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Effect of supplementation of white button mushroom, *Agaricus bisporus* (Imbach, 1946) on disease resistance in white leg shrimp, *Litopenaeus vannamei* (Boone, 1931)

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Abstract
This investigation was designed to evaluate the impact of white button mushrooms on disease resistance of white leg shrimp against *Vibrio parahaemolyticus*. From March to May 2022, the experiment was carried out at the College of Fisheries Science, Kamdhenu University, Veraval. Total six experimental diets with a 35% protein level were prepared. The control diet was prepared without incorporation of white button mushroom (WBM), while the treatment diets were prepared with incorporation of WBM powder at rates of 1%, 1.5%, 2%, 2.5% and 3% level. The post larvae of *L. vannamei* (average weight 0.012g) were stocked in each of 24 aquarium tanks (35 litres) at a density of 20 numbers, and they were fed with a treatment diet for 60 days. The four-day challenge test was conducted. For this purpose, shrimps were randomly selected (10 nos.) from each treatment and gently injected intramuscularly with $0.1x10^4$ cfu/ml of *V. parahaemolyticus*. The data pertaining to disease resistance of *L. vannamei* against *V. parahaemolyticus* were compiled and assessed. According to the study's findings, adding 3% WBM powder to the diet considerably increased the rate of survival following the challenge test against *V. parahaemolyticus*. The less mortality rate (10^d±4.08) was observed in the *L. vannamei* fed with the treatment diet (T_5) that was supplemented with 3% WBM powder while highest mortality rate (60^a±4.08) was found in control group (T_0). This suggests that white button mushrooms may have an effect on boosting the immune system of white leg shrimp that reduced the number of deaths brought on by *V. parahaemolyticus* infection. The result suggests that white button mushroom can be used as an effective feed ingredient that can help shrimp to fight against bacterial diseases in farming practices.

Keywords: White button mushroom, *Agaricus bisporus*, white leg shrimp, *Litopenaeus vannamei*

Introduction
The fishing and aquaculture industries provide a unique and expanding role in giving people access to food, nourishment and employment. The total amount of fish produced worldwide in 2018 was 178.5 million tonnes, of which 82.1 million tonnes came from aquaculture and 96.4 million tonnes from capture fisheries (FAO, 2020) [6]. The Indian fisheries sector contributes 1.07% to the overall GDP of India, whereas 5.30% contributing to the national agriculture GDP (NFDB, 2020) [15]. This clearly implies that aquaculture is crucial to the global economy and market. The main issue with developing aquaculture is emergence of diseases that could be due to increasing production, production in new regions, new candidate species introduction and new cultivation methods. The immuno-stimulatory action of herbal extracts in fish and shellfish cultures has recommended that it might also be utilized as a substitute for vaccinations, antibiotics or chemotherapeutic medicines to provide a less expensive source for treatment with a greater degree of precision, without producing toxicity (FAO, 2016) [5]. Edible mushrooms typically demonstrate substantial nutritional and therapeutic advantages, and are regarded as a valuable health food because they have a low calorific value, lipids, essential fatty acids, rich proteins, vitamins and minerals. The most common and expensive kind of mushroom in India is the white button mushroom (WBM) (Mehta *et al*., 2011) [11]. These are fairly good source of protein, chitin and hemicellulose (rather than starch), very little fat (high in linoleic, palmitic and stearic acid), vitamin C and vitamin B complex (Shao *et al*., 2010; Shrivastava, 1998) [17, 18].
The most extensively researched WBM proteins are lectin and tyrosinase (commonly known as polyphenol oxidase, PPO) (Weijn *et al*., 2013; Yu *et al*., 1999) [21, 22].

Lectins are sugar binding proteins and its activity can be defined by the agglutination of foreign particles and it is one of humoral factors of mobile defense system of crustaceans, and are known to have antitumor, antiproliferative and immunomodulatory properties (Santos *et al*., 2014) [16]. L-tyrosine is changed by PPO into L-3,4 dihydroxyphenylalanine (commonly known as L-DOPA), which is eventually changed into dopaquinone, a precursor in the synthesis of melanin (Weijn *et al*., 2013) [21]. Melanines are responsible for inhibition of the growth of microorganisms by suppressing the activity of extracellular proteinases and chitinases. In addition, PPO also increases phagocytosis, nodule formation, encapsulation and promote haemolymph protein. Small molecules such as phenolic compounds, indoles, statins and other secondary metabolites are present in it. Indoles are related with anticancer and antiaging bioactivity. Ergosterol and ergocalciferol aid to prevent vitamin D efficiency, and ergothioneine is an antioxidant linked to protecting monocyte activity (Muszynska *et al*., 2017) [13].

