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The effects of the supplementation of activated charcoal on the growth, health status and fillet composition-odor of Nile tilapia (*Oreochromis niloticus*) before harvesting

Surintorn Boonanuntanasarn · Pranorm Khaomek · Taratip Pitaksong · Yanling Hua

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Abstract This study shows the effects of dietary activated charcoal (AC) on health status, intestinal morphology and fillet geosmin content of Nile tilapia prior to harvesting (2 and 4 weeks). Four dietary treatments (each diet in six replicates) were formulated to incorporate AC at levels of 0, 10, 20 and 30 g kg^{-1} of the dry diet. Fish were reared in hapas, which were located in earthen ponds. There were not significant differences in growth performances among experimental treatments. The moisture and protein content in the fillet decreased and increased, respectively, as the incorporation level of AC increased. The hematological indices and several immune parameters did not differ significantly among treatment groups. Among the fifteen blood chemicals parameters examined, the significant reductions in protein and cholesterol and the changes in blood minerals were observed in fish fed dietary AC $\geq$20 g kg^{-1}. Dietary AC tended to increase the height of intestinal villi and goblet cell number. Dietary AC also influenced the reduction in geosmin in the fish fillet. Taken together, these findings indicate that AC (at 10 g kg^{-1} diet) could be used as feed supplement for Nile tilapia prior harvesting to reduce geosmin without negative effects.

Keywords Activated charcoal · Nile tilapia · Growth · Health status · Intestinal morphometry · Geosmin

S. Boonanuntanasarn (✉) · T. Pitaksong
School of Animal Production Technology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima 30000, Thailand
e-mail: surinton@sut.ac.th

P. Khaomek
Department of Chemistry, Faculty of Science, Rangsit University, 52/347 Muang Ake, Phaholyothin Road, Lakhok, Pathumthani 12000, Thailand

Y. Hua
The Center for Scientific and Technological Equipment, Suranaree University of Technology, Nakhon Ratchasima 30000, Thailand

Introduction

Activated charcoal (AC), which has non-nutritive and non-digestible components, has been used for medical and veterinary purposes as a universal poison antidote. AC is used as a detoxifying substance due to the influence of both physical and chemical characteristics, including surface area and pore size, on its adsorption capacity (McFarland and Chyka 1993; American Academy of Clinical Toxicology 1999; Bacaoui et al. 2001). AC is effective in the elimination of the mycotoxins, such as aflatoxins (Galvano et al. 1996), as well as pesticide residues (Wilson et al. 1971) that occasionally contaminate the feed's ingredients. The adsorption effect of AC with other detrimental organic compounds would positively influence fish growth, immunity and flesh quality; therefore, AC could be used as dietary health supplement for Nile tilapia aquaculture. Recently, there has been only limited scientific research regarding the use of AC as a feed additive in livestock and aquatic animals (Villalba et al. 2002; Ruttanavut et al. 2009; Majewska et al. 2009; Thu et al. 2010).

The worldwide production of tilapia has been increased intensely to provide a staple source of protein for human consumption. Although Nile tilapia are hardy, easy to farm and fast growing, commercial intensive tilapia production has still been categorized as a type of business risk due to disease outbreaks, fluctuations in the water quality and/or contamination from any agricultural chemical pollutant during the culture period. Tilapia can be commercially raised in many culture systems, including ponds, cages, tanks and raceways. Pond culture is the most cost-effective grow-out system for growing tilapia because the fish can use natural food in addition to complete feed. A musty-earthy flavor is a critical problem of pond-raised tilapia. Geosmin and 2-methylisoborneol (MIB) are secondary metabolic products of some blue-green algae and bacteria (for a review, see Tucker 2000). Fish adsorb geosmin from the surrounding water primarily through their gills (From and Hørlyck 1984). Geosmin is also taken up intestinally, when fish ingest algae. In Nile tilapia, the highest amount of geosmin was detected in the intestine compared with the abdomen, skin and muscle (Yamprayoon and Noomhorm 2000). Several types of AC have been reported to have adsorption effects on geosmin in potable water resources (Ridal et al. 2001; Matsui et al. 2013). Therefore, an investigation of the effect of dietary AC on production performance, health status and fillet composition-odor of Nile tilapia would promote sustainable production of tilapia.

This study aims to investigate the potential use of dietary AC as a feed supplementation for Nile tilapia cultures during the period prior to harvesting. Hematological, blood chemical and immune parameters were evaluated to determine whether AC has detrimental effects on fish health. The intestinal morphology was also investigated. Furthermore, to determine whether AC could produce a reduction in the off-flavor, we analyzed the geosmin content in fillets.

Materials and methods

Experimental fish, design, fish culture and fish performance evaluation

The Nile tilapia (*Oreochromis niloticus*) used in this study were reared at the Suranaree University of Technology Farm (SUT Farm; Nakhon Ratchasima, Thailand). The experimental Nile tilapia were male Nile tilapia that had been produced by feeding the swim-up fry with a 50 mg kg^{-1} 17α-methyltestosterone-treated diet for 4 weeks and then with a

diet consisting of 350 g kg^{-1} crude protein until the start of the experiment. The experimental design was completely randomized into four treatment diets. To test the validity of the conclusions, each treatment was replicated six times with twenty fish per replicate. Twenty-four hapas ($2 \times 2 \times 2.5$ m^3) were maintained in the earth pond of the SUT Farm, and the bottoms of the hapas were embedded 10 cm underneath the bottom of the pond. Prior to the start of the experiment, fish (380–460 g) were randomly distributed in the experimental hapas. The fish were fed Diet AC-0 to adapt to the experimental environment for 2 weeks. During the experiment, the fish were hand-fed twice daily for 4 weeks. Diets were fed ad libitum. Any dead fish was recorded and removed daily. The growth performance and feed utilization were evaluated at the end of weeks 2 and 4. During the experimental period, in order to investigate the effects of dietary AC on health status and fillet composition-odor, stable green water system was implemented to resemble semi-intensive tilapia farming. The daily fluctuation in water temperature was measured daily (at 6:00 and 18:00), and the dissolved oxygen and pH were determined weekly (at 6:00 and 18:00). The water temperatures (mean ± SD) at 6:00 and 18:00 were 28.3 ± 1.4 and 30.4 ± 2.6 °C, respectively. The pH and dissolved oxygen were within acceptable ranges, i.e., a pH of 8.20 ± 0.28 (6:00) and 8.76 ± 0.51 (18:00) and dissolved oxygen of 3.58 ± 0.45 mg L^{-1} [41.1–55.2 % of saturation] (6:00) and 12.25 ± 2.05 mg L^{-1} [135.72–205.58 % of saturation] (18:00). Total ammonia content was measured weekly using Tetratest NH_3/NH_4^+ (Tetra GmbH, Melle, Germany). During the experimental period, total ammonia was maintained at <0.25 mg L^{-1}.

