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Efficiency of dietary synbiotic on hematological, histopathological changes and resistance against *Saprolegnia spp.* in common carp, *Cyprinus carpio* L.

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Abstract
This study was carried out to assess the efficiency of synbiotic on hematological, histopathological changes and resistance against *Saprolegnia spp.* in *Cyprinus carpio*. A total of 100 *C. carpio* weighing 49.55–50.50 g, were randomly stocked into five treatment groups. Fishes were fed different synbiotic concentrations: 0.5% (T1), 1.0% (T2), 1.5% (T3) and 2% (T4), as well as the control group (C) were fed basal diet without any addition of synbiotic. All treatments were fed twice a day at rate of 2% body weight for 56 days. At the end of the feeding trail, all the treatment groups were challenged in a viable fungal suspension of *Saprolegnia spp.* (1×10^5 live zoospores/ml). Considerable changes have been recorded in the differential leucocyte count, percentage of lymphocyte and monocyte showed significantly decrease ($P<0.05$) in T4 compared to the C+ group. Neutrophils (%) was significantly increased in T4 compared to C+ group. Histopathologically, gill sections exhibited epithelial lifting, epithelial hyperplasia and cellular necrosis. Quantitative analysis of gill lesions revealed that the lifting of the epithelium, epithelial hyperplasia were significantly increase ($P<0.05$) in groups treated with synbiotic compared to C+ group. Skin of infected fish (C+) showed loss of the epidermis, necrosis in dermis and penetration of hyphae in underlying musculature, these changes are lesser in extent in fish fed with synbiotic supplemented diet. Although, all levels of dietary synbiotic significantly increase resistance against *Saprolegnia* challenge, the highest survival rate was found in T4 (83%) followed by T3 and T2 (66%), T1 (50%) and C- (16%) respectively. The results indicated that supplementation of dietary synbiotic at 2.0 % in diet are more resistant against infection by *Saprolegnia spp.* Therefore, dietary treatment using probiotic and prebiotic combination had a positive influence on the survival rate and disease resistance in *C. carpio.*

Keywords: *Cyprinus carpio,* Differential leucocyte count, Histopathology, Synbiotic, *Saprolegnia spp*

1. Introduction
The fish pathogenic oomycetes, specifically *Saprolegnia spp*; a member of the family Saprolegniaceae, causing high mortality and economic loss in fresh water fish and fish eggs as well, these pathogenic are widespread and occurred at any stage of fish life cycle [1]. Saprolegniasis (water mold) characterized by a relatively superficial, cottony/woolly white growth on the body surface, or gills, or on fish eggs. Initial lesions are often focal, inconspicuous and small, but these lesions can rapidly extend into dermis and the subjacent superficial musculature layer with time [2].
A number of disinfectants and fungicide such as Malachite green formaldehyde and hydrogen peroxide are frequently used for treatment of Saprolegniasis, however these chemicals are no longer recommended application because of some fungi have developed resistance against these disinfectants, as well as some fungicide are not biodegradable and tend to be accumulate in the environment [3]. Nowadays, the use of probiotics and prebiotics in fish diets have been received great interest mainly for protection against infectious diseases [2]. Probiotic, *Lactobacillus acidophilus* and baker's yeast (*Saccharomyces cerevisiae*) has shown numerous benefits in fish aquaculture and becoming as a promising biological control strategy for improving growth and disease resistance [4, 5]. The prebiotic β-glucan, employed in aquaculture has shown an effective immune-stimulant effect by improving growth, immune response, and resistance against infectious diseases in fish and shellfish [6, 7].
Synbiotic is defined as a combination of probiotic and prebiotic. It is assumed to impart the valuable effect of both components. Synergistic effects can be performed from this combination. Thus, synbiotic can help to improve health status, disease resistance, growth performance,

carcass composition and digestive enzyme activities [8]. Few studies are available regarding the application of synbiotic in aquaculture [8-11]. Therefore, the present study was undertaken to assess the efficiency of synbiotic (combination of probiotic and prebiotic) on hematological, histopathological changes and resistance against *Saprolegnia spp.* in *C. carpio*.

