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RESEARCH PAPER

Impact of Partial Substitution of Soybean Meal by Cottonseed Meal with Iron Sulfate Supplementation in Nile Tilapia Diet

Sara A. Gad[1], Doaa IA Mostafa[1,*], Hend M. Megahed[2], Mohamed A. Bakry[3], Abd-Allah A. Mokhbatly[4], Rehab E. Mowafy[5]

[1]Department of Clinical Pathology, Animal Health Research Institute (AHRI), Zagazig branch, Agriculture Research Centre (ARC), Egypt.
[2]Department of Biochemistry, Animal Health Research Institute (AHRI) Zagazig branch, Agriculture Research center (ARC), Egypt.
[3]Department of Fish health, Animal Health Research Institute (AHRI) Zagazig branch, Agriculture Research center (ARC), Egypt.
[4]Department of Clinical Pathology, Faculty of Veterinary Medicine, kafr El-Sheikh University, Egypt.
[5]Department of Pathology, Animal Health Research Institute (AHRI) Zagazig branch, Agriculture Research center (ARC), Egypt.

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Corresponding Author
E-mail: mohamedabakry79@yahoo.com

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Abstract

Aquatic meals participate in food security and nutrition. The economic dimension greatly influences the sustainability of the aquaculture industry. This study aimed to assess the effects of partial substitution of soybean meal (SBM) with cottonseed meal (CSM) supplemented with ferrous sulfate (FS) on the growth performance and health status of Nile tilapia. One hundred and fifty Nile tilapia (*Oreochromis niloticus*) were split equally into five group (triplicated). Each group was fed one of the following isonitrogenous isolipid diet; diet1 (0% CSM), diet2 (20% CSM), Diets3 (20% CSM with 580 mg/kg (FS), diet4 (50% CSM), and diet5 (50% CSM with 580 mg/kg FS) of total protein for two months. In comparison to the control group, Statistically significant ($p<0.05$) improvements in growth performance, hemogram, and leukogram, as well as, the levels of ALT, AST, urea, creatinine, total protein, MDA, and GSH-px were observed in the third treatment . Histopathologically, it showed the highest values of villus height, width, and absorption surface area (ASA) with normal architecture and cellular details. On the other hand, fish fed diet4 had the lowest growth rat with microcytic normochromic anemia and decreased villus height, width and ASA. The groups that fed CSM showed lower cholesterol levels. In conclusion, CSM inclusion with FS supplementation enhanced growth and health parameters.

Introduction

Aquatic meals are increasingly recognized for their participation in food security and nutrition, not only for their high protein content but also as a unique source for essential omega-3, fatty acids, and bioavailable micronutrients (FAO, 2022). The economic dimension greatly influences the sustainability of the aquaculture industry, where feed inputs cost approximately 40–75% of total aquaculture production expenses (FAO, 2018).

The worldwide aquaculture industry needs ways to reduce the costs and increase the quality of feed to face elevated demand and inadequate supply. Fish nutritionists have concentrated their efforts to catch alternative sources of proteins as substitutes for fishmeal. Among those sources, plant proteins appear to be the most applicable due to their accessibility, safety to the environment, sustainability, and prices (Abdel-Latif et al., 2022).

Cottonseed meal (CSM) is the byproduct of oil extraction from cotton seeds. It is considered the third largest oil-seed meal in the world, after soybean meal (SBM) and rapeseed meal (Li et al., 2023). CSM is categorized next to SBM in Egypt as CSM offers low cost in comparison to fishmeal and SBM per unit protein basis (Gaber et al., 2012). In CSM, the protein and lipid constitute nearly 40% and 5% respectively. Also, it represents a valuable source of arginine for oceanic animals in comparison with fish meal and SBM (El-Saidy and Gaber, 2004).

Herbivorous fish are more efficient in utilizing plant protein than omnivorous and carnivorous fishes. However, the inclusion of CSM in fish diets doesn't rely only on the species, but also, on the concentration of gossypol, and lysine possibility. CSM has long been used as an origin of plant protein in the feeding of terrestrial and aquatic animals (Li and Robinson, 2006; Lim and Lee, 2008). Several studies recorded the permissible level of CSM in Nile tilapia diets, which did not alter their growth performance (Mbahinzireki et al., 2001). Some of these studies mentioned that 40%–50% dietary CSM plus amino acid addition was applicable (El-Saidy and Gaber, 2004; Li and Robinson, 2006).

