http://dx.doi.org/10.1590/1678-4162-12806

Arq. Bras. Med. Vet. Zootec., v.75, n.4, p.744-752, 2023

Propolis extract as a feed additive of the Nile tilapia juveniles

[*Extrato de própolis como aditivo alimentar para juvenis de tilápia-do-nilo*]

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ABSTRACT

This study was conducted to investigate the effects of dietary propolis alcohol extract supplementation on growth performance, digestibility, intestinal morphometry, and physiologic parameters Nile tilápia (*Oreochromis niloticus L.*) juveniles. For performance experiment and intestinal morphometry, 120 fish averaging 1.41±0.3g were randomly distributed into 24 tanks (70L each) in a closed recirculation water system with constant aeration and biofilter. The animals were distributed into four treatments using a completely randomized design with six replicates per treatment. The treatments consisted of four pellet feeds with different alcohol propolis concentrations extract (AEP) (0.0, 0.5, 1.0 and 1.5g kg^{-1}). The performance experiment period was conducted for 45 days. For the digestibility experiment, an inert marker in the feed and the adapted Guelph feces collecting system were used. The results showed no differences in the performance parameters, crude protein apparent digestibility and dry matter of the feeds, intestinal morphometry, and physiologic parameters. However, there was an increasing linear effect on the energy digestibility with increasing PAE concentrations in the fish feed. The usage of the propolis extract supplementation levels of 1% is recommended for optimum effect on villus morphometry in Nile tilapia.

Keywords: aquaculture, additive, digestibility, intestinal morphometry, feeding, *Oreochromis niloticus*

RESUMO

Avaliaram-se os efeitos da suplementação de extrato de própolis na dieta sobre o desempenho, a digestibilidade, a morfometria intestinal e os parâmetros fisiológicos de juvenis de tilápia-do-nilo (Oreochromis niloticus). No desempenho e na morfometria intestinal, 120 peixes (1,41±0,3g) foram distribuídos aleatoriamente em 24 tanques (70L cada), em sistema de recirculação de água com aeração constante e biofiltro. Os animais foram distribuídos em quatro tratamentos, num delineamento inteiramente ao acaso, com seis repetições por tratamento. Os tratamentos consistiram em quatro rações, com concentrações de extrato de própolis (AEP) (0,0, 0,5, 1,0 e 1,5g kg-1). O período experimental foi de 45 dias. Para a digestibilidade, foi utilizado um sistema adaptado de Guelph para a coleta de excretas e um marcador inerte na ração. Os resultados não mostraram diferenças nos parâmetros de desempenho, digestibilidade aparente da proteína bruta e da matéria seca dos alimentos e morfometria intestinal, bem como nos parâmetros fisiológicos. No entanto, houve um efeito linear crescente sobre a digestibilidade da energia com o aumento das concentrações de AEP na ração. Os resultados também indicaram que a suplementação com 1% de própolis teve efeito positivo na morfometria intestinal de tilápia-do-nilo, sendo esse o nível recomendado na dieta.

Palavras-chave: aquicultura, aditivo, digestibilidade, morfometria intestinal, alimentação, Oreochromis niloticus

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Submitted: June 13, 2022. Accepted: March 3, 2023.

INTRODUCTION

The increase in aquaculture activities in Brazil, and around the world, is a growing and long-lasting phenomenon, primarily because of the increase in intensive breeding. Brazilian fish production was 4.7% higher in 2021, compared to the previous year, reaching the mark of 841 million tons of farmed fish, with tilapia being the first most produced group of fish in Brazil, and its production has grown 9.8% in 2021 (Anuário…, 2022). Moreover, the increase in animal production may result in a higher risk of fish diseases because of higher fish densities during storage and management, causing a decrease in natural immunity. High-density fish pens generally produce smaller fishes because of smaller spaces and proliferation of opportunistic pathogens.

The use of chemotherapeutic agents as growth promoters in fish feed formulations has recently gained the attention of fish farming. However, the use of these agents can cause cross-resistance to bacteria in humans (Lulijwa *et al.*, 2020). Therefore, some countries such as those in the European Union and China have restricted or prohibited the use of these substances in fish feeds (Mo *et al.*, 2015). Thus, the search for natural substances that can be used as growth promoters in fish feed formulations is currently a priority.

