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Changes in gut microbiota, hematological parameters, and immune response following dietary administration of oyster mushroom (*Pleurotus ostreatus*) in silver carp (*Hypophthalmichthys molitrix*)

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Abstract
The primary aim was to evaluate the impact of oyster mushroom (*Pleurotus ostreatus*) on the gut microbiota, hematological parameters, and immune response of silver carp (*Hypophthalmichthys molitrix*). The experimental group was given *P. ostreatus* powder at 2% of body weight, while the control group was fed with commercial fish feed. Fish were treated with these two feeds for 8 weeks. Culturing of bacteria, hematological tests, and immunological assays were done to evaluate the effect of these feeds on *H. molitrix*. Physico-chemical parameters of the aquarium were maintained end-to-end during the trial period, such as DO at 5-7 mg L^{-1}, temperature at 24-28 °C, and pH at 7.0-8.2. The quantitative outcomes from the groups were compared using the *t*-test. In all three culturing media, such as nutrient agar (NA), tryptic soy agar (TSA), and eosin methylene blue (EMB), the results indicated a significant decrease in bacterial colonies in the treatment group, when compared with the control group. Compared to the control group, the treatment group had a significantly higher RBC count, higher hemoglobin value, enhanced hematocrit levels, MCV, and MCHC. White blood cells (WBCs), neutrophils, and lymphocytes in the treatment group had a significantly higher count than those of the control group. However, the hematological parameter MCH and immune cells, such as monocytes, remained unchanged in the treatment and control groups. Hence, using immunostimulants such as mushrooms in aquaculture increases the body's natural resistance to infection and facilitates the prevention of various diseases.

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Introduction

Achieving world food and nutrition safety goals within ecological boundaries will need a revolution in food production and dissemination systems globally as the human population heads to 10 billion in the near future (Habib et al., 2025). Global seafood supply has increased from 9 kg per capita to 20.2 kg per capita from 1961 to 2015, and is expected to reach 32 kg per capita in the medium term (2030-2050), with the indication that the price of capture fisheries keeps on increasing as demand per capita increases (FAO, 2018). Thus, for filling this demand gap, aquaculture comes as

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a solution (Gephart et al., 2020; Haque and Mahmud, 2025).

Aquaculture is a rapidly growing food production sector globally and is the cheapest and easiest source of animal protein. Carps are the most important cultured fish, which contribute more than half of freshwater production. Silver carp (*Hypophthalmichthys molitrix*) is widely used in aquaculture; it is present in reservoirs, lakes, streams, and ponds naturally (Zu et al., 2023). *H. molitrix* remains an obligate phyton-plankter through all life stages, and zooplanktons are swallowed incidentally (Tahami et al., 2023). This fish, with its filter-feeding nature, has been used to regulate cyanobacteria blooms in eutrophic aquatic reservoirs (Zhou et al., 2022). Due to rapid growth and frequent breeding of *H. molitrix,* it became the most common fish in freshwaters of various regions of the world, and its production is increasing yearly, and its demand keeps on increasing (Iqbal et al., 2014; Hedayati and Niazie, 2015; Jawdhari et al., 2022).

With the increasing demand for consumption, there is an increase in fish cultivation by intensifying ponds (Rathore et al., 2017). As a consequence of intensifying ponds, the fish are more prone to a stressful environment, which increases the risk of disease outbreaks. The most common way to cope with diseases is the application of antibiotics, which triggers the proliferation of drug-resistant bacteria, sanitary prophylaxis, chemotherapy, and disinfection, leading to the weakening of the immune system, coupled with certain environmental hazards. Further, fish meal is an important source of protein for aquaculture, but there are several problems associated with using fish feed, like instability in fish meal prices and supply affecting the culture practices, and many others (Ahmad and Ibrahim, 2016). Therefore, there is a need to develop alternate ways to increase growth, immunity, and disease resistance in farmed fish. The most positive results achieved are with protein-rich and immunostimulatory feed additives, for instance, natural feeds obtained from agriculture, such as mushrooms that exhibit immunomodulatory and antibacterial properties (Godfray et al., 2010; Hoseinifar et al., 2019). Mushrooms are one of the fishmeal replacers (Katya et al., 2014; Muin et al., 2015).