The three primary polysaccharides found in WBM cell walls are α-glucan, β-glucan and galactomannan (Smiderle *et al*., 2011) [20]. Prebiotics like β-glucan are among those that encourage the development and activity of the desired natural gut microbiota while thwarting the growth of infections, hence enhancing immunity. However, the increasing global demand for shrimp has drawn to the intensification of shrimp culture that is one of the major contributory factors involved in the emergence of infectious diseases. *Vibrio* spp. are involved in several epizootic illnesses that quickly spread to larger populations, posing major challenges to shrimp production and causing large-scale shrimp mortality (Nash, 1992) [14]. It has been observed in aquaculture that β-glucan strengthens the immune system of cultured animals against various bacterial infections. Turbot, *Scophthalmus maximus* immunological response to *Vibrio damsela* was improved by β-glucans from yeast, *Saccharomyces cerevisae* (Figueras *et al*., 1998) [8]. It was discovered that oral administration of β-glucan enhanced *P. monodon* growth and resistance to *Vibrio alginolyticus* (Felix *et al*., 2008) [7]. Additionally, it was noted that β-glucan improved Atlantic salmon, *Salmo salar* antibody response against *Aeromonas salmonicida* (Aakre *et al*., 1994) [1]. The percentage of body weight gain, daily growth index, survival rate and feed conversion ratio all rose when white leg shrimp were supplemented with dried maitake mushrooms, also the number of *Vibrio* spp. was lower in the shrimp than in the control diet (Mochizuki *et al*., 2013) [12].

White button mushroom effect has been shown to improve immune systems and provide a number of other advantages in both humans and animals. And to the best of our knowledge, there isn't much information available about WBM administration in aquaculture. Therefore, the goal of the current study was to assess the effect of WBM on disease resistance in white leg shrimp against *V. parahaemolyticus*.

Singh *et al*. (2022) [23] assessed the effect of *A. bisporus* supplementation on the growth performance and survival of *L. vannamei*. They prepared a control diet (no WBM supplementation) and treatment diets with WBM supplemented at rates of 1%, 1.5%, 2%, 2.5% and 3%. They observed that there was improved body weight gain, SGR, FCR, PER and survival rate in *L. vannamei* as WBM level increased. The highest amongst them was observed in diet supplemented with 3% WBM level. So, there are chances that supplementation with WBM can also significantly improve disease resistance of *L. vannamei* against *Vibrio parahaemolyticus*.

Materials and methods

The research was conducted in the Hands-on Training Center of Department of Aquaculture, College of Fisheries Science, Kamdhenu University, Veraval over a period of 60 days followed by four days challenge test against *Vibrio parahaemolyticus* during March-May 2022.

Diet Formulation

Fishmeal (FM) and fish oil were brought from fishmeal plant, Veraval. Fresh WBM was procured from mushroom farm of Jabalpur. Then cleaned, cut into small pieces, sun dried and powdered in a mixture grinder. Groundnut oil cake (GNOC), wheat flour, tapioca powder, plant oil was brought from local market of Veraval. Vitamins and minerals mixture were brought from local medical store. Standard procedures were performed to determine the proximate composition of ingredients (AOAC, 2019) [2]. The semi-automatic micro-Kjeldahl digestion and distillation systems were used to obtain crude protein levels. Proximate composition of the diets has already been discussed in our previous publication (Singh *et al*., 2022) [23].

Total six diets were prepared, for the experiment including control diet T_0 (without WBM) and five treatment diet T_1, T_2, T_3, T_4 and T_5 feed has been prepared with WBM supplementation as per treatment details (Table 1). Experimental feed was formulated by using Excel feed formulation sheet. In all the experimental diets 35% protein level was maintained. Experimental diet formula (composition) has already been discussed in our previous publication (Singh *et al*., 2022) [23]. The ingredients were mixed well with the required amount of water in an enamel tray. Feed mixture was thermally processed at 121 °C temperature and 15 lbs pressure for 10-15 minutes. After steam cooking of feed mixture, vitamin and mineral mixture was added and mixed well. The feed mixture was run through a mechanical pelletizer and the resulting pellets were spread out on plastic sheets and sun dried until the moisture level fell below 10%. After that, the pelleted feed placed in clearly labelled plastic bottles. The proximate composition of the control and treatment diets were analysed with standard method (AOAC, 2019) [2].

Table 1: The treatments were designated as follows:

Treatment	Amount
T_0	Control diet (without mushroom supplementation)
T_1	Diet supplemented with 1% mushroom powder
T_2	Diet supplemented with 1.5% mushroom powder
T_3	Diet supplemented with 2% mushroom powder
T_4	Diet supplemented with 2.5% mushroom powder
T_5	Diet supplemented with 3.0% mushroom powder

Experimental Setup

A total of 24 rectangular (2x1x1 feet) plastic aquarium tanks were used for the experiment, each containing 35 liters of filtered and disinfected seawater. The plastic aquariums were cleansed with chlorinated water, scrubbed, properly flushed, dried before use and arranged on stands in experimental laboratory. The study was carried out in a completely randomized design (CRD) with 6 treatments and 4 replications.