Feed formulation and pellet preparation

All test ingredients were obtained from animal feedstuff companies. Before formulating the feed, all feed ingredients were analyzed to determine gross composition (moisture, protein, lipid and ash) according to AOAC (1990). Four diets were formulated to incorporate activated charcoal (SKU V-1400, Charcoal House LLC, Crawford, NE, USA) at 0 (AC-0), 10 (AC-10), 20 (AC-20) or 30 (AC-30) g kg^{-1} (Table 1). All diets were formulated to contain same ingredients with little differences in proportions in order to produce isonitrogenous and isocaloric experimental diets. All diets were produced using a hammer grinder, mixer and extruder (Paktongchai Pasusat, Nakhon Ratchasima, Thailand). The dry ingredients were ground by a grinder and mixed by a ribbon screw mixer (22 rpm). The floating experimental diets were produced using a single screw extruder (an extruding temperature of 120–160 °C). All experimental diets were analyzed to determine their chemical compositions according to AOAC (1990) and stored at room temperature until use.

Fish sampling and blood collection

Hematological, blood chemical and immune parameters were evaluated to determine the effects of the activated charcoal on the health status of the Nile tilapia. At the end of weeks 2 and 4, the fish were not fed for 18 h prior to blood sampling. Four representative fish from each diet replicate were randomly selected and anesthetized with 2-phenoxyethanol (0.35 mL L^{-1}). Blood sampling was conducted as described in Pitaksong et al. (2013). Blood was collected by a hypodermic syringe from the caudal vein. The collected blood sample was divided into two sets. One set was added to a tube containing K_2EDTA as an anticoagulant. The second blood sample set was left to clot at 4 °C for at least 3 h and centrifuged at 5,000 rpm for 5 min at room temperature. Plasma was collected by the

Table 1 Ingredients and chemical composition (g kg^{-1}) of the four experimental diets

Ingredient (g kg^{-1})	Diet			
	AC-0	AC-10	AC-20	AC-30
Fish meal	300	302.5	305	307.5
Soybean meal	270	267.5	267.5	267.5
Rice bran	150	145	135	120
Corn meal	145	145	145	145
Cassava chips	120	115	112.5	110
Premix[a]	15	15	15	15
Soybean oil				5
Activated charcoal		10	20	30
Proximate composition (g kg^{-1} dry weight)				
Dry matter	925	910	935	940
Crude protein	326	325	324	324
Crude lipid	64	64	61	59
Crude ash	94	95	102	103
Crude fiber	40	40	42	43
Nitrogen-free extract[b]	516	516	513	514

[a] Vitamin and trace mineral mix provided the following (IU kg^{-1} or g kg^{-1} diet): biotin, 0.25 g; folic acid, 0.003 g; inositol, 0.25 mg; niacin, 0.0215 g; pantothenic acid, 0.03 g; vitamin A, 5,000 IU; vitamin B1, 0.0025 g; vitamin B2, 0.0012 g; vitamin B6, 0.0075 g; vitamin B12 0.00005 mg; vitamin C, 1 g; vitamin D3, 1,000 IU; vitamin E, 100 IU; vitamin K, 0.008 g; copper, 0.02 g; iron, 0.2 g; selenium, 0.3 mg; zinc, 0.32 g

[b] Nitrogen-free extract = 1,000 − (crude protein + crude lipid + crude ash)

centrifugation of the K_2EDTA-blood at 5,000 rpm for 10 min at 4 °C. The serum collected was stored at −80 °C for further analysis. After blood sampling, samples of intestine were collected for histological analysis. Then, the fillet were dissected and frozen for proximal analysis according to AOAC (1990) with slight modifications, such as sample weights (moisture; 2 g, protein; 5 g, fat and ash; 10 g), the fish fillet was minced for geosmin analysis.

Hematological assays

Immediately after blood sampling, the K_2EDTA-blood was used to examine the hematological parameters. The red blood cell count (RBC) was analyzed in duplicate for each sample using a Neubauer hemocytometer after dilution with Grower's solution (Voigt 2000). Hematocrit values (Ht) were measured in duplicate by placing K_2EDTA blood into glass capillary tubes and centrifuging them for 5 min by microhematocrit centrifugation. The hemoglobin (Hb) content was measured using the photometrical cyanohemoglobin method. The white blood cell count (WBC) was determined in duplicate for each sample using a Neubauer hemocytometer after dilution with Dacie's solution (Dacie and Lewis 2001).

Blood chemistry analysis

To investigate the effects of dietary activated carbon on fish health, several blood tests were performed such as glucose, triglyceride, cholesterol, total protein, albumin, blood

urea nitrogen (BUN), total bilirubin (T-bilirubin), direct bilirubin (D-bilirubin), alkaline phosphatase (AP), amylase, serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), calcium, chloride, and iron. Immediately after blood sampling, the K_2EDTA treated blood was used to determine the blood glucose levels in whole blood drawn from the caudal vein using a hand-held glucometer (AccuTrend; Roche). Serum triglyceride was analyzed by the glycerol-3-phosphate oxidase-sodium *N*-ethyl-*N*-(3-sulfopropyl) m-anisidine (GPO-ESPAS) method described by Bucolo and David (1973). Serum was quantitatively determined using the cholesterol oxidase-phenol+aminophenazone (CHOD-PAP) technique described by Flegg (1973). Plasma proteins were estimated by the Biuret method (Gornall et al. 1949). Serum albumin was quantitatively evaluated using the bromocresol green method for albumin (Doumas et al. 1971). BUN was measured by a modified indophenol colorimetric method (Weatherburn 1967). Total and direct bilirubin (T- and D-bilirubin) were measured using the new diazo-DMSO method (Winsten and Cehelyk 1969). AP was examined by the phenolphthalein monophosphate (PMP) method (Babson et al. 1966). Amylase was quantitatively determined using the amyloclastic method utilizing decreases in starch-iodine reactions (Reif and Nabseth 1962). SGOT and SGPT were measured using Reitman and Frankel's colorimetric method (Reitman and Frankel 1957). The calcium content in the serum was measured using the *o*-cresolphthalein direct method (Moorehead and Biggs 1974). Blood chloride was estimated using the thiocyanate method (Hamilton 1966). Iron ferene was examined using an iron quantitative determination in the serum or plasma (IDS, Liege, Belgium).

