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Research Article

Evaluation of the effect of the supplementation of Agaricus brasiliensis mushroom in cytohematological, growth and stress parameters in Nile tilapias (*Oreochromis iloticus*)

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Abstract

Currently, in fish farming, Nile tilapia farming is one of the most widespread types worldwide and is present on all continents. However, stressors are normal in intensive rearing systems, which triggers immune suppression leading to a higher chance of infection, with *Aeromonas hydrophila* being one of the main responsible for the frequent diseases. Inhibition of reproductive and growth performance are also frequently observed in these situations. Thus, it is more than necessary to develop efficient treatments not only against the immune damage generated by stress in fish but also to prevent oxidative stress itself as well as against its side effects related to physiology and infections. The mushroom *Agaricus brasiliensis* is widely recognized in the literature for its immunomodulatory and antioxidant effects, which makes it a potential supplement to counteract the effects of stress on intensive fish farming. In this study, we evaluated the effects of supplementation with the mushroom *Agaricus brasiliensis* through a randomized clinical trial regarding the immunological hematological variables in face of a normal situation and an induced stress situation as well as in the face of an immunological challenge by *Aeromonas hydrophila*. The mushroom proved to have a positive effect under the tested conditions, however, further studies will be necessary to determine its definitive effects.

Introduction

Nile tilapia farming is one of the most widespread types of fish farming in the world and is present on all continents [1]. In this type of farming, cropping systems can be classified as extensive, semi-intensive and intensive systems such as Raceway's, which are characterized by high risk and productivity environments due to the stress caused by the conditions of creation [2]. The stress factors in fish that are commonly found in these breeding systems [3] may be physical, environmental or social [4], with plasma cortisol being the predominant factor in the stress mechanism [5]. The main biochemical effects triggered by stress and release of cortisol in fish are the alteration of osmotic and ionic regulation

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[6], reduction of mobilization of circulating leukocytes and lymphocytes [5] and reduction of the rate of growth and suppression of immune and reproductive functions [7]. Given this fact, a frequent problem in aquatic animal populations is the occurrence of diseases, since water acts as a facilitator of transmission of pathogens in a population of the same species [8]. Many natural products have been described to be safe to use on acquacuture like extracts from clove seed used to sedate fish for different farm operations [9]. This study further revealed that the extracts could be safely applied at 100.0 mg/l, which is sufficient to anaesthetize the fish with little or no changes on its heamatological parameters.

Among the diseases observed in fish, those caused by bacteria are the ones with the greatest risk potential, since bacteria of economic importance for fish farming are present in fish water and microbiota [10], in captivity annual losses associated with streptococcus are approximately 150 million dollars [11].

The Mushroom *Agaricus brasiliensis* has always been reported in the literature as a potent antioxidant and immunomodulator, and currently arouses great scientific and economic interest due to its well documented properties [12], and can be used in fish farming and aquaculture to avoid sanitary problems and excessive use of antibiotics [13]. In the case of direct cytohematological parameters, it is not new that the supplementation of the *A. brasiliensis* mushroom can stimulate responses. In 1998 its potential benefits were already elucidated by stimulating the production of lymphocytes [14]. Supplementation of this mushroom under normal conditions may also increase the number of circulating eosinophils and macrophages [15]. However, in the case of the immunomodulation promoted by this mushroom, the stimulatory effect on monocytes is also described be the activity of isolated fractions of *A. brasiliensis* [12]. A single study evaluated the supplementation of this mushroom in tilapia, however, in a short experimental period, but still, are be observed an increase in immature leukocytes [16].

The objective of this work was to evaluate the cytohematological, growth and weight effects associated with the supplementation with the mushroom *Agaricus brasiliensis*, juveniles of Nile tilapia in normal conditions and the situation of stress and in front of immunological challenge by *Aeromonas Hydrophila*.

Methodology

The present study was carried out at the Pontifical Catholic University of Minas Gerais (PUC-Minas), under the registration of protocol no. 029/2017 of the Ethics Committee on the Use of Animals - CEUA PUC Minas. Nile tilapia (*Oreochromis niloticus*) juveniles, housed in two circular tanks of 300 liters at a density of 15 liters / fish, we used a mean temperature of 27 ± 1.2 in a 12-h photo-light period: 12 h light / 12 h dark and after the acclimation period, the experimental treatments were different for each tank, consisting of a control diet composed of commercial ration and an experimental diet composed of commercial ration enriched with 1% with *Agaricus brasiliensis* during 90 days prior to stress induced by starvation [17]. Feeding was done at will once a day (4:30 p.m.), so that there was not too much leftover in the tanks. A commercial feed with 32.0% digestible protein was used, both for mushroom enrichment and control diet. To perform the enrichment, the feed was ground and the mushroom *Agaricus brasiliensis* was added in 1% dry weight, 60% humidified, extruded and dried by exposure to the sun for 24 hours. For evaluation of hematological parameters, four fish were randomly removed per treatment and anesthetized with 1.0 ml / L Eugenol. After anesthesia, blood was collected utilizing a puncture of the caudal vessel with 1.0 ml syringe, bathed with anticoagulant (EDTA 5.0%) and blood (0.5 ml) collected was stored in heparin tubes and taken immediately for analysis in the laboratory DMVET. The fish submitted to blood collection were separated in another tank with the same environmental conditions and after the effect of the anesthesia were reallocated in the tank of the experiment/control.

