Transactions on Science and Technology, **3**(2-2), 427 - 432, 2016
ISSN: 2289-8786

Comparison of Two Edible Mushroom Extract as Aquaculture Feed Additive to Enhance Immune Response of Asian Seabass

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Received: *29 April 2016*
Revised: *3 August 2016*
Accepted: *1 September 2016*
Online: *27 October 2016*

Keywords:
Asian sea bass; feed additive; *Pleurotus sajor-caju*; *Schizophyllum commune*; *Vibrio harveyi*

ABSTRACT

Limitation of antibiotic practice in aquaculture has created attention to uses of organic immuno-stimulant for growth and survival of juveniles with the development of immune system. The purpose of this study was to evaluate the effects of two edible mushroom extracts, *Pleurotus sajor-caju* and *Schizophyllum commune* as feed additive in the diets of Asian seabass (*Lates calcarifer*) juvenile on serum antibody production. Three experimental diets were formulated with 1% inclusion of *P. sajor caju* (D1), *S. commune* (D2) and commercial diet without inclusion of mushroom additive was used as a control (D0). Asian seabass juveniles with average weight of 3±1 g were fed for 30 days. Each juveniles were challenged with 0.1ml bacteria suspension consisted of 10^8 cfu/ml of *Vibrio harveyi*. Mortality was observed for 10 days after fishes being challenged. Blood was collected before and on third day of challenged, and serum was used to determine antibody titre. Survival rate of D0, D1 and D2 was 60%, 55% and 80%, respectively. Serum agglutinating antibody titer of D2 significantly showed the highest antibody production followed by D1 and D0. Considering the good performance of *S. commune* in the present study, this mushroom can be considered as potential feed additive in the diets for enhancing immune response in Asian seabass juveniles challenged with *V.harveyi*.

Introduction

Asian seabass, *Lates calcarifer* is one of the most cultured marine finfish in aquaculture industry, consequently high demand on this cultured fish increase stocking density. However, intensive farming has causes frequent disease outbreaks among this fish. *Vibrio harveyi* is one of the common pathogen of vibriosis in Asian Seabass (Tendencia, 2002). Disease causes by *Vibrio* spreads rapidly among fish stocked in same cage and cause high mortality led to financial loss. To date, deterrence methods of controlling aquatic disease depend on antibiotics and disinfectant (Harikrishnan *et al*., 2011). Recent interest on mushroom has focused on immunostimulants for controlling bacterial infections and disease control (Brown and Gordon 2003). Mushroom meal as ingredient in feed proven to be safer, environmental friendly, pollution free, improve disease resistance, enhance immunity and decrease mortality (Chang *et al*., 2013).

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Mushrooms are well known for their quality protein such as glutathione, single cell protein and rich amount of essential amino acids and are used as dietary supplement (Mukhopdhay & Guha, 2015). Protein containing polysaccharides extracted from *P. sajor caju* found as promising antitumor activity (Zhuang *et al*., 1993). Beta-glucan a type of polysaccharide immune-stimulant compound can only be found in mushroom, cereal and yeast. Mushroom beta-glucan are capable of exhibiting wide range of biological activities such as antitumor, antimicrobial, anti-allergy, anti-inflammation, cardiovascular regulator, radio-protective, antioxidant, antidiabetic, anti-obesity and immune protective response (Zhu *et al*., 2015). Mushrooms are also low in fat but rich in polyunsaturated fatty acid particularly linoleic acid (Kayode *et al*., 2015) contributes to immune system.

The purpose of this study was to evaluate the effects of two edible mushroom extracts, *Pleurotus sajor-caju* and *Schizophyllum commune* as feed additive in the diet of Asian seabass (*Lates calcarifer*) juveniles.

Methodology

Mushroom preparation

Pleurotus sajor caju was purchased freshly from General Mushrooms *Sdn. Bhd.* General Mushrooms *Sdn Bhd*, a commercial oyster mushroom cultivator located at Jalan Tambunan. Other types of mushroom *Schizophyllum commune* was bought from a farmer from weekend market (Tambunan) who plucked this mushroom from the rubber woods. Fresh mushrooms were brought to laboratory for cleaning and rinsing to remove debris and darts. Mushrooms were chopped into 2 cm^2 and put to dry in the oven at temperature of 40ºC until mushrooms reached complete dryness. Dried mushrooms were then blended into fine powder and stored in zipped plastic bag for future extraction use.

Mushroom extract

Mushroom powder and distilled water with ration of 1:10 was mixed well in flasks and homogenized by stirring with glass rod. Flasks were kept in incubator shaker to shake at 100rpm for 3 days. The mixture was then centrifuged at 5000rpm for 20 minutes to separate the liquid from mushroom debris. Liquid was collected and filtered with Whatman G/C filter paper. Filtrate was concentrate with rotary evaporator at 40°C. The concentrated extracts were freeze at -80°C prior to freeze drying process. Dried extracts were kept in zipped plastic bag and froze at -20°C while not in use.