Exploring innovative methods for preventing contagious diseases has become substantially persuasive in aquaculture after the harmful outcomes of antibiotics and disinfectants (Mohan et al., 2021). Freshwater carps heavily rely on the gut microbiome for several physiological functions, one among these is immune modulation (Navarrete et al., 2012; Banerjee and Ray, 2017). Gut microbiota plays a significant role in the exclusion of pathogenic microbes, maintaining microbial homeostasis (Nayak, 2010; Roeselers et al., 2011; Bretto et al., 2025). As our knowledge of gut microbiome increases, research on probiotics, prebiotics, and symbiotics is also expanding (Sarita et al., 2025). Mostly, these prebiotics are non-digestible oligosaccharides, and now long-chain non-digestible polysaccharides are being focused. One of these novel prebiotics is β-glucan, which is present in many types of fungi. Moreover, β-glucan has the potential to modulate the immune system and is sourced from edible mushrooms (Lam and Cheung, 2013; Mirończuk-Chodakowska et al., 2021).

The oyster mushroom (*Pleurotus ostreatus*) was selected for this research based on its previously known medicinal and beneficial effects (Ulukoy et al., 2016; Sreedharan et al., 2025). Mushrooms stimulate both innate and adaptive immunity by propagating and triggering innate immune cells, namely, neutrophils, natural killer cells, and macrophages (Shyamala and Maheswari, 2021; Toros et al., 2023). In aquaculture, mushrooms have been used as a potent agent against microorganisms as they possess extensive antimicrobial characteristics against both Gram-positive and Gram-negative bacteria. Polysaccharides present in mushrooms exhibit mechanisms of action within the gut microbiota, acting as prebiotics and impacting the digestive system (Anusiya et al., 2021). The oyster mushroom offers a promising avenue for improving nutritional intake and promoting health due to its rich nutritional composition and bioactive properties in fish (Ahmed et al., 2014; Deepalakshmi and Mirunalini, 2014; Bulam et al., 2022).

In fish, the use of mushrooms as feed, or their organic extracts such as *P. pulmonarius, Lupinus perennis*, *Inonotus obliquus, Agaricus bisporus*, *Ganoderma lucidum*, *P. ostreatus*, and *Lentinula edodes* has been studied to increase disease resistance against many different pathogens and overall well-being. The oyster mushroom powder in many different studies significantly enhanced the hematological and non-specific immune responses and growth in fishes like Nile tilapia (Kakavand et al., 2021; Shyamala and Maheswari, 2021), Rainbow trout (Baba and Ulukoy, 2022), and Rohu (Devi et al., 2023; Saha et al., 2023). However, no study has been found on the effect of oyster mushrooms on *H. molitrix*. Thus, the principal objective of carrying out this study was to assess the impact of oyster mushroom (*Pleurotus ostreatus*) on the gut microbiota, hematological parameters, and immune response of silver carp (*Hypophthalmichthys molitrix*).

Material and Methods

Fish and experimental design

Healthy fingerlings of *Hypophthalmichthys molitrix* with an average weight of 5-8 g were collected from the Fisheries Research Farms located at the Department of Zoology, Wildlife and Fisheries, University of Agriculture, Faisalabad, Pakistan. The fish were distributed randomly into two groups, one being the treatment group (n = 20) and the other being the control group (n = 20). The experimental group was given dried mushroom feed at the rate of 2% body weight once a day. Similarly, the control group was given commercial fish feed. The feeding trial continued for eight weeks.