Propolis is a natural additive that has been recently analyzed as a potential growth promoter in the diets of monogastric and ruminant animals. Its increased demand, in both external and internal markets, has encouraged beekeepers to diversify their activities besides collecting honey. The propolis has been used in folk medicine since ancient times due to its numerous biological properties. Recent studies on the chemical composition of propolis associated with its pharmacological activities have also attracted much attention.

Bees collect propolis, a resinous, gummy, and balsamic substance derived from vegetables, from flower buds and plant exudates. It is used by the bees to protect against attacks from other insects and proliferation of microorganisms, including fungi and bacteria (Burdock, 1998). Better health status responses can improvements in the performance of animals fed on diets containing propolis extracts (Farag *et al*., 2021).

Therefore, this study was conducted to evaluate the effects of alcohol extract of propolis as an additive in the diet for Nile tilapia (*Oreochromis niloticus*).

MATERIALS AND METHODS

The fish feeds were formulated according to the nutritional requirements of tilapias (Jobling, 2012). Soybean, corn, and fishmeal containing different concentrations of propolis alcohol extract (0.0; 0.5; 1.0, and 1.5 $g.kg^{-1}$) were used to prepare the feeds. Kaolin was used as an inert material and was mixed with the AEP (Table 1).
For preparing the feeds, the ingredients were pre-dried in an oven with forced air set at a temperature of 55°C for 48h. Subsequently, the ingredients were ground in a Wiley mill, transferred onto a 2mm sieve, and homogenized. The mixture was then pelleted in a meat grinder and dried in an oven with forced ventilation (at 55°C) for 48h, crushed and sorted according to the size of fish. The diet was offered three times a day (08:00, 12:00, and 17:00h) by manual feeding until the fish apparent satiation.

The alcohol extract of propolis was obtained from "Apis Flora Industrial e Comercial Ltda®, Brazil". The alcoholic extract showed the following characteristics: clear liquid appearance without suspended particles, amber colored, and pH 5.35, 56% of alcohol content, 11% of soluble solids and 5.45mgL^{-1} of total flavonoids (information provided by the company). The alcoholic extract was obtained from the mixture of 30g of crude propolis powder with 70% ethyl alcohol until complete 100 mL. This solution was protected from light, under constant agitation for seven days, and then it was filtered to obtain the propolis ethanolic extract.

To evaluate the Apparent digestibility coefficients (ADCs) were used a total of 240 male Nile tilapias (30.0±6.0g) were distributed into 12 modified tanks containing 80L of water and allowed to acclimatization to the laboratory conditions for 15 days prior to the study. After acclimatization, the fishes were maintained during the day in tanks for feeding (three cages in a tank of 500L), each cage containing 20 fishes is one experimental unit, where they received meals between 07:00 to 16:00h, without restraint. After this period, they were transferred to the 300L fecal collection tanks (one cage per tank) made of fiberglass and equipped with a cone-shaped bottom and aeration constant. A container of 200mL clear vinyl was attached through a valve for the collection of feces. The fishes were kept in these tanks until the next day morning (07:00 am) when they were returned to the nourishment aquarium to start a new cycle (Guelph adapted method).

Apparent digestibility coefficients (ADCs) of dry matter, crude protein and crude energy of the Nile tilapia feed formulations containing the different above-mentioned concentrations of AEP (Table 1) and 0.5% Cr_2O_3 as an inert marker were calculated (Santos *et al.,* 2020).