Immunological challenge

For this experiment, 100 Nile tilapia (*Oreochromis niloticus*) fingerlings were used and maintained in two circular tanks of 300 l in the density of 50 fingerlings per tank, at a mean temperature of 27 ± 1.2 in a photo-lighting period of 12 h: 12 h light / dark and after the acclimation period, the experimental treatments were different for each tank, consisting of a control diet composed of commercial feed and an experimental diet composed of commercial feed enriched with 1% with *Agaricus brasiliensis* for 30 days. Shortly after the stress period the fish were challenged by *Aeromonas hydrophila* (ATCC7966) via intraperitoneal injection in low doses (1 x 10-4 CFU) or saline solution to 0.9%. The appearance of clinical signs was observed for 10 days. After this period, the fish were challenged again with a dose of 7.1 x 10-2 CFU of the same bacterium via intraperitoneal injection and were then observed for the next 10 days or until death.

Statistical analyses were performed in the Bioestat 5.0 program, the t-tests of Student are used.

Results

Biometric analysis between groups (90 days)

The tables below (Tables 1,2) present the mean and initial and final standard deviation for the size of the groups tested.

Although not statistically significant, the *A. brasiliensis* mushroom showed a tendency to retard fish weight gain and growth rate. Regarding weight a slight retard was noted at the end of the experiment, but also did not present a significant difference (P> 0.05) (Tables 3,4).

Table 1: Initial fish size comparison (pre-treatment).

Initial size between groups		
Group	**Medium (cm)**	**Standard Deviation (±)**
Control	23.25	1.36
Experiment	22.8	2.6
Experiment starts 0.45 lower		

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Cytohematological effect of supplementation (90 days)

Initially, a baseline study was carried out to determine if both treatments started from the same immunological conditions (Tables 5- 8) and, as expected, no significant difference was observed between the analyzes performed (P> 0.05), indicating that the groups started the experiments under statistically similar conditions.

From the information that the groups did not show differences in the number of initial leukocytes, the experimental period between groups (Table 9-12) was analyzed and we observed that the number of circulating monocytes was statistically lower in the supplemented group (P < 0.05). This result indicates that the supplementation with the *A. brasiliensis* mushroom was able to modify this immunological variable between groups. Regarding eosinophils, they did not present significant numbers for statistical analysis.

Table 2: Final fish size comparison (post-treatment).

Final size between groups		
Group	**Medium (cm)**	**Standard Deviation (±)**
Control	26.6	1.36
Experiment	26	2.6
Experiment ends 0.6 lower		

Table 3: Initial comparison of fish weight (pre-treatment).

Initial weight between groups		
Group	**Medium**	**Standard Deviation (±)**
Control	253	61
Experiment	220.16	69.3
Experiment starts with -33.14 g compared to control		

Table 4: Final fish weight comparison (post-treatment).

Final weight between groups		
Group	**Medium**	**Standard Deviation (±)**
Control	333,2	44.17
Experiment	250,2	101.1
Experiment ends with -83g compared to control		

Table 5: Initial analysis of lymphocyte levels between control and experiment groups.

Initial lymphocytes between groups (%)		
Group	**Medium**	**Standard Deviation (±)**
Control	76.87	11.15
Experiment	82.75	7
P>0.05 (0.40)		

Table 6: Initial analysis of neutrophil levels between control and experiment groups.

Initial neutrophils between groups (%)		
Group	**Medium**	**Standard Deviation (±)**
Control	19.62	9.28
Experiment	13.37	6.89
P>0.05 (0.32)		

Table 7: Initial analysis of monocyte levels between control and experiment groups.

Initial monocytes between groups (%)		
Group	**Medium**	**Standard Deviation (±)**
Control	1.87	1
Experiment	3.25	1.9
P>0.05 (0.25)		

Table 8: Initial analysis of eosinophil levels between control and experiment groups.

Initial Eosinophils between groups (%)		
Group	**Medium**	**Standard Deviation (±)**
Control	0.37	0.47
Experiment	0.62	1.25
P>0.05 (0.77)		

Table 9: Analysis of post-treatment lymphocyte levels between control and experiment groups.