Procurement of *L. vannamei* Post-larvae

The post larvae of *L. vannamei* were purchased from commercial shrimp hatchery and transported in oxygenated polythene bags by road to the experimental site. These were acclimatized in the fibre-reinforced plastic (FRP) tank of 500 L capacity for six days and were fed properly. During that time, the dissolved oxygen, water temperature, alkalinity, salinity, and pH level were maintained with the proper aeration. For the experiment, post larvae with an average weight of around 0.012 g were chosen.

Stocking and Feeding

L. vannamei PL (weight 0.012 g) were stocked in all 24 aquarium tanks at a density of 20 nos. per aquarium and fed two times in a day: morning and evening at a rate of 5% of body weight during experimental period.

Water Quality Management

Every day, experimental tanks were manually cleaned and siphoned to eliminate extra feed pellets and waste before morning and evening feeding. Siphoned seawater was replaced by around 10% filtered and disinfected sea water. To keep the dissolved oxygen level in each aquarium maintained during the experimental period, continuous aeration was offered. Throughout the trial, weekly checks on water quality parameters such temperature, pH, dissolved oxygen, and alkalinity were made. A pH meter and a thermometer were used, respectively, to measure the pH and temperature. Winkler's method and the EDTA method were used, to test the amounts of dissolved oxygen and alkalinity respectively.

Procurement of Pathogenic Bacteria

The bacterial strain of *Vibrio parahaemolyticus* were purchased from the Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India for carrying out challenge test.

Pathogenicity of *Vibrio parahaemolyticus* to *L. vannamei*

Pathogenic *Vibrio parahaemolyticus* strains were checked for its pathogenicity to the experimental animal.

***V. parahaemolyticus* Culture**

V. parahaemolyticus was grown for 24 hours at 25 ºC on tryptic soy agar plates (TSA supplemented with 2.5% NaCl). The culture was then transferred to 10 ml of tryptic soy broth (TSB supplemented with 2% NaCl) and the broth was incubated at 25 ºC for 24 h as a stock culture for the experiment. The *V. parahaemolyticus* culture was centrifuged at 3000 g for 10 minutes in a refrigerated centrifuge. The pellet was resuspended in PBS (phosphate buffer saline) after the supernatant was removed and the optical density of solution was adjusted to 0.8 at 620 nm, which corresponded to 6.4 x 10^8 cfu mL^{-1}. The pellet from this centrifuged standardized bacterial solution was resuspended in 50 µL of PBS after being centrifuged at 3000 g for 10 minutes. From this, 7.81 µL was made into 50 µL using PBS to get 1 x 10^8 cfu/50 µL. To achieve the desired concentration of 10^8 to 10^4 cfu/50 µL, these bacterial cultures were serially diluted with PBS using the usual dilution procedure.

LD_{50} value of *V. parahaemolyticus* in *L. vannamei*

The test was conducted to derive the lethal dose of challenge test. For that purpose, lethal dose of *V. parahaemolyticus* in *L. vannamei* test was carried out in 6 different tanks containing 10 nos. of shrimps in each tank. They were injected with @ 0.1 ml of bacterial suspension containing @ 10^3, 10^4, 10^5, 10^6, 10^7 and 10^8 cfu/ml intramuscularly between the second and third abdominal segment. Shrimp which was injected with 10^4 cfu/ml showed 50% mortality within 96 h duration. So, the lethal dose of bacterial suspension for challenge test was decided to 0.1 x 10^4 cfu/ml.

Injection of *V. parahaemolyticus* to *L. vannamei*

After 60 days of rearing with experimental feeds, animals were used for challenge test. Randomly 10 shrimps were sampled from each replication. *V. parahaemolyticus* culture @ 0.1 ml of bacterial suspension containing 10^4 cfu/ml PBS (LD_{50} dose) were gently injected intramuscularly between the second and third abdominal segment of each *L. vannamei* using sterile 1 ml capacity syringe with 26-gauge needle. In negative control shrimp injected with only PBS. The susceptibility was conducted for 4 days.

Assessment of Mortality

After injection of *V. parahaemolyticus*, the survival of shrimp was monitored at a regular interval of 4 hrs till 4 days. Shrimp not reacting to gentle mechanical stimulation with a soft paintbrush were considered to be dead. The dead shrimp were removed from the respective tanks during each observation and recorded.

Statistical Analysis

Data were subjected to one-way analysis of variance (ANOVA) to test the significance of treatments by using IBM® SPSS® version 22 (IBM Corporation, 2013). The p value <0.05 was considered to be statistically significant.