Diet preparation

The oyster mushroom was obtained from a local source. The fresh mushrooms were cleaned and sliced, then arranged in a single layer on trays. The oven was preheated to 60 °C. The mushrooms were dried in an oven for 24 h until crisp. Once dried, they were allowed to cool completely. The cooled mushrooms were then ground into powder using an electric grinder. Finally, the mushroom powder was stored in an airtight container and used for experimental purposes. Commercial fish feed was purchased from the local market and stored in an airtight container.

Sterilization

We used the moist sterilization method, and culturing media and all glassware were kept in an autoclave at 200 lb, 121 °C for 15 minutes. The inoculation needles were sterilized on a red-hot flame.

Feeding, trial, and sampling procedure

During the feeding trial, the aquarium's water chemistry indices were maintained regularly. After 8 weeks of the feeding trial, the fish were dissected to obtain the gut. Intestinal samples were preserved in Eppendorf tubes already filled with 0.5 mL saline solution, and the obtained samples were shaken well in an electric shaker to make a homogenized solution in each tube. The blood samples were collected from the fish subjected to euthanasia by adding the clove powder solution to water. Blood was collected in a Vacutainer containing EDTA.

Total bacterial count using different media

Solutions of different agar media, such as nutrient agar (NA), tryptic soy agar (TSA), and eosin methylene blue (EMB), were used to prepare accurate jelly-like culture media. For this step, all three agars were dissolved in 100 mL of distilled water contained in conical flasks (Table 1).

Table 1: Different agars used

Agar	NA	TSA	EMB
Weight(g)	2.80	4.00	3.75

Conical flasks were then wrapped with aluminum foil and placed in the autoclave at 121 ºC for 15 minutes. Bacteria in the gut of fish were cultured using media like EMB, NA, and TSA according to the method of Ogunshe and Olabode (2009) and Adejonwo et al. (2020). The samples were placed on the culture media (solidified) using a sterile inoculating loop, and then the loop was moved in a zig-zag (Quadrant Sticking Method) onto a plate (Sanders, 2012). Finally, the Petri plates were placed in an incubator set at 37 ºC for one day. After 24 hours of incubation, these Petri plates were then placed on a colony counter (J-2 Digital Colony Counter), and CFUs were counted by the method of Clarke et al. (2010). This was helpful to find out the effect of mushroom feed and commercial feed on the gut microflora.

Hematological analysis

Blood analysis was performed immediately after the collection of blood using a hemocytometer. For RBCs, the count was done following Parida et al. (2011). Counting was done in an improved Neubauer Hemocytometer by diluting the blood with Hayem's solution. The results were expressed as RBC × 10^6/µL. The hemoglobin test kits were used to determine the Hb levels (g/dL) using the cyanmethemoglobin method. For hematocrit count (HCT), blood samples were placed in glass capillary tubes and then centrifuged at 10,500 rpm for 5 minutes (Adebayo et al., 2007). The secondary hematological parameters, such as MCV, MCHC, and MCH, were calculated by Saravanan et al. (2011)

Determination of immunological parameters

For leukocyte count, blood samples were mixed with the Giemsa stain, and the cells were counted in a hemocytometer under a light microscope by Oluyemi et al. (2008). Differential leukocyte count was made by preparing blood smears stained with the Giemsa stain, methanol, aqua distillate, and methylene blue. Neutrophils, monocytes, lymphocytes, basophils, and eosinophils were identified using a microscope at 100X oil immersion; all cells were morphologically different from each other, and were counted.

Physico-chemical parameters

Consistent monitoring of water parameters was done by maintaining the regulatory control according to the method employed by Manzoor et al. (2023). Electronic devices such as a pH meter and HANNA water testing kit, like HANNA HI-8424, were used to measure pH, temperature, alkalinity, water hardness, and dissolved oxygen (DO). All such measurements are presented in Table 2.

Statistical analysis

The data was subjected to statistical interference at the end of the experimentation. The results were compared using the *t*-test for the final interpretation. The *F*-test was performed first to check the equality of variance, and then the t-pool or t-prime test was applied.