Immune assay

Immune parameters, including total immunoglobulin, lysozyme activity and alternative complement activity (ACH50), were measured. Total immunoglobulin was measured according to Siwicki et al. (1994). Using experimental fish serum, lysozyme activity was analyzed as described by Pitaksong et al. (2013), and ACH50 were determined according to Sunyer and Tort (1995).

Histological analysis

To determine the effect of dietary activated charcoal on intestinal morphology, two fish from each replication of each treatment were sampled and prepared for histological study, as described previously (Phumyu et al. 2012). Portions of the anterior, middle and posterior of the intestines were dissected and preserved in 10 % phosphate-buffered formalin with a pH 7.2. After dehydration, the tissue was embedded in a paraffin wax, cut into slices 5 μm thick and mounted on glass slides. After deparaffinization, the slides were dehydrated and stained with Hematoxylin and Eosin (H&E). The height of the villus was measured on stained sections under a microscope using an ocular micrometer at 100× magnification. The ten longest intact villi in each intestinal position were selected for measurement in duplicate cross-sections for each sample.

Geosmin analysis

Extraction of the geosmin was carried out according to Yamprayoon and Noomhorm (2000), with modifications. Fish fillet samples of known weights (100 g) were minced and mixed with 50 mL of distillated water and 20 g of NaCl. Volatiles were distilled from the

mixture of minced fillet for 3 h. The distillate was extracted with 2 mL hexane containing internal standard [isobornyl acetate (2.5 μg mL^{-1}) and 12 g of NaCl for 15 min]. Anhydrous sodium sulfate was used to remove water from the hexane extract. Geosmin was then analyzed with gas chromatography–mass spectrometry (GC-CP3800, MS-1200, Varian Inc., Walnut Creek, CA, USA) equipped with a capillary column (Varian VF-5 ms, 30 m*0.25 mm ID*0.25 μm film thickness) and a quadrupole mass detector. The injector port temperature was 260 °C, and a 2 μL sample of the extract was used for injection. The flow rate of the helium carrier gas was 1.0 mL min^{-1}, and splitless mode was applied. The transfer line temperature was set at 250 °C. The initial column temperature was maintained at 60 °C for 2 min before being increased to 240 °C at a rate of 8 °C min^{-1} and held for 5.5 min. The mass spectra were obtained by electron ionization (EI) at 70 eV. The scan range was 50–200 *m*/*z* at 0.5 s intervals. The identification of geosmin was performed by comparing retention times with geosmin standards [2.5, 5, 7.5, 10, 15, 20 ppb] and mass spectra with mass spectral libraries (National Institute of Standards, NIST). The amounts of geosmin were calculated from peak areas by comparing them with the geosmin standard curve; isobornyl acetate was used as an internal standard.

Data analysis

All data were analyzed by one-way analysis of variance (ANOVA) using SPSS for Windows (Release 10) (SPSS Inc., Chicago, IL, USA). When significant differences were found among the groups, Duncan's multiple range tests were used to rank the groups. The statistical model utilized was $y_{ij} = \mu + \tau_I + \varepsilon_{ij}$, where y_{ij} was the response; μ, the general means; τ_I, the dietary AC effects; and ε_{ij}, the random error. In addition, regression analysis and goodness of fit (R^2) for the responses (Y) and for the level of incorporated AC parameters (x) were conducted. Throughout the experiment, effects and differences were declared to be significant when their values were less than 0.05 ($P < 0.05$).

Results

The growth performance of Nile tilapia fed different experimental feeds was determined at the end of 2 and 4 weeks (Table 2). Through the whole experimental period, the growth performances, including final weight, weight gain (WG), specific growth rate (SGR) and feed conversion ratio (FCR), of the fish fed all experimental diets showed no significant differences. Fish fed the AC-10 diet had the highest final weight, WG, SGR, feed intake (FI) and the lowest FCR, compared to the other experimental diets; however, due to the high standard deviations, differences were not significant. The condition factor (K) of Nile tilapia did not vary significantly among the groups. Until the end of the experimental period (4 weeks), the survival rates remained high in all experimental groups, ranging from 90 to 100 %.

The effects of supplemental AC on the chemical composition of the fillets are given in Table 3. During the experimental period, an increase in supplemental AC decreased the moisture of the fillets, and a significant reduction was observed at the end of 4 weeks. A significant linear relationship ($y = -0.971x + 778.669$, $R^2 = 0.510$) was found between AC supplementation levels and moisture. At the end of week 2, an increase in supplemental AC significantly increased protein contents of the fillets. By the end of week 4, a significant linear relationship ($y = 0.609x + 140.849$, $R^2 = 0.446$) was observed between

Table 2 Growth performance of Nile tilapia fed experimental diets for 4 weeks (mean ± SD, $n = 6$)

Diet	Initial body weight (g)	Final body weight (g)	Weight gain[a] (%)	SGR[b] (% day^{-1})	Feed intake (g day^{-1})	FCR[c]	K[d]
AC-0	422.5 ± 35.4	487.7 ± 42.8	15.5 ± 5.3	0.54 ± 0.17	5.27 ± 1.14[a,b]	3.80 ± 1.45	1.96 ± 0.19
AC-10	416.3 ± 27.8	503.2 ± 48.2	20.8 ± 6.0	0.67 ± 0.17	6.11 ± 1.00[a]	3.19 ± 0.67	2.04 ± 0.21
AC-20	414.5 ± 28.2	481.0 ± 39.8	16.0 ± 5.1	0.53 ± 0.16	5.10 ± 0.57[a,b]	3.74 ± 1.45	2.02 ± 0.18
AC-30	407.5 ± 23.0	471.3 ± 17.6	15.8 ± 4.6	0.52 ± 0.15	4.96 ± 0.56[b]	3.57 ± 0.99	1.95 ± 0.08