Lymphocytes after 90 days of control test vs experiment (%)		
Group	**Medium**	**Standard Deviation (±)**
Control	77.5	15.84
Experiment	87.75	8.61
P>0.05 (0.29)		

Table 10: Analysis of post-treatment neutrophil levels between control and experiment groups.

Neutrophils After 90 days of control test vs. experiment (%)		
Group	**Medium**	**Standard Deviation (±)**
Control	19	15.34
Experiment	11	7.79
P>0.05 (0.23)		

Table 11: Analysis of post-treatment monocyte levels between control and experiment groups.

Monocytes Post 90 days of control test vs experiment (%)		
Group	**Medium**	**Standard Deviation (±)**
Control	3.5	1
Experiment	1.25	1.25
P>0.05 (0.04)*		

Table 12: Analysis of post-treatment eosinophil levels between control and experiment groups.

Eosinophils After 90 days of control test vs experiment (%)		
Group	**Medium**	**Standard Deviation (±)**
Control	0.375	0.48
Experiment	0.625	1.25
P>0.05 (0.87)		

When the post-stress variables between groups were assessed, no significant differences were observed (Tables 13-16).

Biometric analysis of supplementation (30 days)

Below (Tables 17,18), we compared fish weight at the

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beginning and at the end of the experiment between groups and as expected, a significant difference (p> 0.05) was observed, showing that the mushroom had no negative effect on growth. The same result can be observed with respect to size (Tables 19,20).

Survival challenge

In the challenge of survival against infection we can observe a higher mortality related to the supplementation with the mushroom *Agaricus blazei* (Table 21).

Table 13: Comparison of lymphocyte levels between post-stress groups.

Post-stress lymphocytes between groups (%)		
Group	**Medium**	**Standard Deviation (±)**
Control	86.6	7.5
Experiment	85	7.9
P>0.05 (0.70)		

Table 14: Comparison of neutrophil levels between post-stress groups.

Post-stress neutrophils between groups (%)		
Group	**Medium**	**Standard Deviation (±)**
Control	7.5	3.87
Experiment	6.25	2.62
P>0.05 (0.46)		

Table 15: Comparison of monocyte levels between post-stress groups.

Post-stress monocytes between groups (%)		
Group	**Medium**	**Standard Deviation (±)**
Control	2.75	2.06
Experiment	9	9
P>0.05 (0.26)		

Table 16: Comparison of eosinophil levels among post-stress groups.

Post-stress eosinophils between groups (%)		
Group	**Medium**	**Standard Deviation (±)**
Control	0.25	0.5
Experiment	0	0
P>0.05 (0.60)		

Table 17: Initial comparison of fish weight (pre-treatment).

Initial weight between groups (30 days)		
Group	**Medium**	**Standard Deviation (±)**
Control	3	1.23
Experiment	3.14	1.21

Table 18: Final fish weight comparison (post-treatment).

Final weight between groups (30 days)		
Group	**Medium**	**Standard Deviation (±)**
Control	15.6	5.9
Experiment	16.6	4.7

Table 19: Initial fish size comparison (pre-treatment).

Initial size between groups (30 days)		
Group	**Medium**	**Standard Deviation (±)**
Control	5.5	0.75
Experiment	5.64	0.58

Table 20: Final fish size comparison (post-treatment).

Final size between groups (30 days)		
Group	**Medium**	**Standard Deviation (±)**
Control	9.4	1.26
Experiment	9.3	1.21

Table 21: Mortality observed in challenge of infection.

Day	Mortality rates (%)		Signs
	Control	Experiment	
1 to 5	none	none	not observed
5 to 10	none	none	not observed
Day	Second challenge		
	Control	Experiment	
1 to 5	none	100%	not observed
5 to 10	none	none	not observed

Discussion

In this study, even slightly, the *A. brasiliensis* mushroom proved to be able to modulate the immune response of Nile tilapia concerning the number of circulating monocytes, and in fish, in general, the innate defense is mainly composed of monocytes, macrophages and granulocytes (lymphocytes) [18] and just like in mammals, circulating monocytes give macrophage origin [19]. During an inflammation or an infectious process, monocyte levels can be increased, being one of the major cells involved in acute inflammation as part of the innate immune response [20], thus, the reduction of circulating monocytes observed in the results presented, may indicate that there was a reduction in acute inflammation, which is possibly generated by the capture management itself [21], this idea are reinforced by the confirmation that monocytes are the main producers of cytokines and secrete free radicals [22,23].

In immunostimulatory-based diets containing PAMPs, recognition and activation pathways are unclear [22], but TLRs play a crucial role in both innate and adaptive immunity. Its ability to detect PAMPs is related to the inflammatory response [24] and although there are more than 17 types of TLRs identified in fish, it is possible that the function of TLR4, one of the main monocyte modulation sites of the mushroom *A. brasiliensis* [21,25] has been lost in teleosts [26], thus explaining the low immunomodulatory response to supplementation.