2. Materials and Methods

2.1 *Saprolegnia spp.* strain and spore suspension

The fungus of *Saprolegnia sp.* was isolated from infected common carp, identified and purified based on morphological features and microscopical examination. Spore suspension of *Saprolgenia spp.* was prepared according to method of Chittasobhon and Smith [12]. The concentration of the suspension was adjusted to about 1×10^5 live zoospores/ml**.**

2.2 Diet preparation and feeding

The type of synbiotic used in this research was consist of probiotic [(*Saccharomyces serevisiae* 10^{10} CFU/ml, *Bacillus subtilus* 10^9 CFU/ml and Lactic acid bacteria J6 10^{11} CFU/ml) 0.5%] and ß-glucan (1%) as the prebiotic. A basal diet was formulated (protein 36%, carbohydrates 29%, fat 9%, ash 10%, fiber 5%, moisture 10% and Phosphorus 1.1%); this basal diet served as the control diet and the experimental diets were produced by supplementation of the basal formulation with different concentrations of synbiotic was added to basal diet (0.5%, 1%,1.5% and 2%). The ingredients were ground mixed mechanically in a mixer and pelleted using a grinder. The pelleted diets (1.5 mm) were air-dried, then the feed was stored at 4 °C until feeding trial began.

2.3 Experimental design and husbandry conditions

About 100 of healthy *C. carpio* (mean weight 50.0±2.0g) were collected from a commercial farm (Al–Mahaweel, Babylon/Iraq). The health status of fish were checked for disease and both fish and tanks were disinfected using sodium chloride [13]. After that, fish were acclimatised to laboratory conditions for 2 weeks in ceramic tanks with aeration. Post acclimatization period, 100 fish were randomly selected and divided into 10 tanks trough (measuring 150×80×50 cm) at a rate of 10 fish per tank (two replicates/treatment) filled with (80 L) chlorine–free tap water, Fishes were fed various concentrations of synbiotic as follows: 0.5% (T1), 1.0% (T2), 1.5% (T3) and 2% (T4), as well as the control group (C) were fed basal diet without any addition of synbiotic. Fish were fed experimental diets twice daily at a rate 2% body mass for 56 days. The fish were kept at a natural photoperiod (12 h light /12 h dark), with a mean water temperature 23.2 ± 0.3 °C, pH 7.1 ± 0.2 and DO 6.6 ±0.3 mg/l respectively. At the end of the feeding trail (i.e., 56 days) blood samples were collected from 6 fish which randomly selected from each treatment group for studying differential blood count.

2.4 Challenge test

After 56 days of feeding trail, 10 fish in duplicate from each group were challenged with a viable fungal suspension (1×10^5 live zoospores/ml) of *Saprolegnia spp.* Control groups were divided into two subgroup: negative control (without challenge with *Saprolegnia spp.*) and positive control (fishes were challenged with a viable fungal suspension). Mortalities were recorded in all of the treatments for 2 weeks. Survival rate was calculated using the following formula**:**

Survival rate% =final number of fish survivor/initial number of fish ×100

After 8-14 day of challenge test, blood samples were collected for the differential leucocyte count and three fish from each tank was dissected for histopathological examination.

2.5 Differential leucocyte count

Differential leukocyte counts were determined using Giemsa staining method and detected blood films under light microscope [14].

2.6 Histopathological studies

Tissues of gills and skin with muscles were fixed in formaldehyde solution 10% for 48 hrs. The tissues were then processed regularly and arranged in paraffin blocks. The blocks of the tissues were cut at 5µm thickness and stained with Haematoxylin and Eosin (H&E) and skin sections were stained with Periodic Acid Schiff (PAS). For the gill tissues, histological features were recognized, measured and the number of lamellae was scored. Only those secondary lamellae that were complete from tip to base, were considered for investigation [14].

2.7 Statistical analysis

Statistical analysis was performed using SPSS software (V. 17.0) to verify significant differences among the treatment and the control groups. One way analysis of variance (ANOVA) was applied to scrutinize the data. The differences between means were determined and compared using LSD test with a significance level of $P < 0.05$.

