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Effect of White Button Mushroom (*Agaricus bisporus*) on Immunity and Haematological Parameters of *Oreochromis niloticus*

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SSH and SN planned the research work and wrote the manuscript. SN, IA, AK and AUR conducted the research work. RK, SUK and NN collected the samples. SK and MK analyzed the data and revised the manuscript.

Key words
Immune stimulant, Haematological parameters, *Agaricus bisporus, Oreochromis niloticus,* Aquaculture

ABSTRACT

Nowadays, many challenges are facing by the fish farmer like infections (viral, bacterial and fungal) and environmental stress. Different drugs have been used against several infections, but sometimes they produce toxic effect in fish body and deteriorate the water quality, which in turn decrease the output of the aquaculture. To overcome these issues, herbal products are used in aquaculture and these are the perfect immune stimulator, antibiotic alternative and growth promotor. The current research was conducted to assess the effect of variant level of *Agaricus bisporus* on immunity and haematological parameters of *Oreochromis niloticus*. A total of 120 healthy fishes with average weight 24.0 ± 12.5g were distributed randomly into four fiber glass tanks in triplicates (each tank contains 10 fishes). The experimental diet was prepared with variant concentration of mushroom such as 0% (control group) and 2%, 4% and 6% of extractions for the other three groups. Duration of the experimental work was six weeks. Water quality parameters including temperature was 29 ± 0.5, DO: 5.7 ± 0.04 and pH: 7.5. Results revealed that the 6% *Agaricus bisporus* has shown higher lysozyme activity and ACH50 level, while no significant enhancement was observed in Ig. Beside this all the haematological parameters including WBC, RBC, Hb, Hct and differential leucocyte was observed higher in all the groups as compared to control group. It was concluded that 6% *Agaricus bisporus* can be use as immune-stimulant, and can help the farmers to overcome the emerging aquaculture problems.

INTRODUCTION

Fish is considered as one of the best economical sources of protein. Their meat is rich in omega-3 fatty acid (Sarvenaz and Sabine, 2017). Worldwide, different fish species have been cultured including Neil tilapia which is famous for fast growth and high tolerance range (environmental stress). In Pakistan it was introduced as a farm fish since 2016, and its demand is increasing day by day (Prabu *et al*., 2019). Fish farmers worldwide are facing many challenges regarding health, disease and mortality rate of fish, hence decreasing the output of the aquaculture (Sahoo *et al*., 2020). To overcome these situations many antimicrobial and disinfectants are used but no satisfactory result is achieved so far. Besides this, over use of these drugs can result in development of multi drugs resistance strains of the pathogens, which can pass their resistance genes to the next generation. It not only promotes a drug resistant pathogen but even can harm the consumer health and causes serious environmental hazards (Lúcia and Fernando, 2018; Parrino *et al*., 2020). Therefore, demand for a better alternative to control or prevent fish disease is increased. One of the best method is to use ecofriendly and bio-compatible methods that is using natural immune-stimulants (mostly medicinal herb derivatives). Many researchers successfully use the natural herbal products in aquaculture which can enhance the immune system or can improve the blood parameters for example, pomegranate

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seed oil (PSO) (Acar *et al*., 2018), hot pepper (*Capsicum* sp.) (Parrino *et al*., 2019), essential oil from leaves of the *Cupressus macrocarpa* (Kesbic *et al*., 2020) and essential oil from *Thymus vulgaris* (Zargar *et al*., 2019). Different other plants are also used in the aquaculture practices like garlic (*Allium sativum*), pomegranate (*Punica granatum*), bermuda grass (*Cynodon dactylon*), Indian ginseng (*Whitania somnifera*) and ginger (*Zingiber officinale*) (Reverter *et al*., 2017). Furthermore, fish hematology can be used as a tool to determine the health status of the species, but the blood profile of the fish can be greatly influence by the environment, sexual maturity, age and feed they consume (Fazio *et al*., 2018; Parrino *et al*., 2018; Ahmed *et al*., 2020). Mushrooms are considered one of the most favored healthy foods across the world. Among them *Agaricus bisporus* is well known for its delicious taste and health benefits, hence fetches high price and it ruled the international market. White button mushroom comprises 28-42.5% crude protein, 90-93% moisture, 8.3-16.2% crude fiber, 59.4% carbohydrates, 9.4-14.5% ash and 3.1% fat. It also contains a number of essential minerals and vitamins like 2850 mg potassium, 106 mg sodium, 8.8 mg iron, 912 mg phosphorous, 71 mg calcium, 8.9 mg thiamine (B1), 3.7 mg riboflavin (B2), 26.5 mg ascorbic acid (C), and 42.5 mg niacin (B3). Besides this, *Agaricus bisporus* also contains essential amino acids like lysine and tryptophan. Carbohydrate is stored in the form of glycogen in level 4.5-5.0% and fat is rich in essential fatty acid (linoleic acid) (Harikrishnan *et al*., 2018). Many other pharmaceutical properties are also associated with the white button mushroom like anti-bacterial, anti-fungal, stimulate non-specific immune system, lowering the blood cholesterol and glucose level. However, very limited work was carried out on button mushroom (Rajni *et al*., 2006). For the first time in aquaculture it was used by Harikrishnan *et al*. (2018) and conclude that 5% and 10% of its extract can be used to improve *Clarias gariepinus* hematology and enhance its immunity against *Flavobacterium columnare*. Current study focus to evaluate the effect of the white button mushroom on hematology and immune system of the *Oreochromis niloticus* which can in turn increase the output of the aquaculture.

