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Effects of Dietary Supplemented Shiitake Mushroom Extract on Growth, Non-specific Immune Parameters and *in-vitro* Resistance Against *Aeromonas hydrophila* in Rainbow Trout (*Oncorhynchus mykiss*)

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ABSTRACT

The activity of *Lentinula edodes* (shiitake) medicinal mushroom extract were examined on the non-specific immune response and biometrical performance of rainbow trout (*Oncorhynchus mykiss*). Fish (20 g initial weight) were divided into 3 treatment groups (60 fish/group) and duplicated groups for 6 weeks having two experimental diets supplemented with 1-2% shiitake extract and a control diet. During the feeding process, immunological, biochemical, and biometrical observations were determined using the fish and blood samples taken at weeks 1, 2, 3, 4, 5, and 6, respectively. The results of immunological, biochemical, and biometrical parameters evaluation determined that the maximum influence occurs in rainbow trout fed with 2% shiitake extract. The amount of respiratory burst activity in the blood of fish in the trial groups significantly increased in each diet on the 2nd and 6th weeks compared to controls. The amount of total protein, bactericidal activity were significantly increased in fish being fed a mushroom supplemented diet. Cholesterol level decreased in fish blood, which fed with 2% shiitake extract supplemented diet at 3rd and 6th weeks. These results support the findings that the non-specific immune responses of rainbow trout was stimulated in fish by feeding shiitake medicinal mushroom extract yielding positive results in measured parameters compared to the control group also enhancing the overall growth performance of rainbow trout.

Keywords: Rainbow trout, shiitake, extract, stimulate, innate immunity

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Shiitake Mantar Ekstraktı İlaveli Yemlerin Gökkuşağı Alabalığında (*Oncorhynchus mykiss*) Büyüme, Non-Spesifik İmmun Parametreler ve *in-vitro Aeromonas hyrophila* Enfeksiyonuna Karşı Direnç Üzerine Etkileri

Öz: Bu çalışmada *Lentinula edodes* (shiitake) tıbbi mantar ekstraktının gökkuşağı alabalığının (*Oncorhynchus mykiss*) spesifik olmayan bağışıklık cevabı ve biyometrik performans üzerine etkileri incelenmiştir. Bu amaçla balıklar (başlangıç ağırlığı 20 g) iki tekrar olucak şekilde %1 ve %2 shiitake ekstraktı ilaveli yemlerle beslenen deneme grupları ve bir kontrol grubu olmak üzere üç gruba (60 balık/grup) ayrılmıştır. Deneme 45 gün sürmüştür. Beslenme sürecinde balıklardan 1., 2., 3., 4., 5. ve 6. haftalarda alınan kan ve serum örneklerinden immünolojik, biyokimyasal parametreler belirlenmiştir. Deneme başlangıcında ve sonunda balıklardan gerekli ölçümler yapılarak biyometrik analizler değerlendirilmiştir. İmmünolojik, biyokimyasal ve biyometrik parametrelerin sonuçlarına göre maksimum etkinin %2 shiitake ekstraktı ile beslenen gökkuşağı alabalığında meydana geldiğini belirlenmiştir. Deneme gruplarında balıkların kanında tespit edilen respiratory burst aktivitesi, kontrol grubuyla karşılaştırıldığında 2. ve 6. haftalarda artış gösterdiği tespit edilmiştir. Mantar ekstraktı ilaveli yemlerle beslenen balıklarda toplam protein miktarı, bakterisidal aktivitede önemli ölçüde artış göstermiştir. 3. ve 6. haftalarda %2 shiitake ekstraktı ilaveli yemlerle beslenen balıklarda kolesterol seviyesinin düştüğü belirlenmiştir. Elde edilen sonuçlara göre shiitake tıbbi mantar ekstraktı ilaveli yemlerle beslenen gökkuşağı alabalığının spesifik olmayan bağışıklık cevabının kontrol grubuna kıyasla ölçülen parametrelerde pozitif sonuçlar verdiği, aynı zamanda gökkuşağı alabalığının genel büyüme performansını arttığı belirlenmiştir.