Table 1. Composition percent diets experimental

Ingredients (g kg^{-1})	Treatments (g kg^{-1})			
	0.0	0.5	1.0	1.5
Soy meal	53.97	53.97	53.97	53.97
Corn	29.35	29.35	29.35	29.35
Fish meal	8.91	8.91	8.91	8.91
Propolis	0.0	0.5	1.0	1.5
Soy oil	2.92	2.92	2.92	2.92
Dicalcium phosphate	0.68	0.68	0.68	0.68
Limestone	0.81	0.81	0.81	0.81
Salt (NaCl)	0.35	0.35	0.35	0.35
DL-metionine	0.08	0.08	0.08	0.08
Suplement (vit and min)[1]	0.50	0.50	0.50	0.50
Inert (kaolim)	1.5	1.0	0.5	0.0
Nutrients (on dry matter)				
Digestible energy kcal/kg*	3.144	3.144	3.144	3.144
Dry matter (%)#	96.06	96.20	95.58	95.98
Crude protein (%)**	32.50	32.50	32.50	32.50
Crude protein (%)#	33.35	33.55	33.57	33.61
Crude fiber (%)**	3.83	3.83	3.83	3.83
Fat (%)**	4.70	4.70	4.70	4.70
Starch (%)**	25.58	25.58	25.58	25.58
Methionine +cystine (%)**	1.08	1.08	1.08	1.08
Total methionine (%)**	0.62	0.62	0.62	0.62
Lysine (%)**	1.91	1.91	1.91	1.91
Thryptophan (%)**	0.41	0.41	0.41	0.41
Valine (%)**	1.71	1.71	1.71	1.71
Threonine (%)**	1.27	1.27	1.27	1.27
Arginine (%)**	2.41	2.41	2.41	2.41
Leucine (%)**	2.57	2.57	2.57	2.57
Phenilalanine (%)**	1.55	1.55	1.55	1.55
Hystidine (%)**	0.84	0.84	0.84	0.84
Isoleucyne (%)**	1.54	1.54	1.54	1.54
Calcium (%)**	1.20	1.20	1.20	1.20
Available phosphorus (%)**	0.59	0.59	0.59	0.59

[1] Guarantee levels for kilogram of the product: vit. A = 900.000UI; vit. D3 = 50.000UI; vit. E = 6.000mg; vit. K3 = 1200mg; vit. B1 = 2400mg; vit. B2 = 2400mg; vit. B6 = 2000mg; vit.B12 = 4800mg; folic acid = 1200mg; calcium pantothenate = 12.000mg; vit. C = 24.000mg; biotina = 6.0mg; choline = 65.000mg; niacin= 24.000mg; Fe = 10.000mg; Cu = 600mg; Mn = 4000mg; Zn = 6000mg; I = 20mg; Co = 2.0mg e Se = 25mg). *According Santos *et al.* (2020). **According Rostagno (2005). #Analyzed in Laboratory of Animal Nutrition/Dept. Animal Science/UFRPE.

The performance experiment of the fish was conducted for 45 days. One hundred and twenty Nile tilapia males, with an initial average weight of 1.41±0.3g were used for this experiment. Five animals were used per experimental unit, a tank with 70L capacity each, supplied with constant aeration and biofilter. The tanks were cleaned as required.

The evaluated variables were weight gain, feed intake, feed conversion rate, protein efficiency ratio (PER) (weight gain/protein consumed), and final weight. In addition, hepatosomatic index (HSI) (liver weight/body weight × 100), digestive-somatic index (DSI) (organ weight of digestive system/body weight × 100), total length, standard length, and height were also assessed. The water temperature, dissolved oxygen, pH, total ammonia nitrogen and nitrate nitrogen were monitored daily in situ at 10:00h using a "multivariable water quality instrument" (YSI Professional Plus, YSI Incorporated, Yellow Springs, OH, USA).

Regarding the analysis of the gut histology (intestinal mucosa morphometry), the corresponding values were collected at the end of the experiment performance (45 days). The fishes were euthanized with high benzocaine concentration (193mgL^{-1}) and the initial portion of the intestine, approximately 5 cm in length (located 3 cm below the stomach–intestine junction), was collected randomized from five fishes per treatment. Intestinal samples were opened longitudinally, rinsed with saline, fixed in Bouin's solution for 12 hours, and the material was processed with routine histological technique. Sections were obtained with the aid of a rotary microtome (Leica, Germany). Sections (7µm) thicknesses were stained with hematoxylin-eosin.

Photo-documentation (image capturing) was performed using an Olympus® BX50 light microscope equipped with a 4x objective lens and a computerized imaging system (Image Pro Plus, Version 5.2, Media Cybernetics®). The intestinal mucosa morphometry was performed to measure the mucosa intestinal villus height, using 16 villi per fish with 80 measurements per treatment. All experimental procedures followed the Animal Ethics Committee of the Federal University of Alagoas recommendations (nº 07/2018- CEUA/UFAL).

A completely randomized design was used, with four treatments and six replicates, for performance, physiologic parameters, and intestinal mucosa morphometry, and three replicates for digestibility. One-way analysis of variance and polynomial regression method were performed to identify the differences among the treatments at 5% probability. SAEG software, version 9.1 (SAEG, 2007) was used for the data analysis.

