	$32.09^{c}\pm1.85$	$12.97^{a}\pm0.88$
LPO-Brain	$10.60^{a}\pm0.73$	$31.04^{d}\pm1.78$	$18.86^{c}\pm1.44$	$12.28^{ab}\pm0.98$	$10.55^{a}\pm0.95$
HSP-70-Gill	$12.93^{b}\pm0.37$	$32.37^{d}\pm0.43$	$18.07^{bc}\pm1.57$	$17.73^{b}\pm1.46$	$9.67^{a}\pm0.19$
HSP-70-Liver	$13.47^{b}\pm0.24$	$35.23^{c}\pm0.51$	$23.27^{b}\pm0.36$	$22.93^{b}\pm0.63$	$9.35^{a}\pm0.34$
HSP-70-Muscle	$12.57^{b}\pm0.21$	$38.23^{d}\pm0.42$	$25.57^{c}\pm0.22$	$23.40^{c}\pm0.65$	$10.57^{a}\pm0.43$

Values in the same column with different superscript (a, b, c, d) differ significantly (P<0.05). Data expressed as Mean ±SE (n=6). LPO: n mole TBARS formed/h/mg protein, HSP-70: ng ml^{-1}.

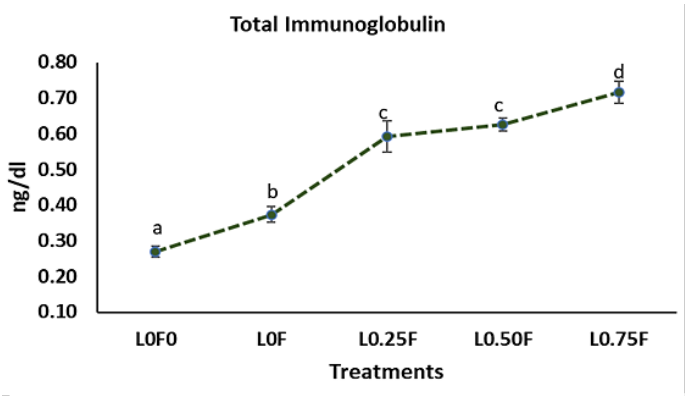

Figure 1. Total immunoglobulin of *Cyprinus carpio* fry fed diteray levan and reared under fipronil stress for 60 day. Data expressed as Mean ± E (n=6). Statistically significant (P<0.05) denoted as superscript letter a, b, c, d, on top of error bars.

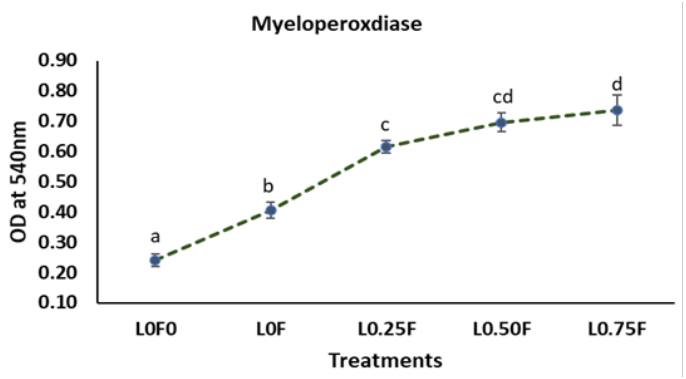

Figure 2. Myeloperoxdiase of *Cyprinus carpio* fry fed diteray levan and reared under fipronil stress for 60 day. Data expressed as Mean ±SE (n=6). Statistically significant (P<0.05) denoted as superscript letter a, b, c, d, on top of error bars.

groups (Fig. 1). Consistent to the total immune-globulin results, the increasing trend of myeloper-oxidase content was observed among the treatment groups and the higher value was observed in the group fed at 0.75% dietary levan which was similar to the group fed with 0. 50% diet (Fig. 2).

Discussions

Microbial levan is a potent functional feed, and its efficacy as enhancer of immune response, heat tolerance, resistance to pathogen, growth performance and nutrient utilization in various species of fishes have been well documented (Gupta et al., 2010, 2013, 2014a, 2015, 2020; Rairakhwada et al., 2007; Huang et al., 2015). In this present investigation, an attempt has been made to probe the protective role of dietary prebiotic levan on immuno-biochemical responses of *C. carpio* fry exposed to sublethal concentration of fipronil.