Anahtar kelimeler: *:* Gökkuşağı alabalığı, shiitake, ekstrakt, stimulate, non-spesifik sistem

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Introduction

Aquaculture is a rapidly developing system of food production. On the other hand, the diseases caused by microorganisms in rainbow trout were becoming serious and resulted in important mortality (Dalsgaard and Madsen 2000). To inhibit the improvement of these bacteria, antibiotics were applied intensely in the fisheries industry. However, long-term use of antibiotics could cause many negative side effects, such as antibiotic-resistant bacteria, antibiotic residues in the environment and fish (Cabello 2006). Research on the usage of dietary supplements in feed has increased lately (Li and Gatlin 2005; Van Hai 2015; Hoseinifar et al 2020). The immunostimulants have been used as feed additives in aquaculture for years (Galindo-Villegas and Hosokawa 2004; Stratev et al. 2018). Some immunostimulants have been exhibited to be effective in fish on the immune system and growth performance (Awad and Austin 2010; Bilen et al. 2011; Binaii et al. 2014; Zahran et al. 2014; Tang et al. 2014; Wang et al. 2015; Hoseinifar et al 2020; Elumalai 2021). *Lentinula edodes* commonly known as shiitake mushroom, belong to the Marasmiaceae family and is widely distributed in Japan, China, and Korea. Shiitake is an edible mushroom with medicinal properties and biotechnological applications. The active components of mushrooms exhibit immunomodulatory, antioxidant and antiviral qualities (Bobek et al. 1991; Mau et al. 2002; Regula and Siwulski 2007). Shiitake chemical constituents are composed of ingredients such as lentinan, L-ergothioneine (Smith et al. 2002; Bernas et al. 2006), several antioxidants (Mau et al. 2002) and minerals (Mizuno 1995). Research suggests that shiitake has high nutritional value. Mushroom raw fruit bodies include 88 to 92% water, protein, lipids, carbohydrates, vitamins, and minerals. Dried shiitake is nutrients, containing 58 to 60% carbohydrates, 20 to 23% protein, 9 to 10% fibre, 3 to 4% lipids, and 4 to 5% ash. There are several studies on using mushroom species in aquaculture such as *Inonotus obliquus* in kelp grouper (Harikrishnan et al. 2012a) and in olive flounder (Harikrishnan et al. 2012b*)*, oyster mushroom in; rainbow trout (Dobšíková et al. 2012), schizophyllan in carp and flounder (Kwak et al. 2003), reishi mushroom in tilapia (Yin et al. 2008) and so on.

The purpose of this study was to assess dietary supplementation of two doses of a mushroom extract derived from shiitake, on immunological, biochemical, and biometrical, observes of rainbow trout (*O. mykiss*) in natural environmental conditions of a rainbow trout fishery.

Materials and Methods

Extraction Of Mushroom

The shiitake was obtained from the manufacturer and extracted with water according to the method described by Yap and Ng (2001). Firstly, 100 grams of dry shiitake pieces were dissolved in 200 mL of water and kept for 24 hours under 60-65 °C temperature in a water bath. Then the extract was filtered with filter papers to remove unwanted residues. Upon completion of this process, the solution was lyophilized and kept at 4 °C until use. For this experiment, lyophilized shiitake extract was added to commercial rainbow trout feed at concentrations of 1% and 2%.

Experimental Diets

A basal diet was prepared following the nutritional requirements of rainbow trout. The composition of the experimental diets is shown in Table 1. No shiitake extract was added to the control group. The trial diets were prepared using the basal diet supplemented with 1% and 2% shiitake extract. The commercial rainbow trout diet was first mixed; the mushroom extract was then added with water (100 mL of water/ kg of diet) to form a paste; then passed through a meat grinder, and pelleted to produce 2.0 mm pellets.

Table 1. Composition of the experimental diet.

Name of diets	Type of diets	Treatment
Control	Basal diet (48% crude protein, 14% crude lipid)	Wihout mushrom extract
L. edodes	Basal diet (48% crude protein, 14% crude lipid)	Shiitake extract (1%)
L. edodes	Basal diet (48% crude protein, 14% crude lipid)	Shiitake extract (2%)

Fish And Experimental Design

Rainbow trout with an average weight of 20 grams were obtained from a commercial rainbow trout farm in 2013. The trial was performed twice with 360 fish allocated into 2000 L ponds (60 fish/pond). Each group were fed *L. edodes* mushroom extract added diets at 0, 1, and 2% for 6 weeks and the replicates consisted of five randomly sampled fish from two mushroom extracts supplemented groups and the control group. The fish were fed twice a day at a rate of 2% of their body weight. Throughout the experiment, water temperature, dissolved oxygen, and pH were monitored daily and maintained at 15.00±0.32 °C, 8.00±0.22 mg L^{-1} and 7.5±0.17, respectively.