Result and Discussion

Challenge test was conducted after the end of 60 days experiment for 4 days. Throughout the 60 days of experimental period environmental conditions were well suited and there was no negative effect of experimental diets on *L. vannamei*. Total weight, mean weight gain (4.152±0.05 g) and specific growth rate (SGR) significantly ($p<0.05$) increased as the WBM supplementation level increased from 1% to 3% with better feed conversion ratio (FCR) and higher protein efficiency ratio (PER) as discussed earlier in our previous published paper (Priya *et al*., 2022) [24]. After completion of 60 days experimental period a random sample of shrimps (10 nos.) was taken from each treatment and challenged with 0.1 x 10^4 cfu/ml of *V. parahaemolyticus* via gentle intramuscular injection with minimal stress to them. Control group shrimps were given 0.1 ml of saline via the same intramuscular injection. All groups were kept under observation for four days, and at every four-hour interval mortality rate were recorded by counting the members. Statistical analysis showed significant difference ($p<0.05$) among the treatments. Treatment T_5 (10^{d}±4.08) had significantly lower mortality rate than all other treatments. Treatment T_3 (25^{c}±0.00) & T5 were found to be at par with treatment T_4 (20^{cd}±4.08).

The result obtained in present study could be may be because of presence of β-glucan in WBM that belongs to the group of prebiotics which stimulate the growth and activity of the desired natural intestinal microbiota, while inhibiting the growth of pathogens. This might have improved the immunity of shrimps that resulted in significantly lower mortality in treatment T_5 (Table 2, Fig. 1).

Results of the present study demonstrated that acceptable growth performance and disease resistance against *V. parahaemolyticus* was observed in diet of post larvae *L. vannamei* fed under treatment T_5 with 3% WBM supplementation as compared to all other dietary groups. Similar result was found by Harikrishnan *et al*. (2018) [9], conducted the research on *Clarias gariepinus* fed with WBM on growth, hematology and immune response against *Flavobacterium columnare* and showed that disease resistance improved significantly with increasing supplementation of WBM in the diet upto 5%. Chelladurai *et al*. (2019) [4] reported that inclusion of 6% milky mushroom extract in the diet of *Babylonia spirata* (Mollusca: Gastropoda) showed significantly higher disease resistance against *Aeromonas hydrophila*. Mochizuki *et al*. (2013) [12] showed *Vibrio* spp. were minimal in *L. vannamei* fed with supplementation of 2% of maitake mushroom as compared to control diet.

Table 2: Mortality rate of *L. vannamei* in different treatments after challenge test against *V. parahaemolyticus* (means of quadruplicate±S.E.).

Treatment	Mean±SE
T_0	60^{a}±4.08
T_1	40^{b} ±4.08
T_2	30^{bc} ±2.88
T_3	25^{c} ±0.00
T_4	20^{cd} ±4.08
T_5	10^{d} ±4.08
Mean	3.043
S.Em. ±	25.000
C.D. at 5%	10.505
C.V. %	29.933

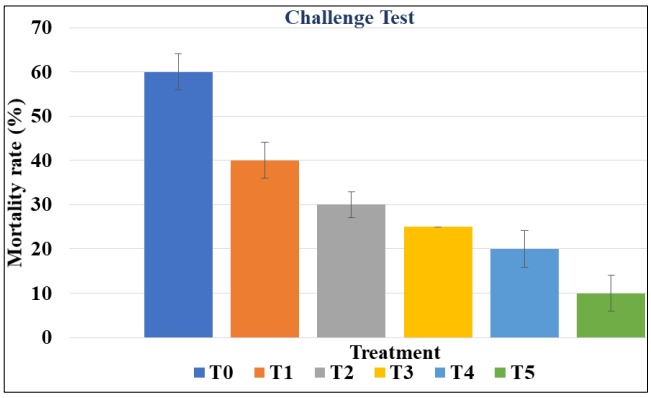

Fig 1: Mortality rate (%) of *L. vannamei* recorded in different treatments during challenge test against *V. parahaemolyticus* after end of 60 days experiment.

Conclusion

On the basis of present study, it is revealed that in the post challenged test significantly lowest level of mortality rate was observed in treatment T_5 (10%) supplemented with 3% WBM powder in feed of *L. vannamei*. This could be mainly because of presence of β-glucan in WBM that belongs to the group of prebiotics that might have improved the immunity of shrimps. This work suggests a new perspective for the use of mushroom in *L. vannamei* which can be applied as a preventive measure against *V. parahaemolyticus* infection to avoid the incidence of high mortalities by provoking innate immunity. The presence of β-glucan, antioxidants and proximate composition in WBM makes it a potential feed ingredient that can increase growth rate and prevent disease incidences in aquaculture.

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