The activity of digestive enzymes is an important indicator of digestive physiology which determines the capacity of digestion and absorption of nutrients, and eventually reflected in better growth. Improved growth is also dependent on the progress of prebiotic fermentation by endogenous gut microbes (Scheppach, 1994; Dimitroglou et al., 2010; Hoseinifar et al., 2011, 2015). The positive influence of prebiotics on growth performance may be associated with improved nutrient digestibility, as a result of enhanced digestive enzyme activity which allow the host to degrade more nutrients (Huang et al., 2015). In the present study, dietary inclusion of prebiotic levan significantly increased digestive enzyme activities of *C. carpio* fry. Fish fed 7.5 g kg^{-1} levan and exposed to fipronil had significantly higher protease and lipase activities compare to other treatment groups except L0F0, which might be due to alteration of local gut microbial communities by

enhancing the proliferation of probiotics such as *Lactobacillus plantarum* and *Bifidobacterium pseudocatenulatum* (Wang et al., 2016). Our results are supported by findings of Akhtar et al. (2012) in *L. rohita* juveniles fed with highest level of pyridoxine incorporation and exposed to endosulfan toxicity. Protease is one of the digestive enzymes responsible for the protein digestion in animal, therefore highest activity in the maximum levan fed group indicates better protein digestion (Swanson et al., 2002; Teitelbaum and Walker, 2002). To corroborate our findings, Xu et al. (2009) reported parallel results of increased protease activity after feeding fructo-oligosacchsride in poultry. Dimitroglou et al. (2010) revealed that dietary MOS (Mannan oligosaccharides) supplementation increased the absorptive surface by promoting longer mucosal folding and increasing the microvilli density and microvilli length in the anterior and posterior gut regions of Atlantic salmon. The results of present investigation are also reflected with our previous finding Gupta et al. (2013) where higher weight gain% and SGR, highest protein efficiency ratio and better food conversion ratio (FCR) were recorded in the $L_{0.75F}$ group fed with (0.75%) levan and exposed to fipronil stress. Huang et al. (2015) suggested that the positive influence of levan on growth performance of fish may be associated with improvements in digestive enzyme activity or the gut morphology, thereby resulting in better degradation or absorption of nutrients in the gut. Increase in enzyme activity correlated with improved growth of *C. carpio* which might be attributable due to increased number of benign bacteria facilitating in the production of organic acids (formic acid, acetic acid, lactic acid), hydrogen peroxide, and several other growth stimulating compounds such as bacteriocins, siderophores and lysozyme (Hoseinifar et al., 2015). However, till to date, no reports are available on the mechanistic approaches on effect of dietary levan on digestive enzymes and microvilli structures in the gut of fish to substantiate our findings.

Alkaline phosphatase, an important regulative enzyme in bio-metabolic processes, plays a vital role in transition and absorption of nutrient in the enterocytes (Harpaz and Uni, 1999; Gawlicka et al., 2000) whereas ACP activity is highly correlated with growth in fish (Debnath et al., 2007). Lowest value of ALP and ACP in the intestine of L_0F group fed diet without levan and exposed to fipronil is in agreement with the previous results (Verma et al., 2007) in *C. carpio* exposed to endosulfan toxicity. Significant decrease in ALP and ACP activity in the $L_{0.75}F$ group might be due to hydrolysis of high-energy phosphate bonds to release phosphate ions to fight stressful situation leading to improved FCR and better growth (Gupta et al., 2013). Our results are supported with the findings of (Sarma et al., 2009) in *Channa punctatus* who fed high protein and high vitamin C diet and Kumar et al. (2012, 2014) in *L. rohita* fed with diet containing lecithin and betaine and exposed to combined effect of endosulfan and temperature.

Antioxidant defense plays a vital part in the response of an organism to pollutants. Numerous processes excite the generation of hydrogen peroxide and production of free radicals or deplete the antioxidant defense, which may cause severe oxidative damage in the organisms if not eliminated effectively (Martínez-Álvarez et al., 2005). In this direction, GST and GPx are the most important antioxidant enzymes, that serves as the first line of defence in the organisms against oxidative stress (Hoseini et al., 2019; Paray et al., 2020; Zargar et al., 2020). GPx and GST are involved in hydrogen peroxide degradation and is responsible for detoxification of harmful xenobiotics. The oxidative stress enhanced after exposure to fipronil in the present study, might be attributable due to the generation of hydrogen peroxide and production of oxygen free radicals. Therefore, to counteract activities of oxygen free radicals, collective action of both enzymatic and non-enzymatic antioxidant is valuable (Pérez-Campo et al., 1993). To corroborate our findings of increased GPx and GST activities in different tissues of fipronil exposed group fed without levan, previous studies also reported enhanced oxidative stress in *L. rohita*, *C. chanos* and *O. mossambicus* exposed to various contaminants (Kumar et al., 2012, 2016, 2017). Significant

reduction in the level of oxidative stress might be correlated with the stress ameliorative efficacy of dietary levan supplementation (Gupta et al., 2014a). Our results are also substantiated with the reduced level of LPO levels observed in the higher levan fed group.

Tissue glycogen is common measured parameter of stress response (Manush et al., 2005). In the present study, the glycogen content of both liver and muscle of *C. carpio* fry decreased significantly in the group exposed to fipronil and fed diet without levan. Our result is in agreement with the findings of Akhtar et al. (2012) who observed decreased glycogen content in *L. rohita* fingerlings fed without pyridoxine and exposed to endosulfan toxicity. Similarly, Rawat et al. (2002) found decreased glycogen content in *Heteropneustes fossilis* when reared under endosulfan stress. Nevertheless, supplementation of levan at 0.75% diet restored the glycogen level to normal in muscle as compared to control (L_0F_0) group. To the best of our knowledge, no literature is available on the effect of levan on glycogen levels in fish to correlate our findings.