Results

Limnological parameters

Fish and other biological organisms grow differently depending on the temperature and dissolved oxygen, which affect their biological productivity. In aquatic habitats, pH is a crucial limnological parameter that controls chemical equilibrium and biological processes. Total alkalinity and total hardness contribute to water quality and consequently, the ecosystem health. Table 2 shows the mean values of the physico-chemical attributes in the treatments, T_0 and T_1 after a period of 8 weeks.

Table 2: Mean physico-chemical parameters

Week	pH	Temperature (˚C)	DO (mg L^{-1})	Total alkalinity (mg L^{-1})	Water hardness (mg L^{-1})
1	7.2	27	4.1	100	150
2	7.3	28	4.3	95	145
3	7.1	27.5	4.2	98	152
4	7.4	27.8	4.4	96	148
5	7.2	28	4.2	97	150
6	7.3	27.5	4.3	94	146
7	7.1	27.8	4.5	99	151
8	7.5	27.7	4.4	95	147
Mean ± SD	7.29 ± 0.16	27.63 ± 0.54	4.29 ± 0.16	97.25 ± 2.49	148.63 ± 2.45

Total bacterial count

The total microbiological content and CFU of the intestinal samples from *H. molitrix* grown on different agar media revealed information on the variety and quantity of microorganisms present in the fish's digestive system. The gut microbiota is affected by changes in diet, and these microbial communities help in immune modulation, thus aiding in aquaculture management strategies. Fish fed with a *P. ostreatus* diet had an impact on the gut colonies, as shown in Table 3.

Table 3: Total viable count of *H. molitrix* (Intestinal samples) cultured on different agars

Agar	CFU-T_0	CFU-T_1	*P*-value	Significant/Non-Significant
NA	258.1 ± 13.42	65.7±12.17	0.0001	Significant
TSA	348.6 ± 9.92	208.3±41.27	0.0001	Significant
EMB	55.3 ± 6.41	11.4±3.89	0.0001	Significant

Hematological parameters

Blood samples were collected from the control and experimental fish after the trial was over. The study consisted of two different groups: T_0 (control group) and T_1 (experimental group). Hematological parameters serve as an indicator of overall health and can reveal physiological shifts in

various conditions.

Primary blood parameters are erythrocyte count, hemoglobin levels, and hematocrit count, and secondary parameters are mean cell volume, mean cell hemoglobin, and mean cell hemoglobin concentration represented as RBC, Hb, HCT, MCV, MCH, and MCHC, respectively (Table 4). Red blood cell count, Hb, and HCT were significantly greater in T_1 than in T_0; however, the reverse was true in the case of MCV and MCHC (Table 4).

Table 4: Hematological parameters of control vs. treatment

Parameter	T_0	T_1	*P*-value	Significant/Non-significant
RBC (x 10^6/µL)	0.31 ± 0.12	1.25 ± 0.24	0.0001	Significant
Hb (g/dL)	2.2 ± 0.42	4.12 ± 0.46	0.0001	Significant
HCT (%)	20.8 ± 6.27	36.9 ± 6.1	0.0001	Significant
MCV (fL)	270 ± 97.30	198.98 ± 69.14	0.042	Significant
MCH (pg)	29.32 ± 6.26	29.41 ± 5.03	0.487	Non-significant
MCHC (g/dL)	25.3 ± 5.96	20.2 ± 2.60	0.02	Significant

Immunological parameters

Leukocytes, the scientific term for white blood cells, lymphocytes, monocytes, and neutrophils, were markedly higher in T_1 than in T_0 (Table 5).

