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Dietary Brown Propolis Extract Modulated Nonspecific Immune System and Intestinal Morphology of Pacu *Piaractus mesopotamicus*

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HIGHLIGHTS

- Brown propolis from Southwest of Parana modulated pacu immune system.
- Dietary supplementation of propolis extract increased fish serum lysozyme concentration.
- Dietary supplementation of propolis extract increased fish circulating organic defense cells.
- Brown propolis extract did not cause toxic effects on pacu.

Abstract: The immunomodulatory and growth promoter effects of brown propolis ethanolic extract (PEE) were determined in pacu. Fish (28.4±0.4 g) were randomly distributed into 12 polyethylene circular tanks (250 L, 30 fish per tank) and fed for 60 days with a commercial diet (32% crude protein) supplemented with 0.0, 1.5, 3.0 and 4.5% (v/w) of PEE in a totally randomized experimental design (n=3). Fish organic defense cells numbers such as thrombocytes and neutrophils increased (p<0.05) in fish fed 3.0% dietary PEE. Serum lysozyme concentration also increased (p<0.05) in fish fed 1.5 and 3.0% PEE supplementation when compared to unsupplemented fish. Growth parameters were not influenced (p>0.05) by treatments. Moreover, dietary PEE decreased (p<0.05) fish intestinal muscular thickness when compared to control treatment. Intestine villi height also significantly decreased in fish fed 3.0% PEE. Long term dietary PEE at 3.0% supplementation level modulated fish nonspecific immune system and it is a non-toxic substance for pacu.

Keywords: fish hematology; fish immunology; nutrition; pacu; phytoterapic.

INTRODUCTION

World aquaculture production has increased in the past 30 years [1] as well as for neotropical fish species in Latin America, based on characiforms such as pacu *Piaractus mesopotamicus*, tambaqui *Colossoma macropomum* and their hybrids [2].

Fish are submitted to several unavoidable stressors (overcrowding and poor water quality) in intensive production systems which impairs fish health and consequently, growth, immune system and disease resistance [3,4]. In addition, the indiscriminate use of antibiotics to control fish disease outbreaks resulted in selection of several resistant pathogenic microorganisms [5,6] and undermine the profitability and sustainability of aquaculture [7]. Thus, the use of natural compounds to modulate the immune system and growth of aquatic organism is a promising method with low-environmental impact for disease control [4,8] since it does not result neither bacterial resistance nor residues in fish flesh [9].

Propolis, also known as “bee glue”, is manufactured product by bees *Apis mellifera*, made from plant exudates and used to building and reparation of hive, as well as protection against microorganisms, with more than 200 bioactive compounds [10,11]. As regards its role on the immune system, propolis has many different biological and pharmacological properties such as antibacterial, antifungal, antiviral, antiprotozoal, antioxidant, anti-inflammatory and immunostimulant activities [11–17]. Due to these characteristics, propolis can be used as additive in aquafeeds to improve fish health and growth.

Promising results on the use of propolis and its extracts (aqueous and ethanolic) to improve growth, immune system, stress and disease resistance was observed in several fish species such as Nile tilapia *Oreochromis niloticus* [18], Mozambique tilapia *Oreochromis mossambicus* [19], rainbow trout *Onchorhynchus mykiss* [20–22], common carp *Cyprinus carpio* [23] and Japanese eel *Anguilla japonica* [24]. However, it is possible that dose response to dietary propolis supplementation in aquafeeds may be species dependent [25].

Pacu *P. mesopotamicus*, an omnivorous Characin native from Parana, Paraguay and Uruguay river basins, is widely produced in Latin American fish farming industry [26]. To our knowledge, there are no previous scientific paper published regarding the effects of dietary propolis supplementation for pacu. Thus, the study evaluated the effects of increasing levels of dietary brown propolis ethanolic extract (PEE) on health and growth of pacu juveniles.