The presence of high amounts of free gossypol in the diet was discovered to be poisonous to monogastric animals, including fish (Zhang et al., 2022). Gossypol can bind covalently to lysine and arginine (Wang et al., 2021), chelate ferrous ion (Prescott et al., 2018), and stimulate the eryptosis of erythrocytes (Zbidah et al., 2012). High levels of cottonseed protein may have deleterious effects on some hematobiochemical parameters and histomorphometric measurements, resulting in reduced villus height and width in the distal intestine (Liu et al., 2022) with damage to the intestinal fold epithelium and increased lymphocyte infiltration and goblet cell malformation (Haokun et al., 2016).

Several attempts were previously studied to reduce the toxic effects of gossypol, such as, fermentation (Zhang et al., 2022), pelleting (Barraza et al., 1991), heating (Xu et al., 2022), and the addition of calcium hydroxide (Kannan et al., 2013) and ferrous sulfate (FS). Iron in the form of FS has been effectively used to ameliorate the toxic effects of free gossypol which present in CSM in the diets of monogastric and terrestrial animals (Martin, 1990). The ferrous ion combine to the active sit of the free gossypol rendering it denatured (inactive) (Barraza et al., 1991). It was hypothesized that the addition FS as a source for iron is a simple, efficient, and cost-effective method to inactivate the free gossypol and so, increases the availability of iron and lysine for the normal physiological function. This study hypothesizes that CSM supplemented with FS will mitigate the negative effects of gossypol and improve growth performance and health status in Nile tilapia.

Our goal was to assess the effects of partial substitution of SBM with graded levels of CSM with and without supplementation of FS on growth performance, hematobiochemical, histomorphometric parameters, and histopathological findings in Nile tilapia *(Oreochromis niloticus).*

Materials and Methods

Fish

One hundred and fifty seemingly healthy male Nile tilapia fingerlings weighing 30±3 g were collected from an earthen fish farm at the Central Laboratory for Aquaculture Research, Abassa, Sharkia province, Egypt. To justify the sample size, the power analysis was calculated by G*Power 3.1.9.7 software with an alpha of 0.05 and the total sample size of 150 while the effect size was 0.4. The generated power was 0.99. They were delivered alive in polyethylene bags filled with water to wet Lab. at Animal Health Research Institute, Zagazig branch. Fish were acclimatized for two weeks in glass aquaria filled with dechlorinated tap water measuring 100 × 40 × 40 cm (100 effective liter each). The aquaria were aerated using an electric air pump, and the temperature was adjusted by heaters at 26°C with a natural photoperiod. The water variables, including dissolved oxygen, pH, ammonia, nitrate, and nitrite concentrations, were maintained at 6.5±0.5, 7.1±0.8, 0.003, 0.31 and 0.42 mg L^{-1}, respectively. A basal diet (30% protein) was offered twice daily at 3% of body weight through the adaptation period.

Experimental Diet

Five 30% protein diets were prepared: diet1 (basal diet), diet2 (CSM represented 20% of total protein), diet3 (20% CSM supplemented with 580 mg/kg FS), diet4 (CSM represented 50% of total protein), and diet5 (50% CSM supplemented with 580 mg/kg FS). The inclusion of CSM was in the place of SBM. The dose of FS was selected after Gaber et al. (2012). All dry component were finely ground and mixed mechanically by a feed mixer. The vitamin premix, mineral premix were added to the oil sources then added to the dry mix of feed ingredients and thoroughly mixed. For each one kg of diet, 150 ml warm distilled water was added to the mixer to produce moist dough. FS was added to the distilled water of diet3&5. The dough was pelleted by pelleting machine with 2mm diameter pore. The produced pellet was dried in a ventilated oven at 40°C until the moisture below 100 g/kg, and all diets were stored at -20°C until use. Table 1 shows the contents of the prepared diets and their chemical analysis (in triplicate) following AOAC (1990) guidelines. Crude protein and crude lipid were estimated using the micro-Kjeldahl method and soxhlet apparatus, respectively. Crude fiber was determined following AOAC 962.09. Ash content was measured by burning 100g of diet at 550^{o}C for 3 hours. Dry matter was measured by placing 100g diet in an oven at 105°C until achieving a constant weight.