Lipoproteins transport the majority of plasma lipids, including cholesterol and triglycerides. LDL and VLDL are the lipoproteins responsible for the vast majority of cholesterol transport in the blood. Supplementation of dietary levan significantly led to reduction in cholesterol, triglycerides, LDL and VLDL of the experimental groups. As the incorporation level of levan increased in the diet, serum cholesterol, triglycerides, LDL and VLDL decreased consequently in the experimental groups. To corroborate our finding, Daubioul et al., (2002) reported decrease in the triglyceride accumulation in the liver of rats fed diet supplemented with dietary fructans. This might be attributed due to high fermentation of fructans which selectively decreased the incorporation of acetate into total lipids that could contribute to lesser triglyceride accumulation. Decreases of serum triglycerides in animals are shown to result from the reduction of very low-density lipoprotein-triglyceride secretion and the inhibition of hepatic lipogenesis by the reduced activity and down regulation of gene expression of lipogenic enzymes (Kok et al., 1996; Delzenne et al., 2001). Feeding of high-molecular weight levan (1-5% w/v) decreased serum total cholesterol in a dose dependent manner and the oral administration of 2% levan reduced adiposity as well as serum lipids in rats (Kang et al., 2002a). The possible mechanism of reduced serum lipid parameters might be attributed to peroxisome proliferation-activated receptors (PPARs) that have important effects on lipid homeostasis, by regulating the expression of genes involved in lipid metabolism (Escher and Wahli, 2000). The supplementation of levan decreased adiposity and postprandial lipidaemia in rats, through the enhancement of uncoupling protein gene expression (Kang et al., 2002b, 2004). The mRNA expressions of hepatic fatty acid synthase and acetyl CoA carboxylase, which are the key enzymes in fatty acid synthesis, are also down-regulated by levan (Kang, 2009).

HSP-70 levels and lipid peroxidation (LPO) are sensitive biomarkers, and found to be highly elevated upon exposure to pollutants like heavy metals and pesticide (Kumar and Singh, 2019). The significant increase in HSP-70 level might be associated due to denatured protein leading to generation and accumulation of reactive oxygen species in response to different kinds of stressors in aquatic animal (Zhang et al., 2009). In the current investigation, HSP-70 has been improved by supplementation of dietary levan which may be due to the stimulation of nonspecific defence mechanism by HSP, which is in accordance to the findings of Sung et al. (2008) and(Gupta et al. (2010).

Lipid peroxidation (LPO) is a process under which oxidants such as free radicals attack lipids containing carbon-carbon double bond(s), especially biological membranes that leads to impaired membrane function, structural integrity as well as inactivation of several membranes bound enzymes (Goel et al., 2005). Thus, LPO is an indicator of principal disorders in structural, functional and enzymatic organization of the biological membranes. In this study, level of LPO has been significantly reduced in levan supplemented groups, hence, it may be inferred that dietary levan

holds this mechanistic property for inhibition of lipid peroxidation and mitigation of stress in *C. carpio*. To supports our results, studies reported that use of other dietary nutraceuticals such as methyl donors (Kumar et al., 2014) and pyridoxine (Kumar et al., 2016) reduced the oxidative stress and maintained their level in the different tissues.

Total immunoglobulin (Ig) and myeloperoxidase content of serum in levan fed common carp were significantly enhanced with concomitant increase in the level of supplementation. The major functions of immunoglobulins in defense-related activities such as destroying the pathogen and amelioration of stress are well recognized (Gupta et al., 2020). Similar to levan, inulin an another prebiotic, has shown to exhibit enhanced IgM level and myeloperoxidase activity in leopard grouper, *Mycteroperca rosacea*, (Reyes-Becerril et al., 2014). The enhanced level of total immunoglobulin and myeloperoxidase content in higher levan fed group at 0.75% suggested that levan incorporation in common carp might have triggered the activation of immune cells which might have led to ameliorate the stress mediated through fipronil exposure. Furthermore, our results are in accordance to studies performed by Ahmadifar et al. (2019) which showed that dietary ginger administration significantly increased total Ig levels in zebrafish. Besides, Hoseini and Yousefi (2019) demonstrated that dietary supplementation of thyme extract significantly improved plasma total Ig in rainbow trout (*Oncorhynchus mykiss*) against oxytetracycline-induced stress, indicating the protective role of prebiotic levan in augmenting the immune status of common carp against pesticide stress.

Conclusion

To summarize the above findings, dietary microbial leavn at 0.75% of incorporation level led to enhancement of the intestinal protease and lipase activities whereas as alkaline and acid phosphatase activity was declined. The ameliorating effect of microbial levan was confirmed by marked improvement in glycogen level, serum cholesterol, triglyceride, LPO, HSP-70, myeloperoxidase content as well as total immunoglobulin level of the fish fed with 0.75% of levan. Overall results showed protective and ameliorative role of dietary microbial levan at 0.75% incorporation level and could be used as potent immuno-additive prebiotic supplements to mitigate the fipronil induced stress in aquaculture. Further investigation using different concentrations of levan should be accomplished to unravel defensive mechanism against fipronil induced immune-toxic impact.

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