MATERIALS AND METHODS

Fish rearing conditions

Trial was set up in indoor (12hr light:12hr dark photoperiod) water recirculation system composed of 12 polyethylene circular tanks (250-L) and biological filtration with continuous aeration and temperature control. The pacu juveniles (28.4 ± 0.4 g) were obtained from a commercial fish farm (Piscicultura Daniela, Francisco Beltrao, Parana, Brazil). At laboratory facilities, fish were previously acclimated to experimental conditions and basal diet for 15 days. Water quality parameters were monitored electronically in a daily basis with portable oximeter (HI 98193, HANNA® Brasil, Barueri, SP) and digital pH meter (MPA 210, MS TECNOPON®, Piracicaba, SP): temperature (26.4 ± 1.3ºC), dissolved oxygen (5.2 ± 0.4 mg/L) and pH (6.9 ± 0.2). Before experimental procedures, this research was approved by the Ethics Committee on Animal Use (CEUA) of UTFPR (protocol nº 2013-007).

Experimental procedures

A commercial extruded (2.0 mm pellets) fish feed formulation (32% crude protein, 5% crude fat, 10% crude fiber, 12% ash, Anhambi Alimentos Ltda., Itapejara do Oeste, Parana, Brazil) was used as basal diet. The basal diet was stored in freezer (-20ºC). Every seven days, the amount of feed sufficient for that period was separated and it was sprayed 0.0; 1.5; 3.0 and 4.5% volume/weight of propolis ethanolic extract (PEE) and stored in dark bottles under refrigeration (4ºC) until use to avoid propolis biocompounds oxidation. The extraction of PEE from brown propolis, and the determination of antioxidant activity, total phenolics and flavonoids content was done according to Oldoni and coauthors [10] (Table 1). The brown propolis was collected from selected colonies of Africanized honey bees (*A. mellifera*) at experimental apiary at Federal Technological University of Parana (Southwest of Parana – latitude: -25.699063^0, longitude: -53.095273^0, altitude: 546 m).

Table 1. Antioxidant activity and bioactive compounds content of propolis ethanolic extract (PEE) from brown propolis.

Antioxidant activity			*Bioactive compounds*	
ABTS	**DPPH**	**FRAP**	**Phenolics**	**Flavonoids**
µmol of Trolox/g		**µmol of Fe^{2+}/g**	**mg GAE/g**	**mg Quercetin/g**
95.2 ± 4.4	40.0 ± 1.9	259.3 ± 9.5	15.5 ± 0.5	0.81 ± 0.04

ABTS: 2-2'-azino-di-(3-ethylbenzthiazoline sulfonic acid) method
DPPH: 2,2-diphenyl-1-picrylhydrazyl hydrate free scavenging method
FRAP: Ferric reducing antioxidant power method

Fish were randomly distributed into 12 polyethylene circular tanks (250-L, 30 fish per tank), each tank representing a replication (n=3). Fish were hand-fed daily with experimental diets for 60 days until apparent satiation (09hr:00min and 17hr:00min).

Sampling

At the end of feeding trial, fish were fasted for 24hr, sedate with alcoholic solution of benzocaine (50 mg/L) and sampled for hematological, immunological and biometrical data. To determine intestine histology, after blood sampling, fish were sacrificed by anesthetic overdose (100 mg/L alcoholic benzocaine solution) and later medullary section.

Hematological and immunological analysis

Blood samples for hematological and immunological analyses of four fish per tank (12 fish per treatment) were drawn from caudal vein using sterilized needles and syringes previously rinsed with heparin. Total red blood cell count (RBC) was performed in Neubauer chamber using formaldehyde citrate buffer as diluent, the hematocrit was determined in microhematocrit tubes after centrifugation for 5 minutes at 10,000 g and hemoglobin content was determined by the cyanmethemoglobin method. Hematimetric indexes was calculated according to Wintrobe [27].

Blood smears from sampled fish were stained with May-Grünwald-Giemsa stain [28] to perform differential and total leukocytes count and thrombocytes count by the indirect method [29]. Total plasma protein concentrations were determined using a portable refractometer (RHC-200/ATC, 0.0~12.0 g/dL) after total blood centrifugation and plasma collection [30].