Experimental Design

Fish were allocated into 5 groups (30 fish/group in three replicates), and the fish weight was recorded individually. Group1 (G1), group2 (G2), group3 (G3), group4 (G4) and group5 (G5) were fed diet 1, diet 2, diet 3, diet 4 and diet 5 respectively, for 60 days. The feeding regime during the experimental period was the same as the adaptation period. One hour after feeding, the uneaten feed was syphoned from each aquarium and

Table 1. Experimental diet composition per 100 g

Ingredients (g)	Diet1	Diet2	Diet3	Diet4	Diet5
ground corn	20.3	20.3	20.3	20.3	20.3
Fish meal (70 % CP)	10	10	10	10	10
Soybean meal (44 % CP)	43	30	30	11	11
Cottonseed meal (40 %CP)	0	15	15	37.5	37.5
Wheat bran	15.5	13.5	13.5	10.0	10.0
Starch	4	4	4	4	4
Corn oil	1.9	1.9	1.9	1.9	1.9
Cod fish oil	2.3	2.3	2.3	2.3	2.3
Minerals Premix[2]	2	2	2	2	2
Vitamins premix[1]	1	1	1	1	1
Ferrous sulfate	0	0	0.058	0	0.058
Total	100	100	100	100	100
Chemical composition					
Dry matter	91.1	91.1	91.1	91.1	91.1
Crude protein	30	30	30	30	30
Crude lipids	10.8	10.9	10.9	11	11
Crude fiber	5.4	6.5	6.5	8	8
Total ash	8.2	8.40	8.45	8.50	8.55
Nitrogen free extract	36.7	35.3	35.3	33.5	33.5
Gross energy (Kcal/100 g)	420.8	417.5	417.5	411.13	411.13

[1]Vitamin premix (per kg of premix): thiamine, 2.5 g; riboflavin, 2.5 g; pyridoxine, 2.0 g; inositol, 100.0 g; biotin, 0.3 g; pantothenic acid,100.0 g; folic acid, 0.75 g; para-aminobenzoic acid, 2.5 g; choline, 200.0 g; nicotinic acid, 10.0 g; cyanocobalamine, 0.005 g; α-tocopherol acetate, 20.1 g; menadione, 2.0 g; retinol palmitate, 100,000 IU; cholecalciferol, 500,000 IU

[2]Mineral premix (per kg of premix): $CaHPO_4.2H_2O$, 727.2 g;$MgCO_3.7H_2O$, 127.5 g; KCl 50.0 g; NaCl, 60.0 g; $FeC_6H_5O_7.3H_2O$, 25.0 g; $ZnCO_3$, 5.5 g;$MnCl_2.4H_2O$, 2.5 g; $CuCl_2$, 0.785 g; $CoCl_3..6H_2O$, 0.477 g; $CaIO_3.6H_2O$, 0.295 g; $CrCl_3.6H_2O$, 0.128 g; $AlCl_3.6H_2O$, 0.54 g;Na_2SeO_3, 0.3 g

dried in oven then its weight was recorded. The feed intake was calculated by subtracting uneaten feed weight from the daily added feed.

Growth Performance and Feed Utilization Indices

At the termination of the trial, the final fish weight was recorded, and the feed intake (FI) was calculated. Growth performance parameters were calculated as followings:

Weight gain (WG) (g) = final body weight (g) − initial body weight (g)

Feed conversion ratio (FCR) = feed intake (g)/ weight gain (g)

Specific growth rate (SGR%) = 100 × (final weight (g) – initial weight (g) / days of the trial)

Blood Sampling

By the end of the experiment, after 24 hour of fasting, 10 fish from each group were picked and anesthetized with buffered tricane-methane sulfonate (E10521-10G; Sigma–Aldrich) (100 mg/L; for 5 min. (Coyle et al., 2004). Blood specimens were collected from the caudal blood vessels into a- 1-mL sterile syringe containing 0.2 mL of EDTA for estimation of hematological parameters and b- dry clean centrifuge tubes for serum separation then left for 15 min. at room temperature then centrifuged at 3000 rpm for 10 min. The supernatant serum samples were stored at -20°C for subsequent biochemical investigations.