Means with different superscripts in each column differed significantly from each other ($P < 0.05$)

[a] Weight gain = 100 × (final mean body weight − initial mean body weight) × initial mean body weight^{-1}

[b] Specific growth rate (SGR) = 100 × [(ln final body weight − ln initial body weight) × experimental days^{-1}]

[c] Feed conversion ratio (FCR) = dry feed fed × wet weight gain^{-1}

[d] Condition factor (K) = 100 × body weight (grams) × L (centimeters)$^{-3}$

Table 3 Approximate compositions of Nile tilapia fillets (g kg^{-1}) fed experimental diets for 4 weeks (mean ± SD, $n = 6$)

Diet	2 weeks				4 weeks			
	Moisture (g kg^{-1})	Protein (g kg^{-1})	Fat (g kg^{-1})	Ash (g kg^{-1})	Moisture (g kg^{-1})	Protein (g kg^{-1})	Fat (g kg^{-1})	Ash (g kg^{-1})
AC-0	762.4 ± 8.7	188.3 ± 5.2^{b}	10.4 ± 1.9	15.3 ± 2.1	777.3 ± 6.8^{a}	158.8 ± 9.8	11.1 ± 3.8	14.7 ± 1.2
AC-10	767.2 ± 10.6	189.7 ± 10.5^{b}	9.5 ± 2.1	15.6 ± 3.9	767.8 ± 8.4^{b}	163.5 ± 12.2	11.1 ± 2.0	15.7 ± 1.4
AC-20	754.9 ± 24.6	197.9 ± 6.5^{a}	12.8 ± 2.2	14.7 ± 1.5	765.6 ± 9.5^{b}	171.3 ± 5.0	10.2 ± 2.0	15.0 ± 1.9
AC-30	767.0 ± 12.7	198.4 ± 4.4^{a}	11.3 ± 3.7	15.0 ± 2.4	745.7 ± 15.1^{c}	179.1 ± 6.7	12.0 ± 4.2	15.1 ± 1.3

Means with different superscripts in each column differed significantly from each other ($P < 0.05$)

AC and protein contents. No significant differences were observed on the fat or ash content in fillets among the experimental groups.

The hematological indices of the Nile tilapia fed different experimental diets are shown in Table 4. Throughout the experimental period, the red blood cell count (RBC), hemoglobin and hematocrit did not vary significantly among diets. By the end of week 2, an increase in supplemental AC led to a significant increase in the white blood cell count. At week 4, white blood cell counts did not differ according to the level of supplemental AC. The effects of AC supplementation on several parameters of humoral, non-specific immunity, including lysozyme activity, total immunoglobulin and alternative complement (ACH-50), were assessed (Table 5). By the end of the experimental period, values for lysozyme activity, total immunoglobulin and ACH-50 did not vary among experimental diets.

Table 6 shows the blood chemical parameters of Nile tilapia fed different experimental diets. Several blood chemical analyses performed, such as glucose, triglyceride, albumin, BUN, T-bilirubin, D-bilirubin, AP, amylase, SGOT and SGPT, did not reveal any significant difference among different diets through the end of the experimental period. However, there were significant differences in cholesterol values which appeared to decrease with an increase in AC supplementation in the diets. By the end of week 4, a significant reduction was determined in Nile tilapia fed diets AC-20 and AC-30, comparing with Diet AC-0. At week 2, the total protein content of Nile tilapia that were fed diets AC-20 and AC-30 was decreased, comparing with those on Diet AC-0. However, at week 4, the total proteins appear to be similar among diets. AC supplementation had effects on the blood minerals of Nile tilapia, by the end of week 2, where a significant decrease in the calcium levels was evident, while had no significant effects on the chloride or iron levels. A significant linear relationship ($y = -0.013x + 3.687$, $R^2 = 0.778$) was observed between AC supplementation levels and the blood calcium content. At the end of week 4, the calcium tended to decrease with an increase in AC supplementation in the experimental diets, although the decrease was not significant. Additionally, significantly higher blood chloride and lower blood iron levels were observed in Nile tilapia fed Diet AC-30 compared to Diet AC-0 ($P < 0.05$).

The intestinal histomorphometry of the Nile tilapia that were fed various levels of AC-supplemented diets is given in Fig. 1 and Table 7. By the end of week 2, AC-supplemented diets led to significant increases in the height of villi in all parts of the intestine ($P < 0.05$). At week 4, fish fed Diet AC-10 diet had higher villi heights in the anterior and middle parts, whereas supplementation with AC at 20 and 30 g kg^{-1} did not generate any further increase in villi height. AC supplementation did not have any effect on the villi of the posterior intestine. At week 2, a significantly higher goblet cell count was observed in only the middle intestine of fish fed Diet AC-30. By the end of week 4, compared with fish on Diet AC-0, the number of goblet cells in all portions of the intestines of fish fed dietary AC was higher. Moreover, dietary AC seemed to increase the thickness of the intestinal muscle in posterior part (Fig. 1) which would indicate the effect of dietary AC on chronic constipation.

Fillet's geosmin content of Nile tilapia fed the AC-supplemented diets are shown in Fig. 2. By the end of week 2, fish fed on AC-30 diet had significantly lower geosmin contents in their fillets ($P < 0.05$). At the end of week 4, significantly lower geosmin contents in the fillets were observed for fish fed the AC-10, AC-20 and AC-30 diets, compared with fish fed the control diet (AC-0) ($P < 0.05$). In addition, a significant nonlinear relationship ($y = 0.016x^2 - 0.830x + 15.856$, $R^2 = 0.702$) was observed between AC and the geosmin content in fillets.