Experiment design

Healthy juvenile Asian seabass (*Lates calcarifer*) average weight of 3±1g were collected from *Perikanan tanjung Badak, Tuaran*. Collected juveniles were acclimatized for 14 days in a tank of BMRI fish wet lab. They were fed with commercial diets. After that, 20 tails of Asian seabass juveniles were stocked in 100L tank supplied with aeration stone in a flow-through system filtered seawater with a flow rate of approximately 2L/min. At the end of 30 days feeding period, each group was divided two with 10 fishes each for replicates. *Lates calcarifer* juveniles were injected

intraperitoneally with 0.1 ml per fish of bacteria suspension consisted of 10^8 cells/ml *Vibrio harveyi* strain VHJR7 according to the method described by Talpur and Ikhwanuddin (2012). Blood was collected before challenge and third day post-challenge from 10 fishes of each group through caudal vein. Blood was centrifuged at 1500 rpm for 5 minutes to obtain serum and was stored in -80°C until serum antibody agglutination assay.

Disease resistance

After Asian seabass juveniles were challenged with the *Vibrio harveyi*, mortality was recorded over a period of 10 days. Survived fishes were counted every day.

Serum Agglutination Test

Antibody titre of challenge fish can be assayed through antibodies presence in serum against pathogen, *V. harveyi* creating agglutination reaction, method according to Biller-Takahashi *et al*., 2014. *V. harveyi* strain VHJR7 obtained from overnight growth in nutrient broth and centrifuged at 3000 rpm for 3 minutes. Supernatant was discarded and bacteria pellet was washed three times with sterile Phosphate buffer saline (pH 7.4). Bacteria pellet was resuspended in sterile PBS and the concentration was adjusted to an O.D= 0.5 (610nm). The absorbance 0.5 was calculated as 1 x 10^8 CFU/ml of bacteria in 1ml suspension

The serum agglutinating titre was determined in a 96-well microtiter plate with round bottom wells. The assay was initiated with a dilution of 1:1 (50 µL of phosphate buffer: 50 µL of serum) and consequently a two-fold serial serum dilutions were made by adding 50 µL of diluted serum into the remaining wells with 50 µL of PBS. Thereafter, 50 µL of inactive *V. harveyi* (1×10^8 Cfu/ml) suspension was added to each well and incubated at room temperature for 18 hours. The agglutination end point was established as the last serum dilution where agglutination was visible. Agglutination antibodies titres were expressed as log2 (x+1) of the reciprocal of the highest serum dilution showing visible agglutination. The last well was used as a negative control, where there was only 50 µL PBS buffer.

Statistic analysis

Microsoft Excel 2010 was used to calculate the means and standard deviations. Antibody titre results were analysed using one-way ANOVA and the comparisons of the mean values were done by using tukey test in software program SPSS version 22. The level of significance setting was $p < 0.05$.

Results and Discussion

No mortality was recorded from all group up to 4th day post challenge (**Figure 1**). First four days, survival of all groups were 100% due to early pathogenicity takes place by presence of symptoms like vasculitis, gastro-enteritis and eye lesions that later lead to death (Austin and Zhang, 2006). Asian

seabass challenged with *Vibrio harveyi* (10^8 cells/ml) showed no mortality in 16 hours when fed with 0.1% to 0.5% of peppermint additive (Talpur, 2014).

Reduced mortalities rate can be seen in *S. commune* extract added diet (D2) as survival was sustained at 80% while *P. sajor caju* extract added diet (D1) and control groups of Asian seabass showed comparison survival of 55% and 60%, respectively. Previous works on *Lates calcarifer* challenged with 10^8 cells/ml of *Vibrio harveyi* suspension improved survival rate at 80% when fed with 0.4% of neem leaves (Talpur & Ikhwanuddin, 2013).

Serum antibody titre was quantified in Log_2 (x +1) indicated the amount of specific antibody produced by challenged Asian seabass juveniles with *V. harveyi*. Quantitative estimation showed that D2 and D1 diet obtained x = 10 and x = 8, respectively. D0 diet obtained antibody titre of x = 5. Both D1 and D2 diet significantly produced higher amount of antibody than D0 diet ($p < 0.05$). Harikrisnan *et al*. (2011) reported that Kelp grouper fed with 1% *Phellinus linteus* extract improved cellular and humoral immune response of fish when challenged with *Vibrio* pathogens. Higher survival rate of *S. commune* group might due to higher amount of antibody produced. Mushroom administration diet at certain dosage tested in aquaculture significantly increased the immune responses (Harikrisnan *et al*., 2012). Optimum production of antibody enable the host to prevent infection and enhance immunity against invasion pathogen (Baba *et al*., 2015).