Hematological Assay

Total erythrocytic and leukocytic counts were applied by an improved Neubauer hemocytometer with Natt and Herrick solution as mentioned by Harrison and Harrison (1986). Packed cell volume (PCV) was calculated by a micro hematocrit (Ht) centrifuge as described by Coles (1986). Hemoglobin (Hb) levels were estimated using the cyanomethemoglobin colorimetric technique as recorded by Van Kampen and Zijlstra (1983). The mean corpuscular volume (MCV) and mean corpuscular hemoglobin concentration (MCHC) were computed. Blood films were prepared, fixed with methyl alcohol, and then stained with Giemsa to calculate the differential leukocytic count as previously described (Feldman et al., 2000)

Biochemical Assays

Total protein was determined by the refractometry method according to (Doumas et al., 1981), albumin values were investigated according to (Doumas et al., 1972), and a mathematical formula was used to determine the serum globulin by subtracting the albumin from the total proteins. Total cholesterol and triglycerides were estimated calorimetrically by commercial kits from Stanbio Company, USA, using the computerized spectrophotometer model Milton Roy 1201. The aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activity were determined

according to (Young, 2001), serum urea was estimated according to (Young, 1995), and serum creatinine was estimated according to (Henry, 1974). Antioxidant biomarkers, malondialdehyde (MDA) was calculated as described by (Satoh, 1987) and glutathione peroxidase (GSH-Px) as described by Miller and Slebodzinska (1993).

Histopathological Investigations

After sampling of the blood, the fish were sacrificed and dissected then tissue samples from the middle part of the small intestine, liver, and kidney were collected, fixed in 10% buffer formalin solution, processed with a paraffin embedding technique, and stained with hematoxylin and eosin (Survarna et al., 2013).

Histomorphometric Measurements

Some parameters of the middle part of the small intestine from previously captured H&E-stained sections were analyzed. Five villus crypt units with intact lamina propria were utilized for all investigations and morphological measurements (Ashraf et al., 2013) with the help of captured photomicrographs (OMAX 40X-2000X) (40X). Images were analyzed using image analysis software (Image J 1.46r, java 1.6.0_20) (64-bit.428 commands, 58 macros). The variables examined for intestinal segments were villus height, width, and absorptive surface area (ASA) of the intestine. ASA was computed using the formula (2π) (VW/2) (VH), where VW represents villus width and VH represents villus height (De Los Santos et al., 2007).

Statistical Analysis

Statistical analysis was applied by analysis of variance (ANOVA). Duncan's Multiple Range (Duncan, 1955) was utilized to detect differences between treatments, mean at a significance level of 0.05. All statistics were analyzed using the SPSS program (SPSS, 2004).

Results

Growth Performance and Feed Utilization Indices

Data explored in Table 2 indicated that WG and SGR of the control group were significantly lower at ($p \leq 0.05$), than the third treatment and were insignificantly lower at ($p \leq 0.05$), than G2. On the other hand, these parameters in the control group were significantly ($p \leq 0.05$), higher than the G4 they were insignificantly ($p \leq 0.05$), higher than G5. Consequently, the elevation of FCR in G1 was significant ($p \leq 0.05$), over G3 but insignificant ($p \leq 0.05$) over G2. The decrement of FCR in G1 was significant ($p \leq 0.05$), in comparison to G4 and insignificant ($p \leq 0.05$), when compared to G5.

The Hematological Studies

Table 3 shows that RBCs count, Hb content, and Ht values were significantly decreased ($p \leq 0.05$) in G4 fed a diet containing 50% CSM compared to the control group with normal MCV and normal MCHC. The fish of G2, which were fed a diet containing 20% CSM in addition to groups 3 and 5, which were fed diets containing CSM supplemented with FS, showed nonsignificant changes in RBCs, Hb, and Ht values compared to the control group.