Leukocyte production of reactive oxygen species (respiratory burst) was determined by nitroblue tetrazolium (NBT) colorimetric assay [31]. An aliquot of 100 µL of total blood was mixed with 100 µL of 0.2% NBT solution (Nitrotetrazolium Blue Chloride, Sigma-Aldrich®, St Louis, MO, USA) and incubated for 30 min at 25ºC. After the incubation period, 50 µL of this suspension was added to 1.0 mL of DMF (N, N-dimethylformamide, Sigma-Aldrich®, St Louis, MO, USA) and centrifuged (755 g) for 5 min. Finally, the absorbance of the supernatant was measured at 540 nm wavelength using a spectrophotometer.

Serum lysozyme concentration was determined based on the lysis of *Micrococcus lysodeikticus* (Sigma-Aldrich, St Louis, MO, USA) as standard [32]. Fish serum samples were heated (56 ºC for 30 min) to inactivate complement system proteins and certify that lysis of *M. lysodeikticus* had occurred only by lysozyme action. Then, 150 µL of fish serum and 150 µL sodium phosphate buffer was added to a glass cuvette and incubated at 26ºC for 2 min in spectrophotometer and 300 µL of *M. lysodeikticus* suspension (0.2 mg/mL sodium phosphate buffer) was added to complete a 600 µL final volume. The difference between the initial and final optical density (Δ OD) was measured between 0.5 and 5 min at 450 nm wavelength using a spectrophotometer. The equation of the lysozyme calibration curve was used to determine the serum lysozyme levels.

Growth performance

Growth performance was calculated as follows: weight gain (WG (g) = final weight - initial weight); specific growth rate (SGR (% body weight/day) = 100 × [(*ln* final weight - *ln* initial weight) ÷ days of experiment]) and feed conversion ratio (FCR (g/g) = total feed intake ÷ weight gain).

Intestinal morphology

A sample of the proximal intestine of three fish per tank (nine fish per treatment) was sampled and fixed for 24h in Alfac solution. The intestine histology was performed according to Sado and coauthors [33]. Intestine sections (5µm) were stained in hematoxylin and eosin (H & E) and documented using digital camera

(AxioCam ERc5s, Carl Zeiss, Germany) connected to a light microscope (Primo Star, Carl Zeiss, Germany). The images were analyzed by using specific software (ZEN, ZEN Blue Edition, Carl Zeis, Germany) for intestinal villi height and muscular layer thickness measures.

Statistical analysis

Significant effects of dietary PEE after 60 days trial was analyzed by one-way analysis of variance (ANOVA) at 5% probability. A polynomial regression analysis was performed for growth parameters. Means of statistically difference were compared by Tukey´s test (α=0.05) after results tested for normality (Cramer Von Mises) and homoscedasticity (Brown-Forsythe).

RESULTS

Hematology and immunology

There was no effect (p>0.05) of dietary supplementation of PPE on some hematological parameters of juvenile pacu (Table 2). Although the absent of significant effect on total leukocytes numbers, 3.0% dietary PEE increased (p<0.05) the number of fish neutrophils when compared to control (unsupplemented) diet. In the same way, thrombocyte number also was significantly higher in fish fed 3.0% PEE diet than control.

Dietary PEE supplementation also affected fish immune system. Serum lysozyme concentration was significantly higher in fish fed 1.5 and 3.0% PEE supplementation when compared to control group (Figure 1). However, no effect (p>0.05) was observed on leukocyte respiratory burst (Table 2).

Table 2. Hematological and immunological parameters (mean ± SD) of juvenile pacu *Piaractus mesopotamicus* fed increasing levels of propolis ethanolic extract in diet for 60 days.