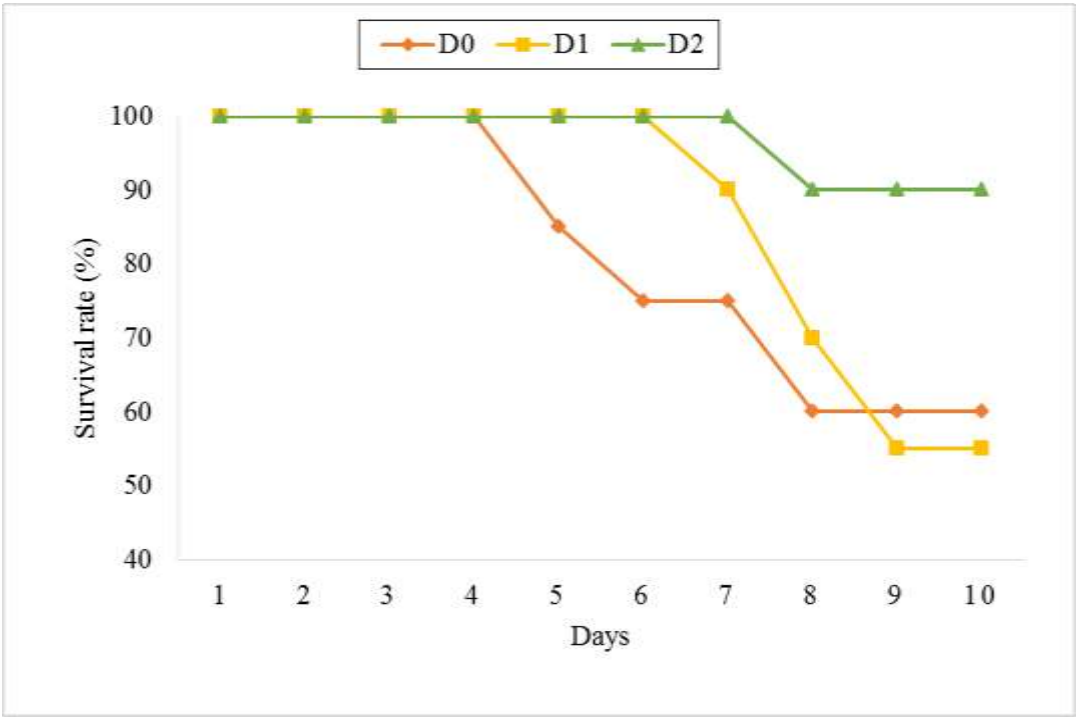

Figure 1. Percentage of survival rate in *Lates calcarifer* juveniles fed with *Pleurotus sajor caju* additive diet (D1), *Schizophyllum commune* additive diet (D2) and control diet (D0) for 10 days post-challenge with *Vibrio harveyi*. Each value is the mean ± SD (n = 20)

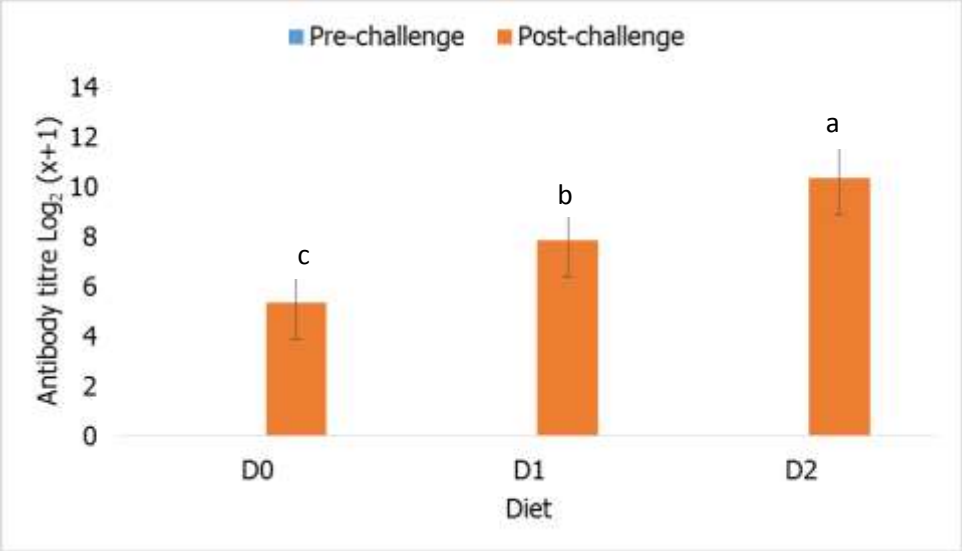

Figure 2. Pre-challenge and post-challenge antibody titre $Log_2(1+x)$ of *Lates calcarifer* fed with *Pleurotus sajor caju* additive diet (D1), *Schizophyllum commune* additive diet (D2) and control diet (D0) for 10 days post-challenge with *Vibrio harveyi*. Each figure was the mean ± SD of 6 tests. Superscript letters above each bar represent statistically significant differences ($P < 0.05$).

Conclusion

Lates calcarifer juveniles fed *S. commune* added diet after being challenged with *V. harveyi* showed significant increased survival rate suggesting that resistance against bacterial diseases correlate with increases in antibody production. *Schizophyllum commune*, non-commercially cultivated mushroom extract could be one of the potential candidate as feed additive to enhanced immune response and disease resistance in Asian seabass juveniles.

Acknowledgement

The study was supported by a project (Project code: ERGS0038-STWN-2013) funded by Ministry of Higher Education, government of Malaysia. Strain used in this study was kindly provided by Assoc. Prof. Dr. Julian Ransangan.

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