RESULTS

The average values for temperature, pH, nitrite, toxic ammonia and dissolved oxygen during the experimental period were 27°C±0.5°C; 6.8±0.3; 0.15 mg.L^{-1}±0.05; 0.25 mg.L^{-1}±0.05 and 6.50mg.L^{-1}±0.25, respectively, and these values remained stable and within the range recommended for the species (Boyd and Tucker, 2012).

Table 2 shows the average values of the performance characteristics obtained at the end of the experiment with Nile tilapia fed diet-supplemented alcohol extract of propolis. Our results show that, after 45 days of feeding, fish productive performance was not influenced by the addition of AEP.

Table 2. Average values of initial weight, weight gain, feed intake, feed conversion rate, final weight, and protein efficiency ratio (PER) of Nile tilapia, according to propolis alcoholic extract levels

	Treatments (g kg^{-1})					
Items	0.0	0.5	1.0	1.5	*P* value	Regression
Initial weight (g)	1.41 ±0.10	1.41 ±0.12	1.41 ±0.12	1.41 ±0.10	0.208	Y=1.41
Weight gain (g)	4.66 ±0.14	4.80 ±0.58	4.78 ±0.46	4.72 ±0.13	0.071	Y=4.74
Feed intake (g)	4.28 ±0.19	4.21 ±0.25	4.46 ±0.29	4.44 ±0.13	0.078	Y=4.35
Feed conversion rate	0.92 ±0.12	0.88 ±0.09	0.93 ±0.10	0.94 ±0.10	0.275	Y=0.92
Final weight (g)	6.07 ±0.55	6.21 ±0.19	6.19 ±0.39	6.13 ±0.56	0.169	Y=6.15
PER	1.58 ±0.19	1.61 ±0.07	1.60 ±0.14	1.58 ±0.19	0.104	Y=1.59

Additionally, index of HSI, DSI, total length, standard length, and height, also there were no significant differences in the average values obtained using the different alcohol extract of propolis concentrations, as showed in Table 3.

Table 3. Average values for the hepato-somatic index (HSI), digestive-somatic index (DSI), total length, standard length, and height of Nile tilapia, according to propolis alcoholic extract levels

Items	Treatments (g kg^{-1})				P value	Regression
	0.0	0.5	1.0	1.5		
HSI (%)	0.79 ±0.13	0.78 ±0.10	0.88 ±0.27	0.72 ±0.12	0.135	Y=0.80
DSI (%)	9.44 ±0.32	9.42 ±0.59	9.64 ±0.35	9.61 ±0.75	0.106	Y= 9.53
Total length (cm)	5.40 ±0.13	5.41 ±0.10	5.36 ±0.14	5.34 ±0.18	0.734	Y=5.38
Standard length (cm)	3.59 ±0.06	3.61 ±0.12	3.58 ±0.12	3.52 ±0.20	0.243	Y=3.58
Height (cm)	1.97 ±0.16	1.93 ±0.15	1.95 ±0.20	1.84 ±0.32	0.509	Y=1.92

There were no significant differences in the ADCs of dry matter, crude protein, and the digestible protein of feeds with different concentrations of alcohol extract of propolis, although there was a positive linear correlation between the digestibility of crude energy and digestible energy and the increasing concentrations of AEP in the Nile tilapia diets. The average values for ADCs of dry matter, crude protein and crude energy and the average values of digestible energy and digestible protein are shown in table 4.

Table 4. Apparent Digestibility Coefficient (ADC) of fed diets with levels of propolis alcoholic extract for Nile tilapia. *P > 0.05

Items	Propolis extract levels (g kg^{-1})				*P*value	R^2	Regression
	0.0	0.5	1.0	1.5			
ADC (%) dry matter	74.60 ±3.40	79.69 ±0.96	82.21±2.64	82.00±7.45	0.190	-	Y = 79.70
ADC (%) Crude protein	89.54 ±1.32	92.19 ±0.63	92.04±1.18	91.64±3.29	0.350	-	Y = 91.35
ADC (%) Crude Energy	79.78 ±1.50	83.76±0.90	85.36±2.21	85.89±4.64	0.021*	0.99	$Y=81.09+6.41x-2.14x^2$
Digestible energy (kcal kg^{-1})	3082.23±57.84	3259.94±35.03	3313.88±71.84	3322.95±179.54	0.019*	0.99	$Y=3086.2+408.18x-168.64x^2$
Digestible protein (g kg^{-1})	29.86±0.44	30.92±0.21	30.89±0.40	30.79±1.11	0.350	-	Y = 30.62

The results of intestinal morphometry analysis showed a quadratic effect with the supplementation of AEP the 1.0 $g.kg^{-1}$ treatment showed the best results (Fig. 1). Results of intestinal morphometry (corresponding villi heights) of 232.06; 293.85; 322.35 and 245.93 µm were observed with the addition of 0.0, 0.5, 1.0 and 1.5 $g.kg^{-1}$ AEP in the fed diet, respectively.