	Propolis ethanolic extract levels (% of diet)				One-way ANOVA
	0.0	**1.5**	**3.0**	**4.5**	**(p-values)**
Htc (%)	34.1±1.6	36.4±1.8	35.5±4.7	37.3±3.9	0.685
Hb (g/dL)	7.21±0.6	7.72±0.53	7.46±0.45	7.32±0.6	0.738
RBC (10^6/µL)	1.64±0.15	1.81±0.21	1.77±0.15	1.70±0.32	0.795
MCV (fL)	213.0±21.2	204.1±17.3	205.9±19.0	243.1±26.4	0.169
MCH (ρg/cell)	45.4±7.3	43.7±8.5	43.3±6.2	48.5±4.3	0.783
MCHC (g/dL)	21.3±1.4	21.2±2.3	21.4±3.7	19.7±1.4	0.811
TPP (g/dL)	6.1±0.3	6.2±0.4	6.3±0.4	6.0±0.3	0.873
Leu (10^3/µL)	16.3±3.6	20.1±2.3	21.4±1.1	17.5±2.5	0.138
Thro (10^3/µL)	30.1±1.8b	37.8±3.9ab	44.4±1.4a	40.0±8.4ab	0.035
Lym (10^3/µL)	7.3±1.6	8.8±1.7	8.8±2.3	6.5±2.3	0.470
Mon (10^3/µL)	3.4±0.6	4.4±0.2	4.9±1.3	4.0±0.8	0.247
Neu (10^3/µL)	3.4±0.6b	5.6±0.9ab	6.3±1.6a	5.8±0.8ab	0.050
Eos (10^3/µL)	0.35±0.1	0.46±0.4	0.34±0.09	0.23±0.1	0.446
SGC (10^3/µL)	0.32±0.1	0.61±0.3	0.49±0.2	0.76±0.4	0.115
LRB (OD)	0.273±0.10	0.270±0.11	0.323±0.03	0.246±0.05	0.721

Different letter in the same row denote difference by Tukey test (α=0.05)
Htc: Hematocrit; Hb: Hemoglobin concentration; RBC: Red Blood Count; MCV: Mean Corpuscular Volume; MHC: Mean Corpuscular Hemoglobin; MCHC: Mean corpuscular hemoglobin concentration; TPP: Total Plasmatic Protein; Leu: Leukocyte number; Thro: Thrombocyte number; Lym: Lymphocyte number; Mon: Monocyte number; Neu: Neutrophil number; Eos: Eosinophil number; SGC: Special Granulocytic Cell; LRB: Leukocyte Respiratory Burst