Table 4 Hematological parameters of Nile tilapia fed experimental diets for 4 weeks (mean ± SD, $n = 6$)

Diet	2 weeks				4 weeks			
	RBC ($\times 10^6$ mm^{-3})	Hemoglobin (g L^{-1})	Hematocrit (L L^{-1})	WBC ($\times 10^3$ mm^{-3})	RBC ($\times 10^6$ mm^{-3})	Hemoglobin (g L^{-1})	Hematocrit (L L^{-1})	WBC ($\times 10^3$ mm^{-3})
AC-0	2.4 ± 0.2	77.3 ± 14.4	0.41 ± 0.06	7.0 ± 1.1[b]	2.3 ± 0.2	111.0 ± 5.0	0.40 ± 0.01	9.8 ± 4.1
AC-10	2.4 ± 0.2	86.3 ± 9.2	0.38 ± 0.02	5.7 ± 1.4[b]	2.4 ± 0.1	110.2 ± 9.0	0.41 ± 0.01	8.9 ± 2.7
AC-20	2.5 ± 0.4	83.7 ± 8.3	0.37 ± 0.02	6.5 ± 1.9[b]	2.3 ± 0.2	114.7 ± 13.5	0.40 ± 0.01	8.7 ± 1.4
AC-30	2.3 ± 0.2	83.0 ± 3.6	0.37 ± 0.03	9.4 ± 2.6[a]	2.2 ± 0.2	116.8 ± 12.1	0.39 ± 0.03	9.8 ± 0.6

Means with different superscripts in each column differed significantly from each other ($P < 0.05$)

RBC red blood cell count, *WBC* white blood cell count

Table 5 Immunological parameters of Nile tilapia fed experimental diets for 4 weeks (mean ± SD, $n = 6$)

Diet	2 weeks			4 weeks		
	Lysozyme (μg mL^{-1})	T-immunoglobulin (g L^{-1})	ACH-50[1] (Units mL^{-1})	Lysozyme (μg mL^{-1})	T-immunoglobulin[a] (g L^{-1})	ACH-50[a] (Units mL^{-1})
AC-0	19.73 ± 0.90	33.7 ± 2.0	31.53 ± 9.47	21.36 ± 0.69	26.2 ± 7.0	41.75 ± 10.63
AC-10	20.57 ± 1.70	29.7 ± 5.9	42.80 ± 13.43	20.38 ± 1.74	31.3 ± 4.8	54.80 ± 4.50
AC-20	20.77 ± 1.25	27.8 ± 2.9	55.99 ± 27.03	21.20 ± 1.27	36.8 ± 11.0	45.22 ± 5.99
AC-30	20.51 ± 1.61	29.9 ± 3.4	55.03 ± 14.55	21.01 ± 0.71	35.3 ± 4.9	37.22 ± 9.72

Means with different superscripts in each column differed significantly from each other ($P < 0.05$)

T-immunoglobulin total immunoglobulin

[a] ACH-50, complementary hemolytic activity

Table 6 Blood chemical parameters of Nile tilapia fed experimental diets for 4 weeks (mean ± SD, $n = 6$)

Blood chemical parameter	Diet (2 weeks)				Diet (4 weeks)			
	AC-0	AC-10	AC-20	AC-30	AC-0	AC-10	AC-20	AC-30
Glucose (mmol L^{-1})	2.87 ± 0.33	2.78 ± 0.16	2.95 ± 0.29	2.96 ± 0.30	2.96 ± 0.28	2.81 ± 0.12	3.11 ± 0.32	3.20 ± 0.30
Cholesterol (mmol L^{-1})	5.01 ± 0.15	4.90 ± 0.20	4.74 ± 0.21	4.75 ± 0.21	5.91 ± 0.11^{a}	5.73 ± 0.28^{a,b}	5.42 ± 0.32^{b}	5.59 ± 0.26^{b}
Triglyceride (mmol L^{-1})	1.53 ± 0.06	1.52 ± 0.05	1.50 ± 0.06	1.51 ± 0.04	1.58 ± 0.06	1.57 ± 0.06	1.54 ± 0.06	1.55 ± 0.03
Total protein (g L^{-1})	41.8 ± 2.3^{a}	40.9 ± 2.8^{a,b}	37.6 ± 1.4^{b}	38.3 ± 3.9^{b}	52.2 ± 3.2	52.5 ± 2.7	52.9 ± 2.7	51.0 ± 2.6
Albumin (g L^{-1})	22.3 ± 1.1	21.9 ± 1.0	20.9 ± 1.2	21.3 ± 1.3	22.9 ± 1.8	23.2 ± 1.3	23.3 ± 1.3	23.0 ± 1.4
BUN (mmol L^{-1})	0.39 ± 0.08	0.42 ± 0.09	0.48 ± 0.12	0.41 ± 0.10	0.50 ± 0.08	0.52 ± 0.07	0.58 ± 0.09	0.63 ± 0.07
T-bilirubin (μmol L^{-1})	3.08 ± 0.68	3.25 ± 0.51	3.59 ± 0.68	3.76 ± 0.68	3.42 ± 1.03	3.76 ± 1.03	4.10 ± 1.03	4.10 ± 1.88
D-bilirubin (μmol L^{-1})	2.05 ± 0.51	2.22 ± 0.68	2.74 ± 1.37	3.08 ± 0.68	2.22 ± 1.71	2.22 ± 0.68	2.57 ± 0.86	2.91 ± 1.03
AP (U L^{-1})	23.42 ± 3.72	23.58 ± 3.71	25.67 ± 2.44	26.00 ± 3.13	26.50 ± 3.48	27.08 ± 2.31	30.00 ± 2.41	30.08 ± 3.26
Amylase (U L^{-1})	58.19 ± 4.13	57.02 ± 3.41	55.89 ± 5.73	54.06 ± 6.05	74.04 ± 1.38	73.3 ± 1.89	73.1 ± 1.69	70.55 ± 3.30
SGOT (U L^{-1})	21.17 ± 3.20	21.50 ± 2.95	22.58 ± 2.08	22.83 ± 1.86	27.13 ± 2.34	28.63 ± 4.71	30.30 ± 4.32	31.97 ± 2.56
SGPT (U L^{-1})	7.92 ± 1.24	8.33 ± 0.98	8.75 ± 1.08	9.58 ± 1.53	8.92 ± 1.53	9.17 ± 2.38	10.50 ± 2.10	11.92 ± 2.27
Calcium (mmol L^{-1})	3.68 ± 0.10^{a}	3.57 ± 0.08^{b}	3.42 ± 0.05^{c}	3.29 ± 0.10^{d}	3.89 ± 0.41	3.71 ± 0.34	3.60 ± 0.50	3.43 ± 0.42
Chloride (mmol L^{-1})	129.08 ± 3.8	130.75 ± 3.54	132.83 ± 4.92	134.92 ± 3.59	129.33 ± 4.45^{b}	129.96 ± 4.29^{b}	133.50 ± 2.24^{a,b}	135.58 ± 2.81^{a}
Iron (μmol L^{-1})	13.29 ± 1.23	1,308 ± 1.51	12.88 ± 1.31	12.25 ± 0.79	14.38 ± 1.05^{a}	13.75 ± 1.77^{a}	12.92 ± 1.29^{a,b}	11.67 ± 1.02^{b}