3. Results

3.1 Differential leukocyte count

The percentage of lymphocyte and monocyte pre challenge with *Saprolegnia spp.* showed a significant decrease ($P<0.05$) in T4, T3, T2 and T1 compared to the control group. Also, lymphocytes (%) recorded significant differences among all treated with synbiotic supplemented diets (T1, T2, T3 and T4). While, the neutrophils (%) recorded significant increases ($P<0.05$) in all treated synbiotic supplemented diets relative to the control group (Table 1). Post challenge with *Saprolegnia spp.* lymphocyte (%) was significantly decreased ($P<0.05$) in T4 compared to the C^+ group. However, lymphocyte (%) showed no significant differences among all treated synbiotic supplemented diets (T4, T3, T2 and T1). While, the neutrophils (%) was significantly increased in treated groups (T3, T2 and T1) in comparison to C+ group (Table 2).

Table 1: Differential leukocyte percentage of *C. Carpio* (pre challenge) in fish treated with synbiotic supplemented diets for 56 days.

Treatment	Mean ± SE (%)				
	Lymphocyte%	Monocytes %	Neutrophils %	Eosinophils %	Basophils %
C	65.00 ± 2.78^{a}	9.3 ± 0.56^{a}	25.3 ± 2.58^{d}	0.30 ± 0.00^{d}	0.06 ± 0.00^{a}
T1	62.67 ± 3.71^{b}	6.33 ± 2.02^{c}	28.33 ± 4.09^{c}	2.00 ± 0.00^{b}	$0. 66 \pm 0.32^{a}$
T2	60.33 ± 0.88^{c}	7.00 ± 1.52^{b}	29.33 ± 0.67^{c}	3.33 ± 0.67^{a}	0.66 ± 0.33^{a}
T3	55.33 ± 1.45^{d}	6.67 ± 1.20^{bc}	35.00 ± 2.88^{b}	2.33 ± 0.33^{b}	0.66 ± 0.33^{a}
T4	53.67 ± 0.33^{e}	5.00 ± 1.52^{d}	39.33 ± 1.76^{a}	1.67 ± 0.33^{c}	0.33 ± 0.12^{ab}
Level of significant	*	*	*	*	*

Values indicated with different superscript letters in the same column are significantly different ($P<0.05$).

Table 2: Differential leukocyte count of *C. Carpio* (post challenge with *Saprolegnia spp.*) in fish treated with synbiotic supplemented diets.

Treatment	Mean ± SE (%)				
	Lymphocyte %	Monocyte%	Neutrophil %	Eosinophil %	Basophil %
C-	62.33 ± 2.86 [b]	11.0±0.70[a]	26.33±2.19 [a]	0.33 ± 0.02	0 ± 0.00
C+	74.3 ± 3.69[a]	8.0± 0.61[ab]	17.3±1.15[c]	0.33 ± 0.00	0 ± 0.00
T1	71.3 ± 3.72[a]	7.3 ± 0.52[b]	21.0±1.08[bc]	0.33 ± 0.00	0 ± 0.00
T2	69 ± 3.64[ab]	7.6 ± 0.66[b]	23.0±1.76[ab]	0.33 ± 0.00	0 ± 0.00
T3	68.3 ± 2.51[ab]	9.0 ±0.40[ab]	22.3±2.09[ab]	0.33 ± 0.00	0 ± 0.00
T4	65.0 ± 2.78[b]	9.3± 0.56[ab]	25.3 ± 2.58 a	0.33 ± 0.00	0 ± 0.00
Level of significant	*	*	*	NS	NS

Values indicated with different superscript letters in the same column are significantly different (P<0.05).

3.2 Histopathological studies

3.2.1 Gills

The gills tissue from the negative control group showed normal structure of primary and secondary lamellae (Figure 1 A), whereas gills tissue in positive control and in fish fed with synbiotic supplemented diets and challenged with *Saprolegnia spp*. exhibited several histopathological findings involving: hyperplasia (Figure 1 B), complete fusion of adjacent lamellae (Figure 1 C), as a result of epithelial hyperplasia. In addition, a detachment of the epithelium, dilation of the central venous (Figure 1D), telangictatisis (Figure 1 E) and necrosis of primary and secondary lamellae were also observed (Figure 1 F). Hyperplasia, fusion of the secondary lamellae and the lifting of the epithelium were significantly increased in fish fed with synbiotic supplemented diets in comparison to negative and positive control groups. While, telangiectasis and necrosis showed significant decrease in fish fed synbiotic supplemented diets than those of control groups (data are not shown).