Leukogram

Regarding the leukogram, Table 4 shows immunosuppression represented by a significantly ($p \leq 0.05$) decreased total leukocytic count and

Table 2. Growth Performance indices of tested groups

Groups parameters	Group1	Group2	Group3	Group4	Group5
Initial weight (g)	37.33±2.50	37.67±2.08	37.07±2.65	37.50±1.50	37.53±1.86
Final weight (g)	68.78±3.96^{b}	70.24±2.30^{b}	76.13±2.97^{a}	63.10±2.79^{c}	67.29± 2.92^{b}
Weight gain (g)	31.53±1.45^{b}	32.57±0.93^{b}	39.13±0.32^{a}	25.7±1.48^{c}	29.76±1.71^{b}
FCR	2.22±0.11^{bc}	2.15±0.06^{c}	1.79±0.02^{d}	2.73±0.17^{a}	2.36±0.14^{b}
SGR	52.56±2.42^{b}	54.29±1.55^{b}	65.22±0.54^{a}	42.83±2.47^{d}	49.60±2.86^{c}

In each row, values followed by different letters are significantly different at ($p \leq 0.05$), FCR = Feed conversion ratio, SGR = Specific growth rate

Table 3. Hemogram of tested groups(n=5)

Groups Parameters	Group1	Group2	Group3	Group4	Group5
RBCs(×10^{6}cells/ul)	3.45±0.15^{a}	3.32±0.04^{ab}	3.7±0.16^{a}	2.17±0.08^{c}	3.68±0.08^{a}
HCB gm%	10.48±0.8^{ab}	11.43±0.98^{ab}	12.34±0.24^{a}	6.68±0.50^{c}	11.05±0.35^{ab}
HCT %	30.36±1.42^{a}	32.49±1.57^{a}	33.24±2.05^{a}	19.38±1.7^{b}	33.58±0.67^{a}
MCV *fl*	88.44±4.02^{b}	98.26±1.06^{ab}	91.45±2.28^{ab}	88.99±4.25^{b}	91.27±0.27^{a}
MCHC %	34.45±1.37	34.72±1.27	36.71±1.42	34.53±0.41	32.91±0.85
PLT(×10^{3}cells/ul)	109.3±3.83^{a}	116.67±4.64^{a}	109.67±3.64^{a}	89.84±5.64^{b}	120.0±4.84^{a}

Values in each row that are followed by different letters are significantly different at ($p \leq 0.05$) n: number of samples; RBCs: Red blood cell count; PCV: Packed cell volume; MCV: Mean corpuscular volume; MCHC: Mean corpuscular hemoglobin concentration; PLT: total platelets count.

heterophilic and lymphocytic counts, in addition to nonsignificantly altered basophilic and eosinophilic counts in G4-fed diet containing CSM 50%. Nonsignificant decrease in total and differential leukocytic counts in G2 fed a diet containing 20% CSM. G3 and G5 showed nonsignificant increases in total and differential leukocytic counts.

The Biochemical Parameters

The present results showed significant increase in liver enzymes (AST and ALT) as well as urea and creatinine concentrations in G4 compared to G1 ($p<0.05$).Also, lipid peroxidase biomarker (MDA) significantly induces, while the activity of GSH-Px significantly diminished in aforementioned treated group compared to the control and the other treated groups ($p<0.05$). Cholesterol concentration significantly decreased as response to the treatment. However the treatment had not significant effects on the levels of triglycerides. A significant decline ($p\leq0.05$) was documented in total protein and albumen levels in fish of G4 compared to those of G1. (Table 5).

Histo-morphometric Measurements Findings

The highest value of surface absorption area was among fish in G2 and G3, while G4 exhibited the lowest value. Villus height and width showed the greatest values in fishes in G2, followed by G3 in the case of villus height and G1 in the case of villus width, while the smallest values of villus height and width were recorded in fish in G4 and G5. Full data were recorded and are shown in Table 6 in addition to the demonstration in Figure 1.

Histopathological Findings

There were no macroscopic changes observed in dissected fish in any of the experimental groups, while the collected liver, kidney and intestine tissues exhibited certain differences among the groups. No abnormalities were detected in the fish of G1. Most liver lesions were observed in G4 fish, which were focal to diffuse degeneration of hepatocytes with vacuolation and sinusoidal congestion (Figure 2a). Some fish revealed perivascular edema and fibrosis in addition to periductal cellular infiltration with inflammatory mononuclear cells (Figure 2b).