MATERIALS AND METHODS

Study site

The experiment was performed in the laboratory of University of Sargodha Sub campus Mianwali located in District Mianwali Punjab, Pakistan and the fish were fed for six weeks.

Feed formulation

The mushroom (*Agaricus bisporus*) commonly known as white button mushroom was purchased from the local super market. The supplemented (control) diet contains crude protein 38%, crude lipids 18%, crude cellulose 1.3%, ashes 8.7%, calcium 1.62%, total phosphorous 1.7% with mineral ash content and vitamin. The mushroom was ground into powder form and added into the basal diet with variant level of 0%, 2%, 4% and 6%. By using vacuum freeze drier the experimental diet was dried for 15 h and was filtered by using 2mm mesh. For further use the prepared feed was stored at -4ºC. The diet was formulated according to the Harikrishnan *et al*. (2018), in which *A. bisporus* replaced maize powder of 1%, 5% and 10% of control diet in groups 1, 2 and 3, respectively and the proximate composition was analyzed for its nutrient content by using the method of Horwitz and Latimer (2005).

Fish sampling and experimental design

A total number of 120 Nile tilapia fish (*Oreochromis niloticus*) of mean body weight 24.0±12.5g were purchased from the Tawakkal fish hatchery (located in district Muzaffar Garh Punjab, Pakistan). All the fishes were healthy and to prevent infection the fishes were treated with 0.2% of $KMnO_4$ solution. The fishes were acclimatized for a period of 14 days with normal L13/D11 and were divided into four groups such as group 1 (control group) fed on control diet with 0% of white button mushroom while group 2, 3 and 4 fed on basal diet with 2%, 4% and 6% of mushroom extract, respectively. Ten fishes in each group were stocked in triplicates in 12 fiber glass tanks (water holding capacity 180 L). During that period basal feed was provided twice a day and water quality parameters were measured on daily basis. The mean water quality parameters including temperature 29±0.5 °C, DO 5.7±0.04 (Hanna Instrument HI9132) and pH 7.5 (Hanna Instrument HI221). The fresh water was added after every three days and the aerator was fixed in each tank. After adaptation the fishes were treated with experimental diet. The feed was provided twice a day of about 3-5% of their body weight.

Blood sampling and haematological parameters

At the end of the experimental diet the blood samples of 0.4 cc were collected from the caudal vein of the healthy randomly selected 4 fishes by using sterile syringe with needle (22 gauges) and stored at 4 ˚C in plastic tubes containing EDTA (anticoagulant) and transport it to the laboratory where they were immediately analyzed. During blood sampling fishes were not anesthetized because it may influence the blood indices (Torrecillas *et al*., 2011). Total red blood cell count and white blood cell count was measured with the help of the Neubauer hemocytometer

as described by Goldenfarb *et al*. (1971). In this method 0.02cc of blood (non-clotted) was taken and diluted with Drabkin solution (5cc) and stored at room temperature for ten minutes. After that the Hb concentration was calculated by the absorbance at 540 nm. Besides this Wright-Giemsa stains was used for the determination of the differential leukocytes count while determination of percentage composition of leukocytes was based on the identification character (Ivanova, 1983). Besides this, on the basis of blood sedimentation the value of hematocrit (Hct) was estimated. In this process 50µL of heparinized blood in micro-heparinized capillary and spun at 12000 rpm for 5 minutes in micro-hematocrit centrifuge (RM-12C, REMI India). Hct values were obtained using hematocrit reader. Findings are presented in percentage (Natt and Herrick, 1952).