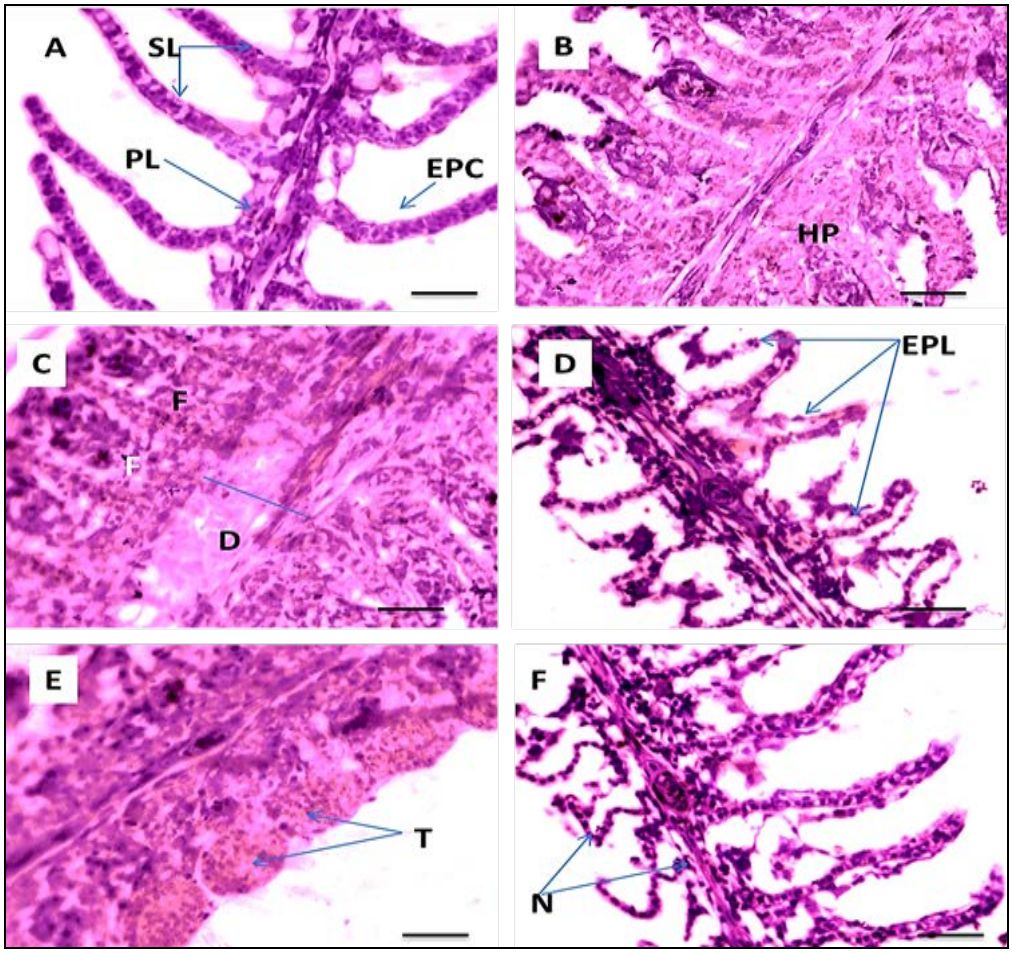

Fig 1: Photomicrographs of the gill tissue of *C. carpio* fed synbiotic supplemented diets for 56 days and challenged with *Saprolegnia spp.* showing: A: normal of gill structure primary lamellae (PL), secondary lamellae (SL) and epithelial cells (EPC); B: demonstrating hyperplasia of secondary lamellae (HP); (C): complete fusion of the secondary lamellae (F) with dilation of the central venous (D); D: showing epithelial lifting (EPL); E: telangictatisis (T); F: necrosis in secondary and primary lamellae (N). H&E stain at 5 µm thickness. Scale bars: 50 µm.