Blood Samples And Serum

Five fish were caught randomly from each group. The 2-phenoxyethanol solution was used as an anaesthetic agent. Blood samples from the fish were taken from the caudal vein with a syringe per week. Some of the blood was taken into the Eppendorf tube for serum samples, kept at 4°C overnight. Then the serum portion was removed. A portion of the blood was taken into heparinized tubes for other tests.

Respiratory Burst Activity

Respiratory burst activity was detected according to the method described by Anderson et al. 1992. NBT (Sigma-Aldrich, St. Louis, MO, USA) solution (0.2%) was freshly prepared in sterile saline solution 0.85% (w/v). Briefly, 50 µL of blood was dropped onto a coverslip and incubated for 30 min at 25 ºC. The coverslip was then gently washed into 0.067 mM sodium phosphate buffer (pH 6.4) to remove the red blood cells. A drop of 0.2% NBT solution was placed onto a slide and washed coverslip was placed on as cell face down onto the drop of NBT solution and incubated again for 30 min at 25 ºC. The cells that showed dark blue colour were counted as NBT positive under the light microscope. Five slides were examined for each fish and five random fields were counted on each slide. For each fish, the 25 fields were averaged and the mean and standard error of values per field was calculated.

Bactericidal Activity

A. hydrophila (ATCC, 7966) bacterial fish pathogen was used as a model to determine bactericidal activity. The colony count method was used to determine serum bactericidal activity (Kajita et al. 1990). *A. hydrophila* was centrifuged and the pellet was washed and suspended in PBS. The bacterial suspension was adjusted to 0.5 Mc Farland at 546 nm. Then 100 µl of serum sample and 100 µl of bacterial suspension were mixed and incubated for 1 hour at 25 °C. 100 µl of serum bacteria mixture was spread on nutrient agar and ıncubated at 25 °C for 24 h before the number of colonies was counted.

Biochemical Assays

Total protein was detected from serum by Bradford (1976) assay using bovine serum albumin (BSA) as the standard in a multiscan spectrophotometer. Albumin, glucose, globulin, triglyceride and cholesterol were determined using Bioanalytic commercial kits.

Biometrical Parameters

The initial and final weights of each fish were measured. Biometrical parameters were calculated according to the following formulae (Laird and Needham 1988).

Weight gain (%) = 100 (final fish weight – initial fish weight) / initial fish weight,

Specific growth rate (SGR, %/day) = 100 (ln final fish weight) – (ln initial fish weight) / experimental days,

Feed conversion ratio (FCR) = feed intake / weight gain,

Diet Analysis

Crude protein, crude lipid, moisture, ash in feed ingredients and diets were determined following standard methods (AOAC 2009). Crude protein was determined by Kjeldahl method and crude lipid by the ether-extraction method. Moisture was detected by oven drying at 105 °C until a constant weight was reached. Ash content was detected after placing the samples in a muffle furnace at 550 °C for 2 h.

Statistics

The data were expressed as arithmetic means standard error (SE). Statistical analysis of data involved one-way analysis of variance (ANOVA) followed by Tukey's pairwise multiple comparison test. Different letters in the figures represent the significant difference at $P<0.05$.

Results

Respiratory Burst Activity

Results determine that the number of NBT-positive cells of the 1% concentration trial group was not as high as the numbers in the 2% concentration trial group but higher than the control group ($P <0.05$). It could be shown in Figure 1 on weeks four, five, and six. The number of NBT positive cells in the 2% concentration group reached the highest peak ($P<0.05$). This result exhibited that the application of shiitake extract caused an increase

in the phagocytic activity of phagocytic cells starting after the second-week post-treatment in both trial groups. The highest level of phagocytic activity was started at the 4th week then kept a similar level in the trial fish up to six weeks.