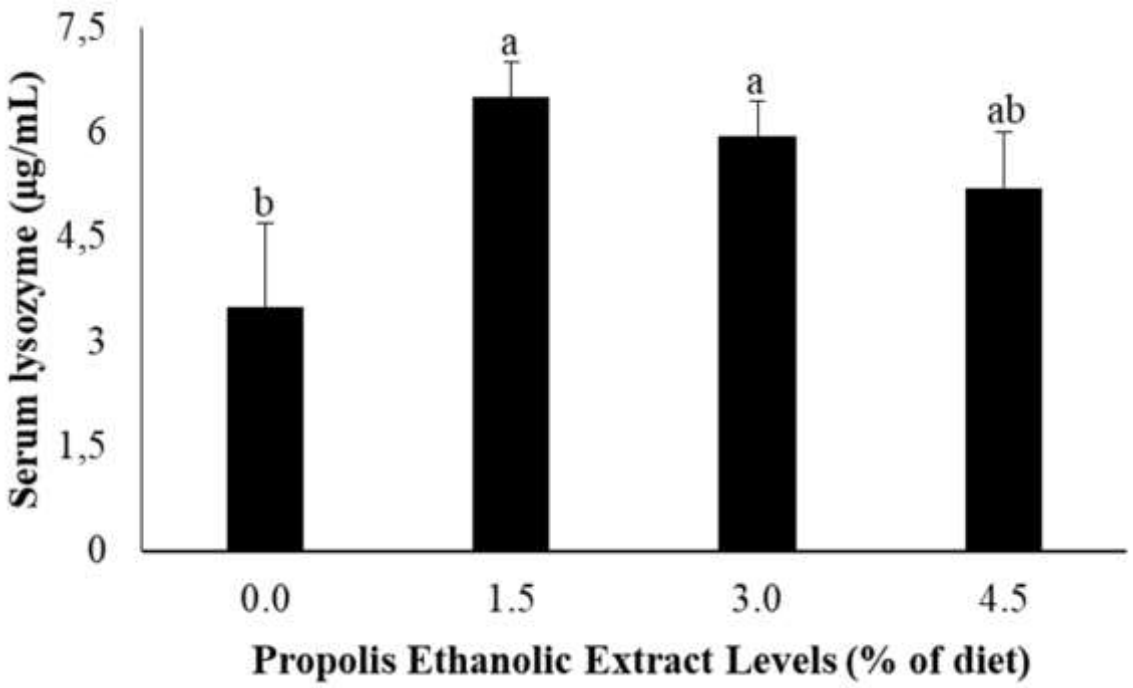

Figure 1. Serum lysozyme concentration of juvenile pacu *P. mesopotamicus* fed increasing levels of dietary brown propolis ethanolic extract. Different letter above each column denote difference by Tukey test (α=0.05).

Growth performance and intestinal morphology

The effects of dietary PEE on fish growth and intestinal morphology are summarized in Table 3. There was no significant effect of dietary PEE on fish growth. On the other hand, intestinal morphology was affected (p<0.05) by supplementation of PEE. Intestine muscle layer thickness decreased significantly in fish fed dietary PEE supplementation when compared to control treatment and intestinal villi height also decreased (p<0.05), but only in fish fed 3.0% dietary PEE.

Table 3. Growth performance and intestine morphology (mean ± SD) of juvenile pacu *Piaractus mesopotamicus* fed increasing levels of propolis ethanolic extract in diet for 60 days.

	Propolis ethanolic extract levels (% of diet)				One-way ANOVA
	0.0	**1.5**	**3.0**	**4.5**	**(p-values)**
Growth performance					
IW (g/fish)	28.5±0.3	28.2±0.1	28.1±0.08	28.7±0.6	0.245
FW (g/fish)	117.6±8.7	134.8±10.9	126.0±12.9	139.5±29.5	0.478
WG (g/fish)	89.1±8.4	106.0±11.1	97.9±12.9	110.8±29.6	0.482
FC (g/fish)	2.62±0.37	3.10±0.16	3.00±0.31	2.94±0.35	0.336
FCR	1.00±0.10	1.13±0.04	1.10±0.10	1.08±0.08	0.357
SGR (%weigh/day)	2.3±0.1	2.6±0.1	2.4±0.1	2.6±0.3	0.474
Intestinal morphology					
Musc. thickness (µm)	95.0±21.7a	63.1±10.8bc	54.9±7.3c	71.9±14.5b	<0.001
Villi height (µm)	202.7±51.5a	189.9±65.2a	129.7±58.1b	198.6±42.0a	0.014

Different letter in the same row denote difference by Tukey test (α=0.05).
IW: Initial Weight; FW: Final Weight; WG: Weight Gain; FC: Feed Consumption; FRC: Feed Conversion Ratio; SGR: Specific Growth Rate

DISCUSSION

To improve fish health and growth and avoid disease outbreaks, farmers can use antibiotics, vaccination and/or immunomodulators. The last one, non-specific immunomodulators represents an important tool in modern aquaculture. Fish welfare and health status after dietary PEE supplementation was accessed by hematological and immunological parameters and results showed that long-term administration (60 days) of PEE in fish diet did not induced adverse effects on fish physiology since there was no effect in erythrogram

and hematimetric indexes when compared to fish fed control diet, representing a desired characteristic for its safe use for feeding protocols in pacu production system. In accordance to our results, rainbow trout supplemented with increasing levels of dietary Iranian PEE for 60 days also presented no intoxication signs of treatments [34].

Immunomodulatory effects of propolis and its extracts are well known in human and animals, including fish [4,12,14,16]. Propolis is rich in bioactive substances such as flavonoids and phenolic compounds that can modulate fish immune system, oxidative stress, and disease resistance. For instance, dietary propolis extracts (aqueous or ethanolic) increased serum lysozyme and immune cells production and activity of tilapia species such as Mozambique tilapia *Oreochromis mossambicus* [19] and Nile tilapia [15,35] as well as for rainbow trout [22,36,37], common carp [23] and Japanese eel [24]. In addition, propolis also mitigated toxic effects and oxidative stress in fish exposed to exogenous chemical compounds or environmental stress [20,25,38–40].