3.2.2 Skin

Skin sections of negative control showed normal arrangements of epidermis, dermis and muscular layer (Figure 2 A). In general, the histopathological alterations of the *Saprolegniasis* were observed in all treatment groups; positive control revealed loss of the epidermis, necrosis in dermis (Figure 2 B), Also, there were enormous infiltration of mononuclear cells (MNCs), massive cellular debris mixed with fungal hyphae, increased in melanophores and extensive edema in the hypodermis and various degree of degenerative alterations in the underlying musculature, resulting in obvious myofibrillar degeneration with loss of nuclei and focal myofibrillar necrosis (Figure 2 C, D &E). However, these changes are lesser in extent in synbiotic diet supplemented groups; T1 showed sloughing, desquamation associated with cytoplasmic vacuolation in epidermal layer. Also, cellular debris mixed with fungal hyphae was seen in epidermal and dermis layers (Figure 2 F&G). While, T2, exhibited infiltration of MNCs, increase the number and size of mucous secreting cells (Figure 3 A&B). T3 and T4 showed cytoplasmic vacuolation and increase of melanophores in epidermis and dermis layers (Figure 3 C, D, E, F &G)

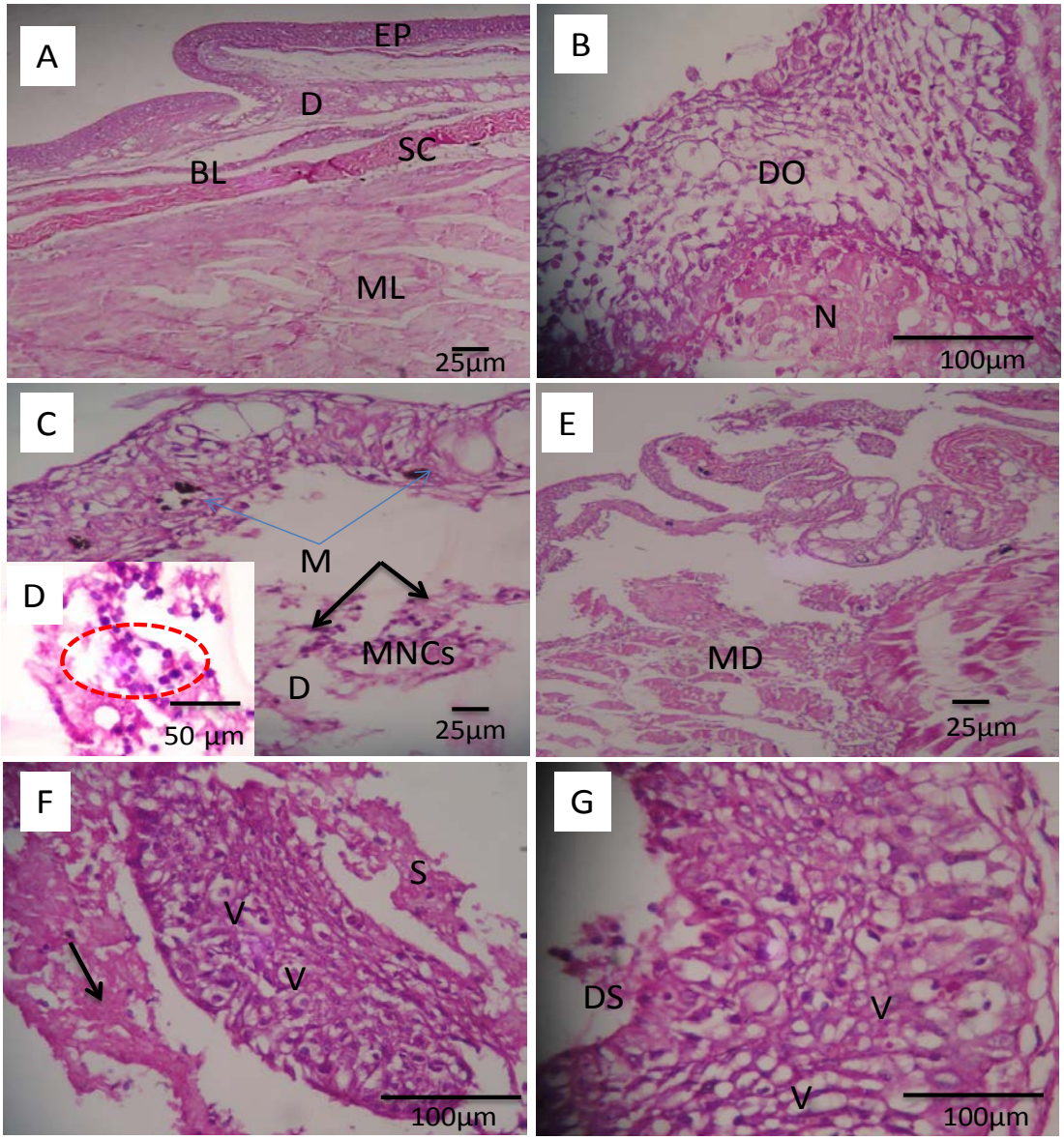

Fig 2: Photomicrographs of the skin tissue of *C. carpio* fed synbiotic supplemented diets for 56 days and challenged with *Saprolegnia spp.* (A): control (normal) showing epidermis(EP), dermis (D) basal layer (BL), stratum compactum (SC) and muscular layer; (B-E) positive control exhibiting disorganized in epidermis (DO), necrosis of dermis (N), desquamation (D) with mononuclear cells infiltration (MNCs) (red circle), increased in melanophore (M) and muscular distraction (MD) (F&G) T1 showing sloughing (S), desquamation of epidermal tissue (DS), cytoplasmic vacuolation (V), cellular debris mixed with fungal hyphae (black arrow). PAS stain; Thickness 5µm.

