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Effects of *Cordyceps militaris* spent mushroom substrate and *Lactobacillus plantarum* on mucosal, serum immunology and growth performance of Nile tilapia (*Oreochromis niloticus*)

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ABSTRACT

An 8-weeks feeding trial was performed to investigate the possible effects of supplementation of Nile tilapia diet with *Cordyceps militaris* spent mushroom substrate (SMS) single or combined with *Lactobacillus plantarum* on immune parameters and growth performance. For this aim, Nile tilapia fingerlings were fed with four experimental diets namely: Diet 1 (0 – control), Diet 2 (10 g kg^{-1} SMS), Diet 3 (10^8 CFU g^{-1} *L. plantarum*), and Diet 4 (10 g kg^{-1} SMS + 10^8 CFU g^{-1} *L. plantarum*). At the end of feeding trial, skin mucus parameters, serum immune parameters, and growth performance were measured. The results indicated that supplementations SMS + *L. plantarum* or/and resulted in a significant increase in skin mucus lysozyme and peroxidase activities compared with the control group after 8 weeks of feeding trial ($P < 0.05$). The highest values of these parameters were recorded for fish fed both SMS + *L. plantarum* supplementations. Nonetheless, no significant difference was recorded between other supplemented groups ($P < 0.05$). For serum immunology, the results showed that serum lysozyme activity, alternative complement, phagocytosis, serum peroxidase, and respiratory burst activities were significantly higher in supplemented groups compared to the control ($P < 0.05$). The highest values were recorded in fish fed both SMS and *L. plantarum* with respect to the individual application. No significant differences were observed between fish fed SMS and *L. plantarum* ($P < 0.05$). Results on growth performance indicated that fish fed supplemented diets showed a statistically significant increase in the specific growth rate (SGR), weight gain (WG), final weight (FW) compared to the control group ($P < 0.05$). The highest SGR and WG values were observed in fish fed both dietary SMS and *L. plantarum*. However, no significant differences in these parameters were observed in fish fed SMS or *L. plantarum* alone ($P > 0.05$). The FCR was significantly lower in fish fed 10 g kg^{-1} SMS + 10^8 CFU g^{-1} *L. plantarum* than in other groups, while control group presented the highest values ($P < 0.05$). The present results suggested that the combination of these natural substances could be considered as potential feed-additives for aquaculture farmed fish.

1. Introduction

The demand for Nile tilapia, *Oreochromis niloticus* has grown tremendously for the last decade since it is a high-quality protein source with high market value [1,2]. Recently, intensive aquaculture system has been expanded and is emerging as one of the most practical and promising tools to meet the increased requirements of Nile tilapia [3]. However, in intensive fish farming, animals are subjected to stress conditions that weaken fish immune systems, leading to increased susceptibility to infectious diseases causing production losses [4,5]. Recently, dietary administration of functional feed additives has been suggested as an environmental friendly alternative approach to enhance the immune response of fish and minimize the risk associated with the use of

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chemotherapeutics [5,6].

Mushrooms and their derivatives have been widely used in aquaculture as immunostimulants because they possess many bioactive compounds such as polysaccharides (β-glucans), dietary fibres, terpenes, peptides, glycoproteins, alcohols, mineral elements, unsaturated fatty acids, antioxidants like phenolic compounds, tocopherols, ascorbic acid etc., [7,8]. *Cordyceps militaris* is the edible mushroom in *Cordyceps* genus with fruit bodies cultivated on a large scale for commercial production worldwide [9]. Considering exponential increase in mushroom consumption; there is increasing volume of the undesirable parts of mushroom disposed by mushroom industries [10–12]. Spent mushroom substrate (SMS) is the by-products of mushrooms culture, which retains a wide range of bio-active compounds such as extracellular enzymes, antibiotics, secondary metabolites, and carbohydrates produced during mycelium and fruiting-body formation [13]. An approximately of 500,000 tons of cereal substrates have been used to produce 4000 tons of *C. militaris* mushroom in China [7,12–15]. To the best of our knowledge, no published work has been conducted using SMS as a feed additive in aquaculture.

Probiotics are live microbial cells that could improve the growth performance and welfare of fish when consumed in adequate amounts [14]. Several reports suggested that the supplementation of probiotics can improve the growth, immune response, and disease resistance of fish [6,15–18]. *Lactobacillus plantarum* is a gram-positive lactic acid bacterium which plays a beneficial role in the fish gut by producing antibacterial substances to inhibit the increment of harmful intestinal bacteria that suppress growth of competing bacteria [19–21]. Therefore, the supplementation of *L. plantarum* induced immune modulation, enhanced the growth performance, and increased disease resistance in several fish species [22–26].

In recent years, the literature on the use of spent mushroom substrate and probiotics in the diet and their effect on growth, mucosal, and serum immunology of fish are very limited. Hence, the present investigation was carried out to evaluate the effects of dietary supplementation of *C. militaris* spent mushroom substrate or/and *L. plantarum* on the growth, and immune parameters of Nile tilapia, *O. niloticus*.