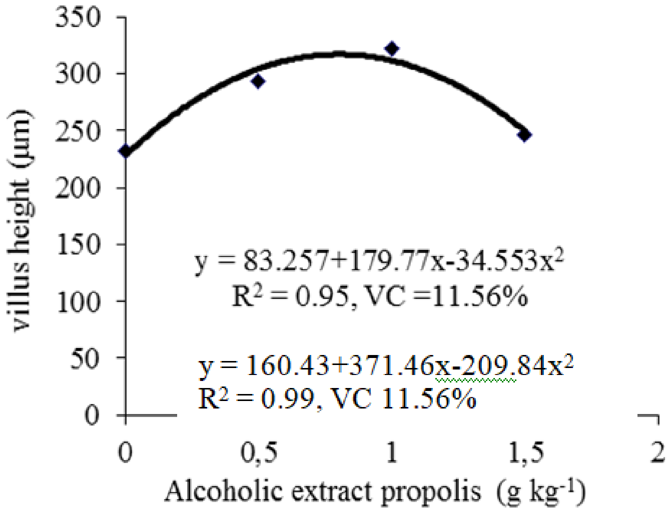

Figure 1. Average values of villi height for Nile tilapia fed with increasing levels of propolis alcoholic extract in the diet.

DISCUSSION

Propolis is a prebiotic that has been widely used in human and veterinary medicine for the treatment of various infections involving the gastrointestinal tract. The primary mechanism of the action of probiotics is the beneficial modulation of the natural microbiota of the host. It is also speculated that some specific prebiotics could directly act on the intestinal translocation of pathogens, thereby preventing their adherence to epithelial cells and activation of the acquired immune response (Ashaolu, 2020).

It has been reported by De la Cruz-Cervantes *et al.* (2018) that in Nile tilapia, the application of prebiotics results in the growth of beneficial microbial populations and improvements in the intestinal immune system and anatomic features of the gastrointestinal tract. Therefore, in some cases, it is expected that this will be positively reflected in the productive performance of the animal. Conversely, propolis for fish diet is effective against Gram-positive bacteria and can act as a growth promoter.

However, in the present study, there was no apparent health challenge that propolis alcohol extract could potentiate. Therefore, the addition of propolis in the feed formulations did not result in any positive effect on performance, morphometric parameters of HSI and DSI and the digestibility of crude protein and dry matter, probably because the conditions were optimal for fish growth.

Corroborating these results, Mattos *et al.* (2017) also did not observe any positive results in the performance of juvenile angelfish (*Pterophyllum scalare*) fed with diets containing propolis extract (0-1.220 mg.kg^{-1}) for 33 days. Gunathilaka *et al.* (2015) obtained a similar result using olive flounder (*Paralichthys olivaceus*), which confirmed that diets with propolis (0.0; 0.25, 0.5; 0,75 and 1.0%) did not differ from that of the controls (without the addition of propolis) based on the performance parameters, for 4 weeks.

Nevertheless, results should also consider the fact that there is a great variation among the different types of propolis and bee flora, primarily depending on where they were produced (Mountford-McAuley *et al.*, 2021). These authors also stated that excessive amounts of propolis could result, inclusively, in negative collateral effects. These negative results may be related to the assimilation of phenolic compounds, as they may reduce the appetite and growth of the fish. On this account, the propolis also contains some compounds that can cause toxic effects when intake in high levels (Lavinas *et al.*, 2019). Already, Burdock (1998) reported that higher doses of propolis maybe impact a negative effect on animals and may interfere with the intracellular process, whereas the lowest doses often showed better results.