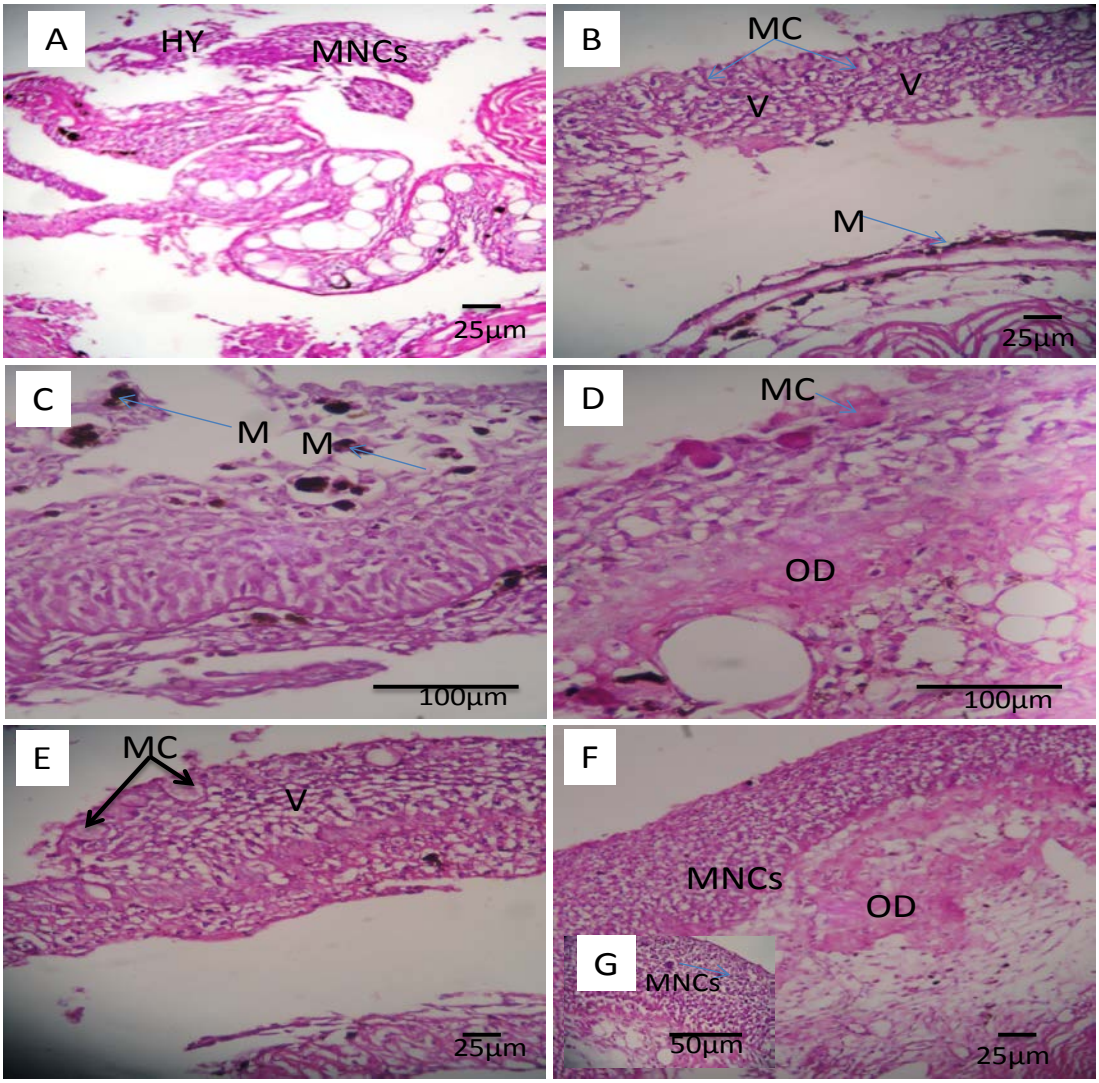

Fig 3: Photomicrographs of the skin tissue of *C. carpio* fed synbiotic supplemented diets for 56 days and challenged with *Saprolegnia spp.* (A&B): T2 showing cellular debris mixed with fungal hyphae, MNCs infiltration, increase mucous cells (MC), cytoplasmic vacuolation and increased melanocyte (M); (C&D) T3 exhibiting increase in melanophores (M), increase mucous cells (MC) and edema in epidermis (OD); (E-G) T4 showing extensive increased in mucous cells (MC), cytoplasmic vacuolation In epidermis (V), MNCs infiltration, and edema in epidermis (OD); PAS stain; Thickness 5µm.

3.3 Disease resistance

After the challenge of the fish with *Saprolegnia* mortality rate was registered for 14 days. All levels of dietary synbiotic showed significantly increase the resistance against *Saprolegnia* relative to C+ group. The highest survival rate was observed in C- group (100%) followed by T4 (83%), T3 and T2 (66%), T1 (50%) and C+ group (16%) respectively (Figure 4).

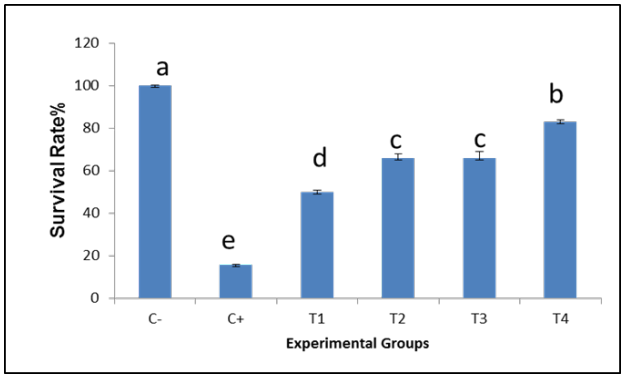

Fig 4: Survival rate of *C. carpio* fed different treatments of synbiotic and challenged with *Saprolegnia spp*. Different superscript letters indicated significant differences (P<0.05) among treatments.

4. Discussion

Through differential counting of leukocytes, the results showed significant differences among treatments compared with the control**.** The enhancements in WBC in the present study specify the immune-stimulant and potential effects and anti-infective properties of probiotics and prebiotics. However, combination of β-glucans with bacterial supplements as synbiotic has been scarcely considered. Synbiotic/probiotics are additionally able to multiply fishes granulocytes and macrophages the same as higher vertebrates [15].These results are in line with previous studies that obtained by AL- Saphar, [16] who found that administration of probiotic demonstrated significant differences of WBC count in common carp. Selvaraj *et al.* [17] observed that yeast glucan administration significantly increased in total blood leukocyte counts and an increase in the proportion of neutrophils and monocytes of *C. carpio*. In contrary, Firouzbakhsh *et al.* [18] noticed that dietary synbiotic Biomin IMBO (*Enterococcusfaecium* and Fructooligosaccharide) (FOS) at three levels 0.5, 1.0 and 1.5 g kg^1 for 60 days significantly increased percentage of lymphocyte in rainbow trout, *Oncorhynchus mykiss*.