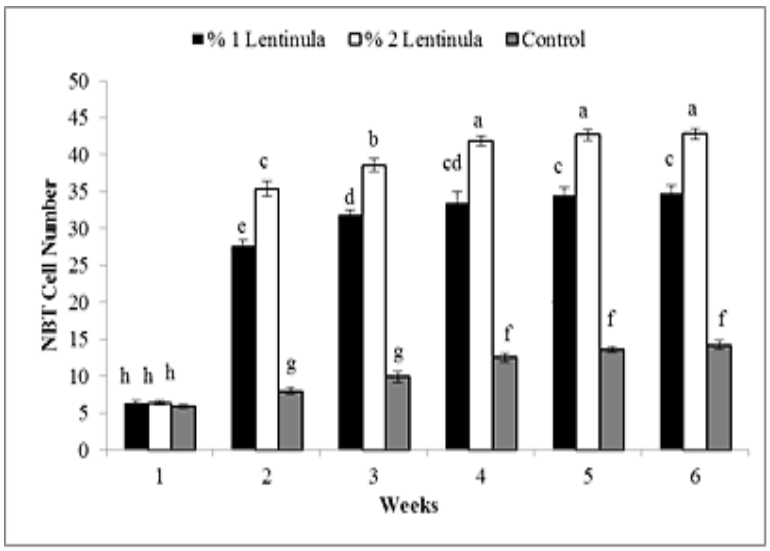

Figure 1. NBT positive cells in the blood of *O. mykiss* fed with shiitake extracts diet at different concentrations. Values are expressed as mean ±SE (n=10). Mean values at bars with different superscript letters at the same stage were significantly different (P<0.05) from the control.

Bactericidal Activity

The serum bactericidal activity significantly increased in fish fed with two concentrations of supplemented diet against *A. hydrophila* bacterial pathogen when compared with the control group (Figure 2).

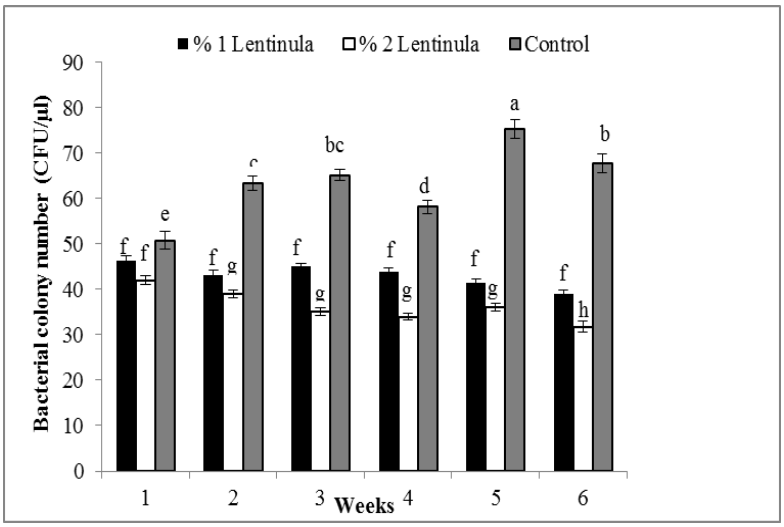

Figure 2. The serum bactericidal activity of *O. mykiss* fed with mushroom extract supplementation diets against *A. hydrophila*. Values are expressed as mean ±SE (n=10). Mean values at bars with different superscript letters at the same stage were significantly different (P<0.05) from the control.

Biochemical Profile Of Serum

The effects of two doses of shiitake extract supplemented diet on rainbow trout detected through serum biochemical parameters are shown in Table 2. Significant increases in serum total protein value and globulin level were found in 1% and 2% groups compared to the control from 3nd to 6th week and mushroom extract supplemental groups at the end of this experiment. There were no changes in albumin, glucose, and triglyceride of all treatment groups compared to the control on weeks 1, 3 and 6. There were no changes in the cholesterol level between treatment and control groups on a 1 week, whereas they were significant decreases in 1% and 2% shiitake extract enriched diets on week 6 (Table 2).