Table 5: Immunological indices of control vs treatment

Immune Cells	T_0	T_1	*P*-value	Significant/Non-significant
WBC (x 10^3 /µL)	21.3 ± 6.61	61.9 ± 9.7	0.0001	Significant
Lymphocytes (%)	60.5 ± 3.67	90.52 ± 4.72	0.0001	Significant
Monocytes (%)	3.3 ± 0.63	4.6 ± 2.78	0.1101	Non-significant
Neutrophils (%)	11.52 ± 3.89	31.82 ± 4.46	0.0001	Significant

Discussion

Oyster mushrooms have been used for centuries for various treatments and are also called “Green medicine”. Mushrooms contain 8.6% to 22.6% protein; additional nutrients include 15% vitamin C, 40% riboflavin, thiamin, and niacin, and 55% linolenic acid (Assemie et al., 2022). Oyster mushrooms are particularly rich in the complex polymers called glucans, which are found in the cell walls, and include β-glucans, which are known to strengthen innate immunity, thereby benefiting fish (Mirończuk-Chodakowska et al., 2021). The mushrooms as feed can be used in place of fish meals since the levels of essential amino acids they contain equal the recommended daily intake of fish (Ayimbila and Keawsompong, 2021).

Chowdhury et al. (2015) investigated the antimicrobial effect of oyster mushrooms, and their findings confirmed the results of the present research. Samples cultured on different media, like nutrient agar, tryptic soy agar, and eosin methylene blue agar, suggested a lower bacterial count in the experimental group as compared to the control group. This decrease might be linked to the antimicrobial compounds found in the oyster mushroom, as found in our findings. The bacterial colonies were significantly reduced in the treatment groups as compared to the control group. Younis et al. (2015) and Ahmad et al. (2014) investigated similar results as suggested in our findings, such that different bacteria, such as *Pseudomonas aeruginosa*, *Escherichia coli*, *Bacillus atropaeus*, *Staphylococcus aureus*, *Bacillus subtilis*, and *Salmonella typhi,* were inhibited from growing in the experimental group fed with 2% oyster mushroom as compared to the control group, which was fed with commercial fish meal. Shyamala and Maheswari (2021) documented similar results, supporting our findings that immunostimulants such as oyster mushrooms could enhance the resistance of fish to several bacterial pathogens.

The control group fed with commercial fish feed had different types of bacteria found in their gut, but the treatment group had only a few beneficial bacteria in their gut. This signified that the antibacterial properties of the mushroom had inhibited the growth of many bacterial strains and promoted the growth of probiotic bacteria due to the presence of a vast quantity of prebiotics in the oyster mushroom. These findings are parallel to those documented in other studies (Bawadekji et al., 2017; Ogidi et al., 2021; Hamad et al., 2022; Lesa et al., 2022; Vlassopoulou et al., 2022) on different animals that were fed with *P. ostreatus* diets. Furthermore, the bacteria inhibited by the oyster mushroom diet were also the same, i.e., Gram-positive were *Bacillus pumilis*, *B. cereus*, *B. subtilis*, *Micrococcus luteus*, *Staphylococcus aureus,* and *Enterococcus faecalis,* and Gram-negative bacteria such as *Enterobacter aerogenes*, *Burkholderia pseudomallei*, *Klebsiella oxytoca*, *Pseudomonas*

aeruginosa, *Moraxella* spp., *Salmonella pullorum*, *K*. *pneumonia*, *Escherichia coli*, *S*. *typhi*, *Vibrio* spp., and *Shigella* spp. These findings were similar to those reported in different earlier published studies (Adejonwo et al., 2020; Liang et al., 2021; Toros et al., 2023).

The variations in hematological parameters of *H*. *molitrix* being fed with dietary oyster mushroom after 8 weeks were assessed. The RBCs were significantly enhanced by the dietary oyster mushroom. These findings are analogous to those reported elsewhere (Enyidi and Nwosu, 2023), who investigated the effect of oyster mushroom on hematological variables of African catfish, and the levels of RBCs were significantly higher than those in the controls. Habib et al. (2022) reported similar outcomes in the Nile tilapia and verified our conclusions that dietary intake of oyster mushrooms evidently enhanced the levels of hemoglobin. Safari and Sarkheil (2018) documented parallel results in *Cyprinus carpio* and upheld our conclusions that dietary supplementation with oyster mushroom significantly impacted HCT levels in the treatment group as compared to the control group. Hematological parameters such as MCV and MCHC were significantly different when compared to the control group. However, mushroom feed did not affect the serum MCH indices significantly. These findings align with the results of the study conducted by Binaii et al. (2014) on Beluga fish and Chitsaz et al. (2018) on juvenile Great sturgeon. Ahmed et al. (2014) investigated the leukocyte levels of the Nile tilapia, and their findings were similar to our results. The WBC levels in *H*. *molitrix* fed with 2% of dietary oyster mushroom were significantly elevated compared to those in the control group.