Means with different superscripts in each column differed significantly from each other ($P < 0.05$)

BUN blood urea nitrogen, *T-bilirubin* total bilirubin, *D-bilirubin* direct bilirubin, *AP* alkaline phosphatase, *SGOT* serum glutamic oxaloacetic transaminase, *SGPT* serum glutamic pyruvic transaminase

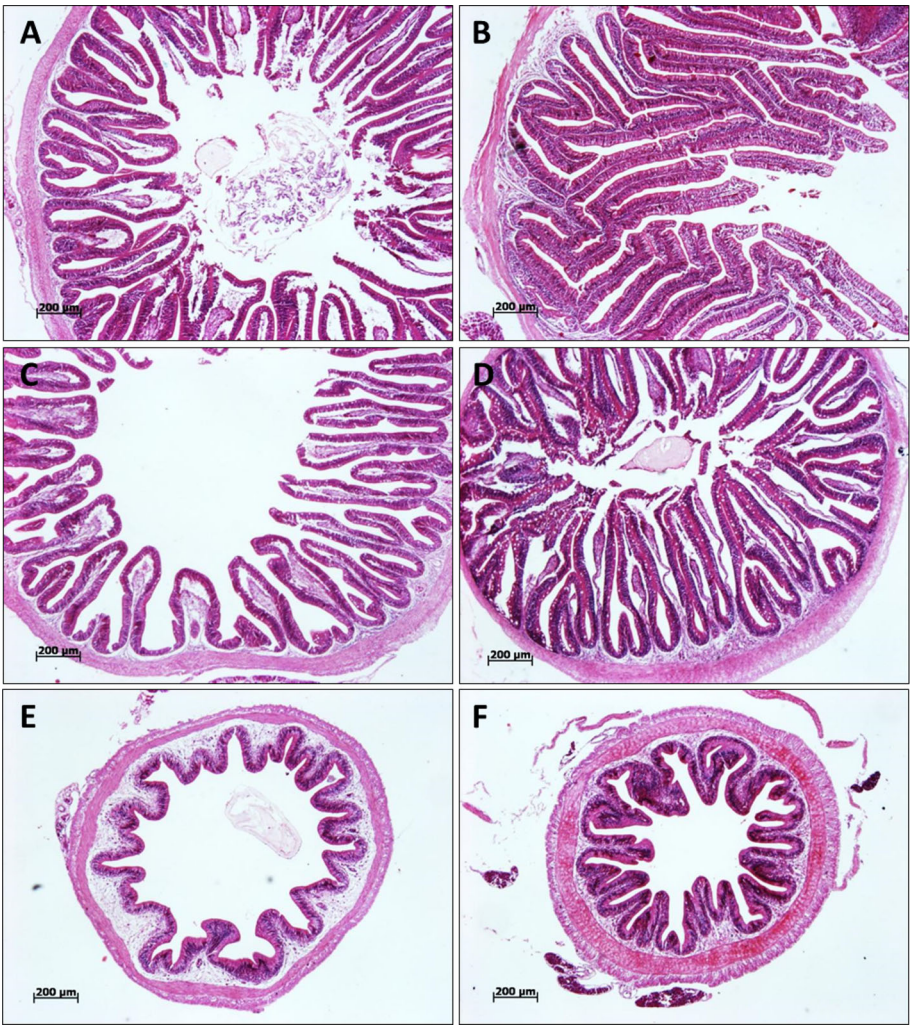

Fig. 1 Morphology of villi obtained from Nile tilapia fed on AC-0 (**a**, **c**, **e**) or AC-10 (**b**, **d**, **f**). Different sections of intestine include the anterior (**a**, **b**), the middle (**c**, **d**) and the posterior (**e**, **f**) parts

Discussion

AC, a non-nutritive substance, has the potential to be used as feed additive due to its detoxifying effects. In pond-reared fish, potential areas of concern for fish production include contamination with harmful substances, such as mycotoxins and/or pesticides in feed ingredients, and an off-flavor. To address the latter concern, AC has been widely used in medical, veterinary and wastewater treatments, as well as in water treatments for aquaculture. So far, studies in the utilization of AC in animal feed have been controversial. For example, while nonsignificant protective effects against fumonisin have been observed in pigs, dietary AC marginally alleviated the growth performance-inhibitory effects of aflatoxin in growing chickens and increased the FI of shrubs in small ruminants (Edrington

Table 7 Intestinal villi height and number of goblet cells in different parts of the intestine of Nile tilapia fed experimental diets for 4 weeks (mean ± SD, $n = 6$)

Diet	Anterior		Middle		Posterior	
	Villi height (μm)	No. of goblet cells	Villi height (μm)	No. of goblet cells	Villi height (μm)	No. of goblet cells
2 weeks						
AC-0	355.0 ± 149.0^{b}	18.5 ± 11.5	241.0 ± 134.6^{b}	25.4 ± 12.9^{a}	202.3 ± 51.7^{b}	18.0 ± 9.7
AC-10	617.3 ± 208.8^{a}	18.9 ± 8.7	423.0 ± 116.7^{a}	28.6 ± 14.1^{a}	304.0 ± 105.8^{a}	26.2 ± 12.2
AC-20	586.3 ± 89.0^{a}	20.5 ± 11.2	434.7 ± 157.7^{a}	29.8 ± 9.0^{a}	306.7 ± 65.9^{a}	28.5 ± 17.4
AC-30	746.7 ± 123.5^{a}	22.2 ± 10.2	466.3 ± 90.3^{a}	40.8 ± 14.5^{b}	341.7 ± 93.0^{a}	26.4 ± 21.6
4 weeks						
AC-0	894.0 ± 112.4^{b}	28.8 ± 18.1^{b}	540.0 ± 117.9^{b}	37.5 ± 26.7^{b}	397.0 ± 143.2	19.2 ± 12.1^{b}
AC-10	1,202.3 ± 135.9^{a}	45.6 ± 19.7^{a}	881.3 ± 97.6^{a}	40.2 ± 25.7^{a,b}	476.0 ± 232.4	30.7 ± 17.1^{a}
AC-20	1,049.7 ± 203.4^{a,b}	41.8 ± 18.4^{a}	710.7 ± 246.3^{a,b}	52.4 ± 24.5^{a,b}	403.0 ± 148.5	28.4 ± 13.0^{a}
AC-30	1,043.0 ± 139.7^{a,b}	44.8 ± 18.6^{a}	502.7 ± 172.1^{b}	56.0 ± 24.3^{a}	406.7 ± 63.4	29.8 ± 16.9^{a}