Table 4. Leukogram of tested groups (n=5)

Groups Parameters	Group1	Group2	Group3	Group4	Group5
WBCs (×10^{3}cells/ul)	9.73±0.16^{ab}	9.3±1.33^{ab}	10.56±1.5^{a}	8.29±0.65^{b}	10.44±0.96^{a}
Heteropils (×10^{3}cells/ul)	5.32±0.05^{a}	4.43±0.2^{ab}	5.2±0.18^{a}	4.13±0.51^{b}	5.91±0.58^{a}
Eosinophiles (×10^{3}cells/ul)	0.02±0.002	0.05±0.02	0.03±0.001	0.02±0.005	0.02±0.002
Basophiles (×10^{3}cells/ul)	0.03±0.004	0.13±0.01	0.04±.003	0.1±.007	0.04±0.007
Lymphocytes (×10^{3}cells/ul)	4.13±0.12^{a}	4.38±0.19^{a}	4.87±0.01^{a}	3.78±0.2^{b}	4.20±0.08^{a}
Monocytes (×10^{3}cells/ul)	0.29±0.15^{bc}	0.31±0.03^{b}	0.42±0.005^{a}	0.26±0.08^{c}	0.35±0.026^{b}

Values in each row that are followed by different letters are significantly different at ($p\leq0.05$) n: number of samples; WBCs: white blood cell count.

Table 5. Biochemical and antioxidant variables of tested groups (n=5)

Groups Parameters	Group1	Group 2	Group 3	Group4	Group 5
AST(U/L)	27.46±1.18^{b}	26.88±1.31^{b}	27.13±1.65^{b}	37.11±0.90 ^{a}	26.89±0.92^{b}
ALT(U/L)	24.6±0.99 ^{c}	23.92±0.87^{c}	22.84±0.54 ^{c}	31.41±0.68 ^{a}	27.01±0.85^{b}
Urea (mg/dL)	4.71±0.29^{b}	4.77±0.20^{b}	4.96±0.13^{b}	6.1±0.11^{a}	5.18±0.16 ^{b}
Creatinine(mg/dL)	0.59±0.01^{b}	0.62±0.04^{b}	0.55±0.05^{b}	1.27±0.07^{a}	0.68±0.02 ^{b}
MDA (nmol/mL)	5.82±0.19^{b}	5.71±0.24^{b}	5.55±0.35^{b}	7.04±0.16^{a}	6.27±0.20^{ab}
GSH-Px (U/ml)	6.47±0.21^{a}	6.92±0.11^{a}	6.82±0.22 ^{a}	5.73±0.30 ^{b}	6.32±0.13^{ab}
CHO (nmol/mL)	134.67±2.7^{a}	95.16±2.11^{bc}	98.83±2.28^{b}	84.18±1.31^{d}	89.13±2.3^{cd}
TG (nmol/mL)	28.32±0.57^{a}	26.66±0.78 ^{a}	27.54±0.79^{a}	26.68±0.34^{a}	27.77±0.20^{a}
T. protein (g/dl)	6.4±0.05^{ab}	6.43±0.05^{ab}	6.52±0.06^{a}	5.61±0.15^{c}	6.08±0.08^{b}
Albumin (g/dl)	3.67±0.09^{a}	3.36±0.03^{ab}	3.65±0.07^{a}	2.76±0.05^{c}	3.26±0.19^{b}
Globulin (g/dl)	2.73±0.12^{a}	3.07±0.24^{a}	2.87±0.09^{a}	2.85±0.12^{a}	2.82±0.09^{a}
A/G Ratio (g/dl)	1.36±0.09^{a}	1.09±0.03^{bc}	1.27±0.06^{ab}	0.90±0.03^{c}	1.18±0.07^{ab}

Group with different letters within the same column are significantly different at ($p\leq0.05$) AST: aspartate aminotransferase; ALT: alanine aminotransferase; MDA: Malondialdehyde; GPX: Glutathione peroxidase; CHO: Cholesterol; TG: Triglycerides; T. protein: total protein.