Another fact that should be taken into account is that for immunostimulants, such as β-glucan, the route of administration is a determinant factor in the immune response, especially in the case of tilapia, since the supply of immunostimulants via feed produces an effect attenuation in the cells of the immune system when compared to the supply via immersion and intraperitoneal injection, in regard to monocyte, leukocyte, and lymphocyte counts. This result is

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also observed when we compared the oral immunostimulant intake against a control diet [27].

The results of our study corroborate with another results presented [16], thus bringing the concept that low doses of this mushroom did not present a strong immunomodulatory activity in tilapias, similar results were observed in FOS supplementation studies [28].

Recently, it was evidenced that hypoxia stress can generate alterations in the immune system of fish [29], but to date, the effect of starvation on the count of circulating leukocytes in fish has not been investigated. Thus, based on the results presented in this paper, we suggest that the response to starvation may not trigger significant immunological changes in adult *O. niloticus*, or that fish may present different mechanisms of non-cortisol-dependent immune response.

Ichthyologists consider changes in leukocyte differential counts as one of the most sensitive indicators of acute stress in fish because there is a close link between leukocyte profiles and glucocorticoid levels. High neutrophil rates for lymphocytes in blood samples strongly indicate high levels of glucocorticoids as acute responses to stress [30]. In this work we can observe a higher neutrophil: lymphocyte ratio for both groups post stress period when compared to the non-stress period, we observed that the treatment with *A. brasiliensis* was also able to maintain this score calculated by the neutrophil: lymphocyte ratio at 0.7 after the stress period, whereas in the control group the score was approximately 0.9 which may indicate a lower stress in the supplemented group.

It was elucidated that there was an interesting difference, although not statistically significant among the means of the weight of the groups, where *A. brasiliensis* tended to delay the weight gain in approximately 20% in the supplemented group, also, we observed a higher mortality rate for the experimental group.

This mushroom has 33.14 to 54.22% of carbohydrates [31], which represents a considerable increase (0.3g to 0.54g / kg) of carbohydrates in the supplementation since the diet control is carbohydrate-free. The low capacity of fish to use dietary carbohydrates is reported by inadequate regulation in the use (glycolysis) and production (gluconeogenesis) of hepatic glucose [32], which can also lead to a higher mortality or lead to losses in fish growth and weight gain [33]. In fish fed a high-carbohydrate diet compared to a high-protein diet, a lower rate of weight gain and a specific growth rate can be observed [34]. The studies are not conclusive as to the carbohydrate content; however, apparently Nile tilapia supports high carbohydrate dosages [35]. In our second attempt we observed that the mushroom had no negative effects on the physiological aspects of tilapia growth and weight. However, the effects on growth and weight may be dependent on the amount of ß-glucan incorporated into the diet, duration of feeding, room temperature and the species under study [36].

Infection challenge

Although knowledge about *Aeromonas hydrophila* is limited, its virulence has already been elucidated against aquatic populations. Among the virulence factors already documented for *A. hydrophila*, hemolysins, arabinasins, proteases, adhesins, enterotoxins, phospholipase and lipase, enzymes leading mainly to hemorrhagic septicemia in stressed fishes [37].

We observed a systemic response to the second challenge of bacteria, where the fish supplemented died soon after the application. One of our hypotheses is the hyper reaction caused by supplementation, however, glucans alone or in combination with PAMPs or bacterial injection do not cause apoptosis in organs related to the immune system [38]. Another plausible biochemical pathway is the interaction between the biochemical mechanisms of the pathway of *Agaricus brasiliensis* and Eugenol. It has recently been shown that Eugenol can act synergistically with cisplatin increasing its chemotherapeutic potential by mainly inhibiting the transcription factor pathway (NF-kB) [39]. The mushroom, on the other hand, modulates the immune response of enterocytes, reducing the translocation of NF-kB in Caco-2 cells [40].

In addition to acting as a stress mediator, NF-κB is constitutively present in neurons participating in physiological functions of the CNS, such as synapse, development and neural plasticity, therefore, an exacerbated physiological response to a stressor agent, resulting in a drastic reduction of the level of this neurotransmitter, could result in death.

Highlights

This first clinical trial randomized presents a new supplementation with potential benefits; this is the first study of Agaricus brasiliensis mushroom supplementation in fish; this study brings new possibilities for study.

Conclusion

Based on our findings, we can conclude that the supplementation with the mushroom *Agaricus brasiliensis* is justified in the pisciculture by the potential immunomodulatory activity and, possibly, antioxidant effect, without bringing any physiological losses in terms of yield and biomass conversion in short periods of supplementation.

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