Means with different superscripts in each column differed significantly from each other ($P < 0.05$)

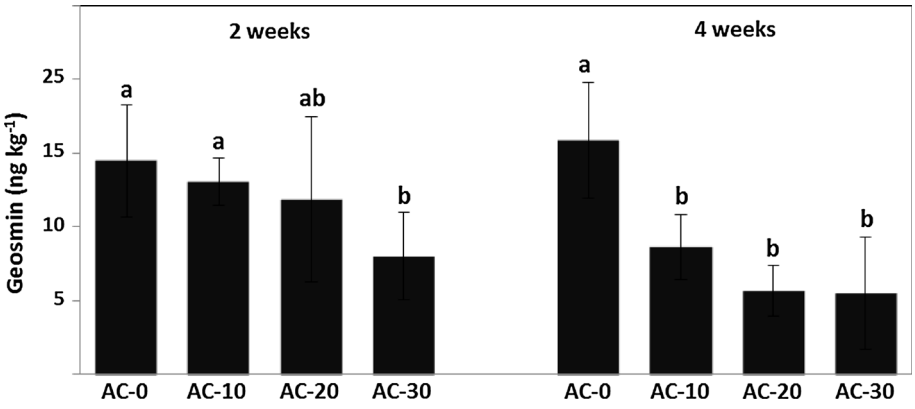

Fig. 2 Geosmin content in the fillets of Nile tilapia fed on activated charcoal (AC) at 2 weeks and 4 weeks. *Different superscript letters* in the *bar graph* indicate significant differences at $P < 0.05$. Note that a nonlinear relationship was predicted for the AC and geosmin contents at week 4 ($y = 0.016x^2 - 0.830x + 15.856$, $R^2 = 0.702$)

et al. 1997; Piva et al. 2005; Rogosic et al. 2006). Because AC is a universal adsorbent, the administration of AC could have both positive and negative influences on the digestive physiology and metabolism. In addition, long-term consumption of AC would induce adverse effect on Nile tilapia due to its non-nutritive and non-digestible substance. In this study, we demonstrated both positive and negative effects of AC supplementation on the growth, health status, chemical composition and geosmin content in fillet and intestinal morphology of Nile tilapia during marketable size (an ideal market size for pond-reared Nile tilapia is generally greater than or equal to 400–500 g).

The ability of AC to absorb a wide range of noxious substances and gases (McFarland and Chyka 1993; American Academy of Clinical Toxicology 1999; Bacaoui et al. 2001) would most likely increase feed efficiency, thereby improving the growth performances of the animals. The present study showed that the growth performance (WG, SGR, FCR) of Nile tilapia at the grow-out stage appeared to be similar in all diets. However, an improvement was observed in fish fed diet at 10 g kg^{-1} AC supplementation level, but differences were not significant because of the high standard deviations observed. In our experience, although the experimental diets were formulated to contain similar levels of nutritive chemical composition, the incorporation of AC at high levels affected the physical characteristics of the extruded diets, including palatability, color and smell. These qualities could influence the fish's appetites. The variable effects of charcoal-supplemented diets on growth performance have been demonstrated in chicken and fish. For example, growth performances (FI, WG, FCR) were linearly improved as the supplementation level of wood charcoal increased from 0 to 100 g kg^{-1} in broiler chicken at 28 days of age; however, the positive effects were limited at the older ages (Kutlu et al. 2001). Charcoal supplementation up to 6 g kg^{-1} could improve growth performances in broilers during the 21st to 49th days of age (Kana et al. 2011). Supplementation with bamboo charcoal at 5 g kg^{-1} significantly improved the growth performances (FE, WG, SGR) of flounder, *Paralichythys olivaceus,* at juvenile stages (Thu et al. 2010). In addition, a mixture of dietary charcoal and wood vinegar at 5 and 10 g kg^{-1} for 8 weeks was shown to increase feed efficiency and WG, respectively, of flounder at later stages (Yoo et al. 2005).

Combining these data, it seems that whether charcoal influences growth performance depends on the supplemental dosage and growth stages of the animals.

The approximate composition of fish body and fish fillet has generally been determined to assess the nutritional status of the fish. Supplementation of AC at the level of 20 and 30 g kg^{-1} effected changes in some proximal composition parameters of Nile tilapia fillet. AC supplementation linearly increased the protein and decreased the water contents in the flesh of Nile tilapia. This finding was similar to the results of Thu et al. (2010) who reported a positive effect of bamboo charcoal supplementation on digestibility, leading to an increase in the protein content of the fish bodies. However, this positive result did not significantly affect moisture, lipid or ash levels in the carcasses of the flounder. In contrast, the mixture of dietary charcoal and wood vinegar did not influence the approximate composition in flounder (Yoo et al. 2005). The reduction in the proportion of moisture in the fillets was most likely caused by an increase in other contents such as protein, fat and ash. Because AC adsorb significant amount of water (Deng 2006), it remains to be further investigated whether dietary AC, particularly at high levels and/or over long periods, may affect the fluid homeostasis processes in fish.