Table 6. Histomorphometeric measurements of some mid intestine parameters in experimental groups: (n=5)

Groups parameters	Group1	Group2	Group3	Group4	Group5
Villus high (VH) (µm)	344.45±16.25^{c}	563.36±25.3^{a}	436.60±18.3^{b}	195.26±9.29 ^{e}	255.02±13.6^{d}
Villus width (VW) (µm)	98.69±3.64^{b}	112.91±2.61^{a}	80.22±3.93^{c}	76.93±3.48^{c}	75.38±2.39 ^{c}
Absorption Surface Area (ASA)(mm)	106.80±6.45 ^{b}	199.12±4.83^{a}	109.85±5.88^{b}	47.44±8.67^{c}	60.48±4.09^{c}

Means ± standard error with different letters at the same column were significant at $p\leq0.05$

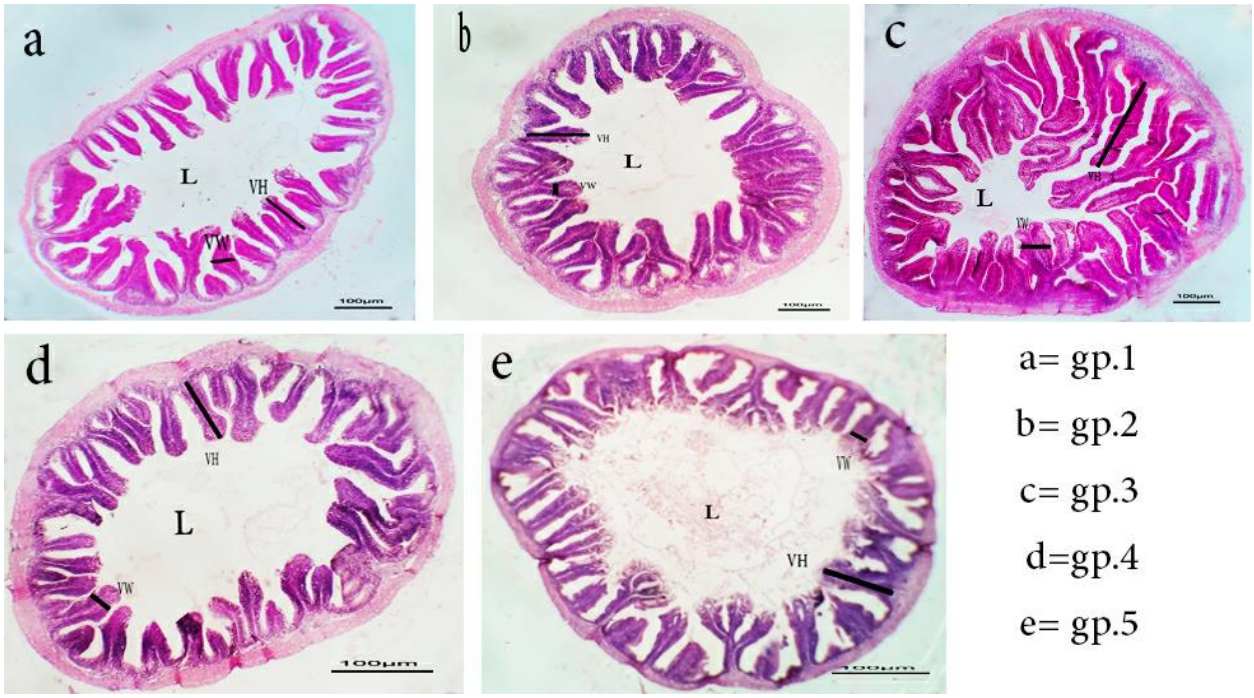

Figure 1. Photomicrograph of some mid intestine sections analyzed for histomorphometeric measurements: VH (Villus height) VW (villus width) L (lumen) in different experimental groups.