2. Materials and methods

2.1. Preparation of spent mushroom substrate

The *C. militaris* strain CMRU1 was kindly supplied by Faculty of Agricultural Technology, Chiang Mai Rajabhat University, Thailand, which maintained on Potato Dextrose Agar (PDA) slants and subcultured monthly [27]. The slants were inoculated with mycelia, incubated at 20 °C for 7 days, and then were used to prepare inoculum culture (15 g L^{-1} dextrose, 5 g L^{-1} sucrose, 0.5 g L^{-1} $MgSO_4 \cdot 7H_2O$ in silkworm pupae juice 50 g L^{-1}). Four 6-mm diameter discs were punched out from PDA plates and the mycelium of *C. militaris* discs was transferred to 250 mL flask containing 50 mL of the inoculum medium. The culture was incubated at 20 °C for 5 days in a reciprocating shaker at 100 rpm [27]. The rice medium was made up of 1 kg of rice in 1.5 L of nutrient solution (35 g L^{-1} sucrose, 5 g L^{-1} peptone, 1 g L^{-1} KH_2PO_4, 0.5 g L^{-1} $MgSO_4$, and 0.05 g L^{-1} vitamin B_1). The inoculation quantity was 5% (v w^{-1}). After inoculation, the fermentation medium was incubated at 20 °C for 4 weeks. After fermentation, fruiting bodies of *C. militaris* were harvested and the SMS was dried in an oven at 60 °C for 2 days. The dried spent substrate was then ground and sieved pass through a 0.1-mm sieve. A single dose of SMS (10 g kg^{-1}) was considered for inclusion to basal diet based on the results obtained from our previous study [28].

2.2. Animal and experimental design

The experimental fish were Mono-sex (male) Nile tilapia fingerlings, which was supplied from Chiang Mai Pattahana Farm, Chiang Mai, Thailand. Upon arrival to the Department of Animal and Aquatic Sciences (Chiang Mai University) fish were stocked in 1000 L tanks and acclimatized with lab conditions for 2 weeks. Thereafter, fish (28.10 ± 0.04) were randomly allocated to 16 glass aquarium (150 L) at density of 20 fish per tank and fed on experimental diets for 8 weeks. During the feeding trial fish were fed on experimental diets twice a day (at 8:00 a.m. and 5:00 p.m.) up to apparent satiation. Thirty percent of water was daily exchanged to maintain water quality in each aquarium. The physiochemical parameters of water include water temperature, pH and dissolved oxygen were monitored daily and maintained at 22.25 ± 0.95 °C, 7.81 ± 0.27, and 5.59 ± 0.41 mg $litre^{-1}$, respectively.

Lactobacillus plantarum CR1T5 was kindly provided by Dr. Saowanit Tongpim, (Department of Microbiology, Faculty of Science, Khon Kaen University; Thailand). The administration dose of *L. plantarum* (10^8 CFU g^{-1}) in this study was selected based on previous studies Son, Chang, Wu, Guu, Chiu and Cheng [26] and Giri, Sukumaran and Oviya [24]. *L. plantarum* supplemented diets were daily prepared according to the method described by Irianto and Austin [29].

A basal diet was formulated based on modification of diet used in previous study Tiengtam, Khempaka, Paengkum and Booanuntanasarn [30]. The basal diet has been proved to be suitable for Nile tilapia in our previous studies [31]. For preparation of the experimental diets, the basal diet was supplemented with different levels of SMS and *L. plantarum* as follows: 0 (Diet 1-Control), 10 g kg^{-1} of SMS (Diet 2), 10^8 cfu g^{-1} *L. plantarum* (Diet 3), and 10 g kg^{-1} of SMS + 10^8 cfu g^{-1} *L. plantarum* for Diet 4 (Table 1).

2.3. Measurement of innate immune response

2.3.1. Samples preparation

At the end of the feeding trial (8 weeks), five randomly selected

Table 1
The formulation and proximate composition of experimental diet (g kg^{-1}).

Ingredients	Diets (g kg^{-1})			
	Diet 1	Diet 2	Diet 3	Diet 4
Fish meal	270	270	270	270
Corn meal	200	200	200	200
Soybean meal	270	270	270	270
Wheat flour	60	60	60	60
Rice bran	150	150	150	150
SMS[a]	0	10	0	10
Lactobacillus plantarum (CFU g^{-1})	0	0	10^8	10^8
Cellulose	30	20	30	20
Soybean oil	5	5	5	5
Premix[b]	10	10	10	10
Vitamin C[c]	5	5	5	5
Proximate composition of the experimental diets (g kg^{-1} dry matter basis)				
Crude protein	322.1	322.3	322.1	322.3
Crude lipid	74.8	75.3	74.8	75.3
Fibre	52.5	53.5	52.5	53.5
Ash	106.7	107.3	106.7	107.3
Dry matter	927.0	927.5	927.0	927.5
GE (Cal g^{-1})[d]	4105	4101	4105	4101

[a] SMS = *Cordyceps militaris* spent mushroom substrate.
[b] Vitamin and trace mineral mix supplemented as follows (IU kg^{-1} or g kg^{-1} diet): retinyl acetate 1,085,000 IU; cholecalciferol 217,000 IU; D, L-a-tocopherol acetate 0.5 g; thiamin nitrate 0.5 g; pyridoxine hydrochloride 0.5 g; niacin 3 g; folic 0.05 g; cyanocobalamin 10 g; Ca pantothenate 1 g kg^{-1}; inositol 0.5 g; zinc 1 g; copper 0.25 g; manganese 1.32 g; iodine 0.05 g; sodium 7.85 g.
[c] Vitamin C 98% 5 g.
[d] GE = Gross energy.