Hematological parameters could be used as indices of the health status of the fish. The present study demonstrated that dietary AC did not significantly affect such hematological parameters as RBC, hemoglobin or hematocrit throughout the experimental period (Table 4). These findings were similar to the results performed in turkeys (Majewska et al. 2009). Because AC is considered a non-specific detoxifier, its use should contribute to improve health conditions. Certain immune parameters, including the white blood cell counts, lysozymes, total immunoglobulin and ACH50, were determined in order to evaluate the effects of supplementation of charcoal on immune status. White blood cell count of Nile tilapia in the present study was lower when compared to the values reported by Phumyu et al. (2012) and Li et al. (2011). The different number of white blood cell number may be due to age- or size-related changes. It seems that white blood cell decreases with fish size increases. It was reported that tilapia exposed to pesticide and mycotoxin led to decrease in white blood cell count (Hart et al. 1997; Kumar et al. 2011; Selim et al. 2013). AC supplementation at high levels (for a short-term feeding period) had positive effects on increasing the white blood cell number. An increment in white blood cell number by dietary AC could be explained by the acute effect of AC as non-specific detoxifier when the diet contained a high level of AC. In addition, in Nile tilapia, diet contaminated with aflatoxin led to decrease in white blood cell number, and mycotoxin adsorbent showed to increase number of white blood cell (Selim et al. 2013). Dietary AC did not significantly influence the tested humoral immune parameters. In general, fish immune system comprises of complex network of circulating cells, organs and physiological systems. Therefore, further investigation is required to determine whether dietary AC has any effects on other immune parameters in order to clearly understand the effect of dietary AC on fish health.

Blood chemicals parameters measured as health indices of Nile tilapia were presented in Table 6. Supplementation with bamboo charcoal was reported to increase protein digestibility (Thu et al. 2010). Because AC is considered a universal adsorbent, it also has the potential to adsorb many nutrients. Plasma protein was consequently manifested in the effect of AC supplementation on nutrition status, immune and liver function in fish. The present result showed that although supplementation with AC $\geq$20 g kg^{-1} (2 weeks) had significantly negative effects on plasma proteins, its values are in the reference interval for tilapia (Hrubec et al. 2000). Additionally, the plasma protein levels after 4 weeks of experimentation appeared to be similar among the experimental groups. These evidences

suggested that dietary AC had no obviously adverse effects on protein assimilation. The present results showed that supplementation of AC $\geq$20 g kg^{-1} influenced blood cholesterol levels. Supplementation with a mixture of charcoal and wood vinegar was demonstrated to alter fatty acid profiles and to decrease saturated fatty acids in flounder (Yoo et al. 2005). Moreover, AC interfered with the enterohepatic circulation of bile acids and cholesterol; therefore, ingestion of AC was demonstrated to reduce serum cholesterol in patients with hypercholesterolemia (Neuvonen et al. 1989). The present result demonstrated that dietary AC had effects on the mineral content of blood, including calcium, chloride and iron. The absorptive properties of AC with mineral (Olsen 2010) and the release mineral content would affect plasma electrolyte in fish bodies.

Mycotoxins were demonstrated to affect the morphology of intestine by a decrease in villi height in pig and chicken (Awad et al. 2006; Kolf-Clauw et al. 2009). Intestinal integrity affects the physiological metabolism of nutrient absorption. Dietary AC contributes to the excretion and elimination of pathogens, toxins and noxious gases, and thereby, it is considered a gastrointestinal decontamination agent. Therefore, AC would have positive effect on intestinal morphology. Nevertheless, medical case studies have reported adverse effects from the administration of AC on gastrointestinal obstruction (Watson et al. 1986; Atkinson et al. 1992; Green and McCauley 2006). The present study demonstrated that AC supplementation significantly increases the villus height in all parts (for 2 weeks) and in the anterior and the middle parts (for 4 weeks) of intestines. These results were similar to the results of studies in piglets, which involved supplementation with charcoal powders and wood vinegar compound liquids (Mekbungwan et al. 2004). A positive correlation between villus height and growth performance was reported in Nile tilapia (Vechklang et al. 2011; Phumyu et al. 2012), and this positive correlation was not observed in the present study. Due to its non-specific adsorptive capacity, AC most likely binds to and excretes certain essential nutrients. Thus, this study suggests that AC has positive effects on the intestinal integrity and negative effects on metabolism of nutrient absorption, and thereby, dietary AC had nonsignificantly positive effects on growth performance. Goblet cells play an essential role in the synthesis and secretion of mucin into the mucus layer, destroying pathogens (Blomberg et al. 1993). Mycotoxins were also revealed to reduce number of goblet cells in pig (Bracarense et al. 2012). The present study demonstrated that AC supplementation led to increases in the goblet cell counts. The AC adsorption of pathogen and/or noxious substances in the intestine leads to increases in the number of goblet cells along the intestinal villi.

Musty-earthy odors, which are primarily caused by *trans*-1,10-dimethyl-*trans*-9-decalol (geosmin) and 2-methylisoborneol (MIB), have become a problem not only in potable water resources but also in pond-based farming systems. Tilapia collected from commercially freshwater and brackish ponds which were revealed to contain geosmin at 1.56–9.85 μg kg^{-1} (Yamprayoon and Noomhorm 2000). Compared to the previous report, lower geosmin concentration in Nile tilapia in the present study might be due to the different in pond condition and culture system. For instance, comparing to fish ingest algae, Nile tilapia in this study was fed practical diet ad libitum which resulted in low geosmin content in flesh. Several types of AC, including granular, powdered and superfine powdered AC, have been demonstrated to be suitable for use in the removal of geosmin and MIB from drinking water (Ridal et al. 2001; Cook et al. 2001; Matsui et al. 2013). It is impractical to remove both of these earthy-odor substances from the water in fish ponds. Fish primarily adsorb geosmin through their gills and intestines via the ingestion of algae and bacteria on the pond bottom, and the highest geosmin content was found in the intestine (Yamprayoon and Noomhorm 2000). Our results showed that AC

supplementation for 4 weeks led to a significant decrease in the geosmin content of fish fillet, following a quadratic response, while MIB was not detected. It was revealed that reduction (96–98 %) in geosmin from Nile tilapia flesh took 16 days when fish were reared in aerated clean water without geosmin (Yamprayoon and Noomhorm 2000). The present study provides a method to reduce off-flavors in fish fillet using dietary AC at 30 mg kg^{-1} for 2 weeks or at 10 mg kg^{-1} for 4 weeks caused by geosmin.

In conclusion, this study demonstrated that AC supplementation at low levels (10 g kg^{-1}) for 4 weeks prior to harvesting did not have any detrimental effects on the health status of Nile tilapia. Our findings provide evidence of the effect of the use of AC as feed additive and of the potential benefits of utilizing AC to reduce off-flavors in pond-based tilapia production.

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