However, Deng *et al*. (2011) reported that the administration of an alcohol extract of propolis to rainbow trout, in levels dietary 2-4 $g.kg^{-1}$, for 10 weeks, significantly improved the growth performance and feed efficiency, as compared with controls diets. They also described that propolis prevented digestive disorders and served as the better feed. These findings were also confirmed by Meurer *et al*. (2009), who showed that the administration of 2.22 $g.kg^{-1}$ brown propolis extract to Nile tilapia fingerlings (4.1 ± 0.1 g) resulted in better performance, probably because of the private properties of brown propolis, which was produced in Serra do Araripe Cariri in the southern region of the state of Ceará (Brazil).

The propolis used in the present study was collected from the South-eastern region of Brazil and had major differences in terms of bee flora compared with that of the brown propolis used by Meurer *et al.* (2009). However, the extract used in the present study was similar to that used by Arauco *et al.* (2007), whose results were contradictory to those of the present study, when the addition of propolis extracts in the feeds of bullfrogs results in an improved weight gain and percentage of monocytes in peripheral blood, thereby possibly showing an immunostimulatory effect by using lower doses (0.2% and 0.5%) for 8 weeks.

Abd-El-Rhman (2009) also reported the positive effects of using green and raw AEP from Egypt in the feed formulations of juvenile Nile tilapia that were infected by *Aeromonas hydrophila*, thereby showing a better productive performance, for 28 days. Farag *et al.* (2021) reported that comparative studies have revealed that, although different propolis may have a different chemical composition, always this propolis have considerable high biological activities.

In the present study, despite the absence of significant effects on performance and digestibility values of crude protein and dry matter, an improvement in the raw energy digestibility of the feeds was observed with an increase in the concentrations of propolis extracts, indicating a positive effect. This is probably due to the selection of beneficial bacteria that helps in the utilization of fibers and more complex carbohydrates. The mechanism of antibacterial activity is considered complex and difficult to understand and may be attributed to the synergism among flavonoids, hydroxy acids, and sesquiterpenes. (De la Cruz-Cervantes *et al.*, 2018).

The proportion of these substances in propolis varies with the location and time of its collection (Lavinas *et al*., 2019). Therefore, its effects as a growth promoter could also differ for doses of propolis and/or its origin especially. These contradictory results suggest that further studies should consider different propolis processing and composition.

The intestinal mucosa showed foliaceus villi of irregular height and integrity. We did not observe the presence of intestinal crypts, suggesting that the enterocytes are proliferating at the base of the villi. These characteristics have been previously observed for teleost and specifically for tilapia (Gargiulo *et al.,* 1998).

According to Estruch *et al*. (2020), the intestinal mucosa is of great importance in the digestive, absorptive, and metabolic processes in teleost fish. However, this effect did not interfere positively with performance in the present study. Presumably, the best results of intestinal morphometry with the supplementation of AEP the 1.0 $g.kg^{-1}$ in the diet for tilapia, it was possibly due to intestinal microbiota modulation, occurring consequently to act as nutrients for beneficial bacteria in the gut, prioritizing its growth. Additionally, the microorganism's metabolites will be reducing pH through the increase of organics acids and decreasing the fixation capacity of the gut pathogenic bacteria, reflected on villi heights of intestinal mucosa and fish health thereafter.

The quadratic effect involving the increase in villi height was observed in the present research, probably because of enterocytes having better access to energy sources and improved microbial selection. It is possible that flavonoids in propolis enhance food ingestion and absorption, and nutrient metabolism in fish. The alcohol extract of propolis also has other active compounds in its composition such as: phenolic compounds, aromatic acids, esters, terpenoids, fatty acids, among others that can influence its beneficial action as an additive, however flavonoids are the most studied compounds and with more consolidated results of propolis (Lustosa *et al*., 2008).

Future studies should focus on the identification of the active ingredient in propolis and the possibility of using propolis as a natural antioxidant in food and feed.

Studies on the incorporation of natural additives in the fish feed as growth promoters and their effects on production have generated variable results, being necessary additional studies to validate the previous findings.

CONCLUSIONS

The use of propolis alcohol extract as a growth promoter in the Nile tilapia fish feed did not result in an improvement in performance, although an improved use of energy resources and improve on villus morphometry was observed. Thus, the use of propolis extract supplementation is recommended at levels of 1% in diet.

ACKNOWLEDGMENTS

The Alagoas Research Foundation, (FAPEAL) and Pernambuco Research Foundation (FACEPE), Brazil supported this study.

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