Table 2. Serum biochemical parameters of *O. mykiss* fed different levels of shiitake extracts supplemented diet

W	C	Glucose (mg/L)	Albumin (mg/L)	Globulin (mg/L)	Triglycerid (mg/dl)	Cholesterol (mg/L)	Protein (mg/mL)
1	1%	90.61±3.23^{a}	0.42±0.010^{a}	4.50±0.37^{c}	94.78±1.40^{a}	168.92±3.47^{a}	34.35±0.90^{f}
	2%	89.07±3.12^{a}	0.40±0.012^{a}	4.12±0.28^{c}	95.71±1.80^{a}	173.99±2.26^{a}	34.53±1.06^{f}
	Control	93.10±1.05^{a}	0.41±0.017^{a}	4.45±0.22^{c}	99.53±2.26^{a}	177.00±2.15^{a}	35.49±0.84^{f}
3	1%	87.51±2.75^{a}	0.41±0.01^{a}	5.19±0.25^{b}	87.78±1.51^{b}	119.44±4.96^{b}	41.75±0.77^{d}
	2%	88.46±2.29^{a}	0.41±0.012^{a}	6.85±0.35^{a}	83.91±2.06^{b}	114.29±5.80^{b}	45.85±0.50^{c}
	Control	90.30±1.31^{a}	0.40±0.040^{a}	4.72±0.14^{c}	86.80±1.61^{b}	172.21±5.50^{a}	37.84±0.84^{e}
6	1%	86.57±2.42^{a}	0.40±0.07^{a}	5.40±0.34^{b}	81.80±2.49^{c}	119.21±4.98^{b}	47.50±0.42^{b}
	2%	84.35±1.57^{a}	0.40±0.013^{a}	6.98±0.41^{a}	79.28±1.94^{bc}	111.28±2.33^{b}	52.18±0.63^{a}
	Control	87.01±1.29^{a}	0.42±0.015^{a}	4.88±0.17^{c}	84.42±1.54^{c}	176.21±3.15^{a}	38.18±0.52^{e}

Data are represented as mean±SE (n=10). ^{a,b,c,d,e,f} Different letters represent significant differences at P<0.05. (W: week, C: concentration).

Biometrical Parameters

The promoting effect of shiitake extract in the diet on the growth performance of rainbow trout is shown in Table 3. The average initial body weight (*IW*) did not differ among all groups. At the end of the trial, the average final body weight and the specific growth rate (*SGR*) in the experimental groups were significantly higher than those in the control group (P<0.05). The weight gain rate (*WGR*) in groups increased compared with that of the control (P<0.05). The feed conversion ratio (*FCR*), especially in the group, 2%, was significantly lower compared to the control (P<0.05).

Table 3. Effects of shiitake extract on the growth performance of *O. Mykiss*

Group	*IW* (g)	*FW* (g)	*WGR*(%)	*SGR*(%)	*FCR*
1% Shiitake	20.22±0.11^{a}	50.85±4.42^{b}	151.48±1.6^{b}	2.04±0.43^{bc}	1.24±0.15^{a}
2% Shiitake	20.26±0.26^{a}	55.03±3.94^{a}	168.29±2.95^{a}	2.19±0.28^{a}	1.19±0.21^{b}
Control	19.34±0.17^{a}	46.88±4.72^{c}	142.39±2.45^{c}	1.96±0.42^{b}	1.25±0.24^{a}

Data are represented as mean±SE. ^{a,b,c} Different letters represent significant differences at P<0.05.

Discussion

Using immunostimulants in farm animals as well as in aquaculture has been an upcoming area in recent years. Herbs containing bioactive compounds health, increase the body's natural resistance to infection and facilitate in prevention and treatment of various diseases (Sivaram et al. 2004; Basha et al. 2013). To develop alternative practices for growth promotion and disease management in aquaculture, attention has also been focused on the identification of novel drugs, especially from natural sources. The present trial evaluated the effects of the medicinal shiitake extract on growth performance and non-specific immune parameters in rainbow trout. Fish were fed with food, including 1% and 2% shiitake extract for a total of six weeks. Results showed that in both concentrations, the shiitake extract was able to stimulate some parameters on the non-specific immune system in fish. The NBT reduction product obtained after reaction with superoxides is a very good indicator of the health status or the immunization effectiveness in fish (Anderson et al. 1992). The present study results detected that the mushroom extract did significantly enhance the number of respiratory burst activity of experimental groups and they were significantly different from that of the control group. Also, parallel results have been documented in different fish species such as Mozambican tilapia (Logambal et al. 2000) rainbow trout (Dügenci et al. 2003; Bilen et al. 2011), Indian major carp, (Rao and Chakrabarti 2005), (Kumar et al. 2013), *Oreochromis niloticus* (Laith et al. 2017), and *S. aurata* (Baba et al. 2014; Guardiola et al. 2018). Serum bactericidal activity is a mechanism noted for the killing of pathogenic organisms in fish (Ellis 2001). *A. hydrophila* was used as a model in this experiment. The lowest number of bacterial colonies indicated the efficiency of immune cells in serum to kill the pathogen. The results of this work showed significantly higher serum bactericidal