Lysozyme activity

To determine the lysozyme activity a slight modification was made in the lysoplate method described by the (Siwicki and Anderson, 1993). In this procedure, 2 ml of suspension comprises of *Micrococcus lysodeikticu*s (Sigma, USA: 0.375 mg ml-1, 0.05 M sodium phosphate buffer, pH 6.2) and 50 ml of serum were mix thoroughly and then concentration was measured in spectrophotometer at 450 nm (Biochorm Libra S12, UK). Optical density was measured at different interval of time i.e. 15, 30 and 270s. Lysozyme activity was measured by the reduction in absorbance. Value obtain from the experiment was expressed in the unit of time (min^{-1}).

Serum Ig level

To determine the Ig level, we have to precipitate it from the serum, for this 100 ml of 12% polyethylene glycol was mixed well with the 100 ml of plasma sample was incubate for 2h at 25°C. After incubation it was centrifuge at 5000rpm for 15 min. Then the supernatant was discarded and level of Ig was determined by difference between remaining and total concentration of the serum.

Alternative complement activity (ACH50)

For this, procedure proposed by the Ortuño *et al*. (2002) was followed. In this method the 500 µl of test sample was serially diluted with the Hank's buffer to achieve the final concentration of the 10-0·078%, then 500 µl of SRBS was added. And incubate for 1 hour at 22°C. After that it was centrifuge at 800rpm for five mints at 4°C and un-lysed RBC was removed. By using 450 nm optical density of spectrophotometer (Biochorm Libra S12, UK). The value of the Hb content in the supernatant was measured. The minimum and maximum value of hemolysis was determined by adding the 500 l HBSS to 500 l of SRBC. The hemolysis degree was obtained by plotting a curve (Y) against the total volume of the serum added on log-log scaled graph. The 50% hemolysis produce by the volume of the serum was determined and ACH50 % ml^{-1} was obtained for each set of experiment.

Statistical analysis

The data was analyzed statistically (SPSS Software version 17.0) by applying ANOVA (one way) with significant level 5%. Data are depicted in tables as mean ± standard deviation. Among the groups the significant difference was compare by applying Duncan test.

RESULTS

The immunological and hematological parameters of *Oreochromis niloticus* fed on variant level of *Agaricus bisporus* for six weeks are shown in Table I. The result showed that there was notable increase ($P < 0.05$) in lysozyme activity and ACH50 in treated groups than control group. But in the Ig level there was no significant difference ($P > 0.05$). There was also notable change in the hematological parameters of the fish when compared with those of control group.

DISCUSSION

The present study was conducted to investigate the effect of the basal diet with various level of white button mushroom (*Agaricus bisporus*) on immune response and haematological parameters of *Oreochromis niloticus*. The result revealed that the fish fed on 6% of white button mushroom is effective than other treatments of the diet especially lysozyme and ACH50. Lysozyme is involved in phagocytic activity, increases fish immune response and causes the destruction of bacteria cell wall.

Chitsaz *et al*. (2018) carried out study on juvenile of great sturgeon and study the effect of *Lentinula edodes* on fish immune response and they observed that the lysozyme activity and SOD (superoxide dismutase) was significantly higher in fish fed on 2% of mushroom. Another study revealed that the *Pleurotus eryngii* and *Lactobacillus plantarum* enhance the Pangasius catfish lysozyme activity, respiratory burst activity and phagocytosis (Van *et al*., 2016). Besides this Akrami *et al*. (2015) reported that in beluga the basal diet with onion powder of 1% increased the lysozyme activity. According to Uluköy *et al*. (2016) the rainbow trout fed on extract of 1 and 2% of *Pleurotus ostreatus* which significantly increase the fish lysozyme activity, phagocytic activities, but no significant increase was observed in Ig (total serum immunoglobulin). Similar findings were observed in our study in which the Ig level was not significantly increased.

Table I. Effect of *Agaricus bisporus* for six weeks on immunological and haematological parameters of *Oreochromis niloticus*.