In fact, dietary PEE modulated pacu immune system regarding serum lysozyme concentration and organic defense cells numbers, such as thrombocytes and neutrophils and this result, reflect a better health condition of fish and capacity to respond an infectious disease. Lysozyme is an important bactericidal enzyme produced by leucocytes, including neutrophils, that hydrolyzes the β-1,4 linkages between N-acetylmuramic acid and N-acetylglucosamine of gram-positive bacteria [41]. Fish thrombocytes are blood cells involved in hemostasis as platelets in mammals, unlike the last one, is only fragments of cells [42]. Moreover, this cells in fish can be related to immune response, since it participates in morbidity process and disease resistance and, also presents phagocytic activity, production of pro inflammatory factors (IL-1β) and MHC class II molecules in cell surface that suggests their function as cells presenting antigen to T lymphocytes and participation on the linkage between innate and adaptive immunity [43–47].

Propolis bioactive compounds modulates the immune system via macrophage activation and cytokines production and microbicidal proteins production by macrophages [12] and could explain our results, since the brown propolis herein used was collected from Southwest Region of Parana, Brazil and presented elevated concentration of bioactive compounds [48].

Despite significant effects on fish health, contradictory results can be observed. Nile tilapia supplemented dietary propolis and *Aloe barbadensis* extract at 0.5, 1.0 and 2.0% inclusion levels did not present *in vivo* effects on nonspecific immune system, but *in vitro* tests showed increased leucocyte phagocytosis activity [49]. In the same way, propolis showed bactericidal properties against *Aeromonas hydrophila in vitro*, but not necessarily *in vivo* [50]. The chemical composition of propolis as well as bioactive compounds concentration can vary extensively [9,51], hence, it also can explain variable results on fish immune system.

The growth promoter effect of propolis in fish generally is justified by better absorption and digestion of the dietary nutrients as consequence of the improvement of intestinal health due to its antimicrobial activity [18,19,36,52] and presence of some vitamins and minerals that improve the action of some digestive cofactors [15]. However, as herein observed, some studies have reported no growth-promoting effect in fish fed diets supplemented with propolis [23,34,51,53,54]. It is usual to justify the non-promotion of growth due to factors such as the variability of the chemical composition and palatability of propolis [50,51], time of experimentation, fish species, environmental factors [23]. In this study, the PEE palatability can be excluded, since it was observed no effects on fish feed consumption between treatments.

The effective concentrations of propolis supplementation for growth promotion are very variable, even when supplied for the same fish species. For example, the growth-promoting effect of PEE for Nile tilapia was recorded in minimum doses ranging from 5 g/kg (55) to 10 g/kg (15) and for rainbow trout from 2 g/kg (36) to 10 g/kg (52). Moreover, this research is pioneers on the use of propolis in neotropical fish pacu and point the need for further studies on this topic for specie.

Fish digestive system and its integrity at tissue and cellular levels can directly influence nutrient absorption and growth. Its suggested that the antibacterial activity of propolis compounds improves intestinal health and mucous cell numbers [9]. The increase in mucus production by enterocytes also improve the digest viscosity and could stimulate the intestine muscular layer development to move the alimentary bolus trough digestive tract [33]. However, our study did not confirm this hypothesis and significant effect on intestinal villi height and muscular layer thickness herein observed are inconclusive.Fish digestive system shows high phenotypic plasticity in response to diet composition [56] and this, can be considered. In addition, Nile tilapia post larvae and fingerlings fed increasing levels of dietary PEE did not show changes in intestinal villi height [51] and reinforces that, the mode of action of propolis on fish gut morphology are still unclear.

CONCLUSION

This study is pioneering in use of dietary PEE for neotropical fish pacu. In fact, bioactive compounds at 3.0% PEE supplementation modulated pacu immune system and corroborate the use of this natural compound as health promoter and does not present toxic effect for fish. However, sequential studies on time, dose and administration route and reliable chemical standardization of different propolis samples as well as research on pacu physiology and immunology are scarce and still necessary for its safe use in aquaculture.

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