Alkinani and Al-Obaidi (2020) reported enhanced levels of lymphocytes and neutrophils in the treatment group supplied with 2% of oyster mushroom powder compared with the control group. However, there was no significant difference in monocyte level in both groups fed with the experimental diet and the control diet in *H*. *molitrix.* Similar results were reported in Rainbow trout (Ulukoy et al., 2016) and Nile tilapia (Ahmed et al., 2014). In contrast, the total number of leucocytes increased in the group fed with the oyster mushroom-supplemented diet compared with the control group, similar to what has been documented earlier (Talpur and Ikhwanuddin, 2012; Yeganeh et al., 2015). In our investigation, there was a significant rise in the total white blood cell count (TWBC) in the experimental group. This increase could have been due to the β-glucan found in mushrooms, known to be a potent immunomodulator (Lave et al., 2010; Chang et al., 2013). The escalation in blood leukocytes indicated a boost of the cellular component of the innate immune system. Leukocytes are pivotal immune cells engaged in defending the body against infectious diseases and foreign invaders. These findings align with those of Vetvicka et al. (2013) and Falco et al. (2014).

Currently, a limited number of studies have assessed the impact of the oyster mushroom on the overall composition of fish. Therefore, the available findings have a few extensive comparisons to draw upon. Nevertheless, these findings strongly indicate the positive influence of incorporating specific mushrooms, such as oyster mushrooms, into fish diets. This addition significantly enhances gut microbiota, improving the fish quality in terms of their entire body, thereby contributing to their overall health and fitness.

Conclusion

The study highlights the beneficial effects of incorporating oyster mushrooms into the *H*. *molitrix* diet, revealing significant reductions in bacterial colonies and notable increases in RBC and WBC counts. Enhanced hemoglobin and hematocrit levels further underscore the nutritional and health-promoting properties of mushrooms, advocating for their inclusion in fish diets to support antibacterial effects and growth promotion.

Author(s), Editor(s) and Publisher's declarations

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Contribution of authors
Conceptualization and design of the study: MH, SP, FR. Conduction of experiments: MH, SP, A. Data collection, visualization, and interpretation: MH, SP, FR, A, SH, AA. Formal statistical analysis: MH,

SP, A, SH, NM, AmA. Writing of first draft: MH, SP, FR, A, AmA. Proofreading and approval of the final version: MH, SP, FR, A, SH, ZG, NM, AA, AmA.

Permissions and ethical compliance
This work was approved by the Institutional Ethical Review Board/Committee (IERB/C) of the University of Agriculture, Faisalabad, Pakistan (Approval number 3162-72).

Handling of bio-hazardous materials
The authors certify that all experimental materials were handled with great care during collection and experimental procedures. After completion of the study, all materials were properly discarded to minimize/eliminate any types of bio-contamination.

Supplementary material
No supplementary material is included with this manuscript.

Conflict of interest
The authors declare no conflict of interest.

Availability of primary data and materials
As per editorial policy, experimental materials, primary data, or software codes are not submitted to the publisher/Journal management. These are available with the corresponding author (s) and/or with other author(s) as declared by the corresponding author (s) of this manuscript.

Authors' consent
All authors have critically read this manuscript and agreed to publish in IJAaEB.

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It is declared that the authors did not use any AI tools or AI-assisted services in the preparation, analysis, or creation of this manuscript submitted for publication in the International Journal of Applied and Experimental Biology (IJAaEB).

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