Parameters	Control group (0%)	Group 1 (2%)	Group 2 (4%)	Group 3 (6%)
Immunological parameters				
Lysozyme activity (u mL^{-1})	24.2 ± 1.13^{b}	27.6 ± 2.47^{b}	28.7 ± 3.48^{b}	32.9 ± 1.25^{a}
ACH50 (u mL^{-1})	141.4 ± 13.25^{b}	142.5 ± 13.51^{b}	143.7 ± 14.31^{b}	150.6 ± 13.07^{a}
Ig (mg / L)	31.4 ± 2.34^{a}	32.3 ± 4.02^{a}	34.5 ± 3.07^{ab}	36.9 ± 2.51^{ab}
Haematological parameters				
WBC (10^3 ml^{-1})	10.9 ± 0.7^{a}	11.30 ± 1.3^{a}	12.5 ± 1.4^{b}	13.2 ± 0.1^{b}
RBC (10^6 ml^{-1})	00.9 ± 0.08^{c}	00.10 ± 0.07^{c}	00.12 ± 0.06^{ab}	00.16 ± 0.06^{b}
Hct (%)	32.7 ± 1.16^{b}	34.4 ± 1.6^{b}	36.4 ± 1.9^{b}	38.5 ± 1.3^{a}
Hb (g dl^{-1})	03.8 ± 0.73^{b}	04.5 ± 0.76^{ab}	04.9 ± 0.71^{ab}	05.4 ± 0.51^{a}
Lymphocyte (%)	62.2 ± 1.3^{a}	63.8 ± 3.1^{a}	64.3 ± 1.5^{a}	66.7 ± 3.21^{b}
Neutrophil (%)	22.3 ± 1.11^{a}	22.8 ± 1.2^{a}	23.1 ± 2.1^{a}	25.6 ± 4.4^{b}
Monocyte (%)	10.7 ± 0.4	12.3 ± 0.6^{a}	13.7 ± 2.0^{a}	14.9 ± 1.4^{b}

Values are shown as mean ± SD. Values of the different superscripts in the same rows are significantly ($P < 0.05$) different. *Abbreviations: ACH50 (Alternative complement activity), Ig (serum total immunoglobulin); WBC (white blood cell), RBC (red blood cell), Hct (hematocrit), Hb (hemoglobin).

Harikrishnan *et al*. (2011) reported the higher level of ACH50 when fish was treated with experimental diet contains prickly ash extract, pyrethrum and pomegranate. According to Bazari *et al*. (2017) the 1.5% of *Aloe vera* extract can boost the immunity of Siberian sturgeon such as lysozyme activity and ACH50 than control group. Awad *et al*. (2013) study the effect of *Managifera indica, Lupinus perennis and Urtica dioiaca* on rainbow trout and they reported that the extract of 1% and 2% of these plants are effective in fish complement activity (ACH50). In several studies the haematological indices have been used as they are important to identify the nutrients and pathogenic condition of the animal body (Jahanbakhshi *et al*., 2013). In the current study the haematological parameters such as WBC, RBC, Hct, Hb. Lymphocytes, monocytes and neutrophils are significantly higher in *Oreochromis niloticus* which fed on variant concentration of *Agaricus bisporus*. Chitsaz *et al*. (2018) reported that the effect of 2% of *Lentinula edodes* can enhance the RBC, Hct and Hb value in sturgeon juvenile which is similar to our study, but WBC and differential cells were significantly decrease than control group which is contrary to our study. According to Ndong and Fall (2007) the WBC counts was higher in hybrid tilapia which fed on basal diet with garlic at 0.5% and 1%. Another study reported that the *G. cambogia* extract can enhance the WBC counts (Dada and Ikuerowo, 2009). According to Bazari *et al*. (2017) who conducted study on Siberian sturgeon (*Acipenser baerii*) by introducing alovera extract to evaluate the haematological parameters and concluded that the WBC, RBC and MCV showed significant increased. According to Nwabueze (2012) the effect of garlic increases the WBC counts, Hb, packed cell volume (PCV) and RBC in the fingerlings of *Clarias gariepinus* fed on 0.5% of garlic extract. Sahu *et al*. (2007) described that the fingerlings of *Labeo rohita* which fed on *Magnifera indica* kermel, their RBC counts were increased and this intensification of RBC is sign of higher cellular immunity. Binaii *et al*. (2014) reported that the differential leukocytes were observed increased in beluga body on treatment with *Urtica dioica*.

CONCLUSION

Current study concluded that the 6% of white button mushroom (*Agaricus bisporus*) has shown progressive increase in fish immunity and hematology. Hence their use can be effective in order to reduce the mortality rate and to maintain the healthy aquaculture environment. Many mushrooms possess therapeutic properties. Therefore, these are the best alternative to the antibiotics used in aquaculture. So, result of current research open a new perspective of the mushrooms use in the aquaculture and further studies are needed to evaluate cost-benefits and molecular pathways.

Statement of conflict of interest

The authors have declared no conflict of interest.

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