It is well known that the gills contribute in the respiration, osmoregulation and excretion in fish. However, due to their

close contact with the external environment, these are particularly sensitive to the changes in water quality [19]. The observed gill epithelial hyperplasia and the lifting up of the epithelial cells beside the partial to complete lamellar fusion are considered non-specific reactions of the gills to toxic irritants or to infection [20], which were natural attempts by the exposed fish to increase the diffusion distance between their blood and the toxic external environment (i.e., the epithelia of skin, gills act as external barriers separating the fish from its environment). These epithelia work as mechanical barriers to invading pathogens, but they also contain chemical (antibodies, lysozyme, etc.) and cellular (immune cells) defenses. In fact, a wide variety of chemicals has been reported to impact immune parameters of teleost fishes [21, 22]. Histopathological manifestations due to mycotic infection in fishes have been documented by Chauhan *et al.* [23] and Songe *et al.* [24]. The results of the present study in the gills are in agreement with El Genaidy *et al.* [25], Refai *et al.* [26] and Ashour *et al.* [27]. Similar results were observed by Hussian *et al.* [28].

The histopathological changes observed in the skin tissues of carp fish challenged with *Saprolegnia spp.* in the current study were similar to those described in a salmon infected with *Saprolegnia spp.* by Hussein and Hatai, [29]. It is well known that the skin of the fish is metabolically very active, and it rapidly responds to stressors [30]. Because it is a living tissue, fish skin is a common target for many opportunistic pathogens that are present in the environment. These pathogens could also be ubiquitous on healthy skin resulting skin damage [31].

The presence of degeneration in the muscle bundles and the presence of cellular debris mixed with fungal hyphae produced by Saprolegnia [32]. Peduzzi and Bizzozero [33], explained that the enzymatic activity is possibly a causative factor for the pathogenesis of *Saprolegnia.* The increased number of mucous secreting cells in synbiotic supplemented diets groups are perhaps due to enhanced release of mucous cell contents as a defense protecting against *Saprolegnia spp*. Fish epidermis is in direct contact with the environment and mucus is regarded to be a first line of defense mechanism the epidermis. Stimulation of mucus secretion is a characteristic stress stimuli in fish and has been documented for other stressors such as heavy metals including: copper, mercury, lead, copper and chromium [28]. Similar patterns of manifestations in the skin of *Saprolegnia* infected fish has been reported by Hatai and Hoshiai [34], Hussian *et al.* [28] and Chauhan *et al.* [24].

The combination of probiotic and prebiotic, was reduced fish mortality from 83% in the control to 16 – 50 % in the synbiotic-treated groups. These results are in accordance with Firouzbakhsh *et al.* [18] who noticed that rainbow trout, (*Oncorhynchus mykiss*) fed synbiotic Biomin IMBO (*Enterococcus faecium* and FOS) and after a challenge by *S. parasitica*, reduced fish mortality significantly from 88% in the control to 27–44% in the synbiotic-treated groups. Also, Gang *et al.* [35] found that cobia (*Rachycentron canadum*) fed diets supplemented with various levels of probiotic and prebiotic (chitosan) for 56 days challenged with *Vibrio harveyi* reduced fish mortality significantly. The beneficial effect of dietary supplements in challenge studies is also related to the fact that both probiotic and prebiotic supplements reduce or eliminate the incidence of opportunistic pathogens by blocking their attachment and thus inhibiting host colonization [36].

5. Conclusion

From the results obtained in the present investigation, dietary supplementation of the synbiotic at 2% significantly enhanced survival rate and disease resistance of common carp against *Saprolegnia*, suggesting a potential use of this synbiotic in fish aquaculture. The results of this study require to be studied in depth in order to further adjust the use of these additives in the diet for common carp as combination (probiotic and prebiotic) and or in sole and/or with other feed additives for disease control plans in farmed fish.

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