activity in trial groups. Especially in higher doses 2% of shiitake extract. As our study is shown in parallel ginger (Nya and Austin 2009a) lupin, mango and stinging nettle (Awad and Austin 2010) garlic (Nya and Austin 2011) decaffeinated green tea (Sheikhzadeh et al. 2011) *Saccharomyces cerevisiae* (Sheikhzadeh et al. 2012) and black cumin seed oil and nettle extract (Awad et al. 2013) have enhanced serum bactericidal activity in rainbow trout.

The increase in the levels of serum protein, albumin, and globulins in fish is thought to be associated with a stronger non-specific immun response (Wiegertjes et al. 1996). Plasma proteins include the humoral factors of the non-specific immune system (Magnadottir 2006). By examining previous studies, it was found that the serum has different total protein amounts depending on the fish species and environmental factors in which they lived. The present experiment determined an enhancement of total protein in groups fed with the highest doses of mushroom extract that indicated the highest significant value compared to the control group. This is in agreement with ginger, mistletoe and nettle (Dügenci et al. 2003), garlic (Nya and Austin 2011), tetra (Bilen et al. 2011), black cumin seed oil, and nettle extract (Awad et al. 2013) have enhanced serum total protein level in rainbow trout. Also, Binaii et al. (2014) reported increases in total protein level in juvenile beluga fed with nettle. These reports suggested that a high concentration of total protein in fish serum was likely to be a result of the enhancement of the non-specific immune response. The present results show that the albumin and glucose did not increase while globulin certainly increased. A similar study was reported to have an increase of total protein and globulin in rainbow trout after feeding ginger, garlic (Nya and Austin 2009a; Nya and Austin 2009b), cumin seed oil and nettle extract (Awad et al. 2013). High cholesterol levels in the first week of the experimental groups in the present study showed a decrease compared to the control group after six weeks. In animal studies, oyster mushrooms significantly enhanced plasma cholesterol turnover by 50% with a corresponding 25% decrease in liver cholesterol levels as compared to controls (Bobek et al. 1995). Other animal studies have documented significant reductions in serum and liver cholesterol levels when dried and powdered mushrooms were included in the animal diets (Bobek et al. 1991). Xu et al. (2008) detected that the administration of polysaccharides from shiitake significantly reduced serum total cholesterol, triglyceride level in high-fat rats. Similarly, Hwang et al. (2012) showed that dietary supplementation with shiitake mushroom cholesterol level reduction of eggs in layer chickens. In another study, the effect of *L. edodes* in a mouse model of hypercholesterolemia was investigated by Yang et al. (2013). They determined that *L. edodes* promotes fat removal in hypercholesterolemic mice by supplemented fed feeding. Several herbs were tested for their growth-promoting activity in aquatic animals. Zahran et al. (2014) showed that Astragalus polysaccharides could promote the growth of Nile tilapia. Wang et al. (2015) observed that dietary supplementation of *Rehmannia glutinosa* increased the growth rate in *Cyprinus carpio*. By examining specific growth rates, it can be concluded that the different concentrations applied to the fish did not bear any negative effect on any parameters of the non-specific immune system. They usually have a positive effect on the growth and improvement of performance (Dobdikova et al. 2012; Talpur and Ikhwanuddi 2013; Kanani et al. 2014). Also, Guo et al. (2004) reported several mushroom and herb polysaccharides, on the growth performance of broilers, and found shiitake to be a significant growth performance in broilers. The results are shown in the present study also indicates that mushroom extract included in the diet is useful for improving the growth performance of rainbow trout.

In conclusion, the present study demonstrates the effect of the mushroom extract on the growth and non-specific immune parameters of *O. mykiss*. Results indicate that shiitake mushroom may be a potential immunostimulant for enhancing non-specific immune response and disease resistance in juvenile rainbow trout.

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