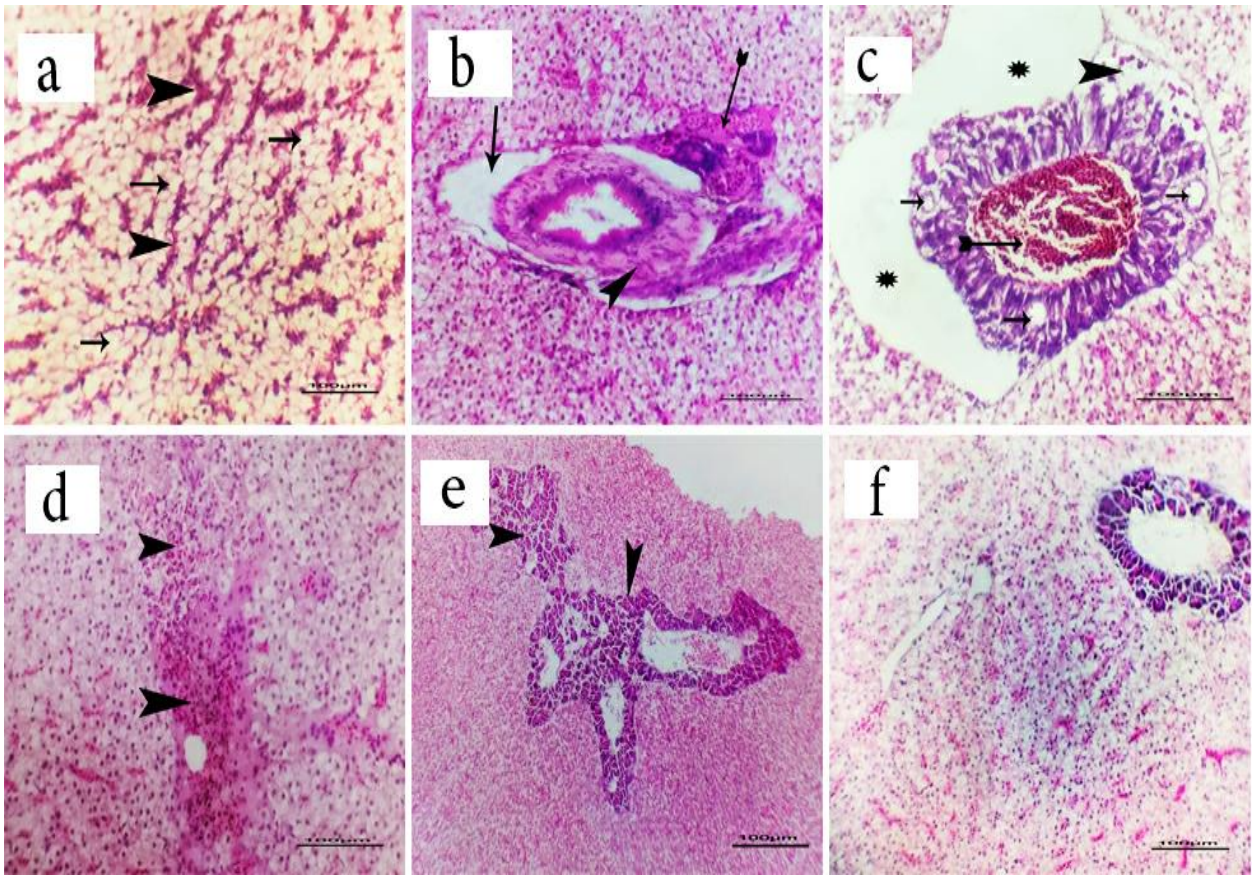

Figure 2. Photomicrograph of H&E stained liver sections of tested G s at the end of experiment showing. a) liver (gp.4) with diffuse degeneration of hepatocytes with vacuolation (arrows) and sinusoidal congestion (arrows head). b) liver (gp.4) with perivascular edema(arrow) and fibrosis (arrowhead) and periductal cellular infiltration with mononuclear cells (tailed arrow). c) liver (gp.4) with vacuolation (arrows) to partial necrosis of hepatopancreas (arrowhead) in addition to sever vascular congestion (tailed arrow) and perivascular edema (stars). d) liver (gp .5) with focal perivascular inflammatory cells infiltration (arrowhead) e) liver (gp .3) with hyperplasia of hepatopancreatic tissue f) liver (gp.2) with apparently normal architecture and cellular details. (scale bar=100µm)

Vacuolation to partial necrosis of hepatopancreatic tissue with severe congestion and perivascular edema was also observed (Figure 2c). Hepatic tissue of G5 showed focal perivascular inflammatory cell infiltration (Figure 2d) with mild inflammation, while in G3, fish suffered hyperplasia of the hepatopancreas (Figure 2e). Most fish in G2 appeared to have normal architecture and cellular details (Figure 2f). Renal tissue exhibited the most changes among fish in G5, which demonstrated renal medulla with intratubular eosinophilic cast formation, which was the characteristic lesion seen in all fish, and intertubular mononuclear cell infiltration (Figure 3a). The renal cortex of the same group appeared to have focal degeneration of renal tubules and intratubular eosinophilic casts in addition to cloudy swelling of some renal tubules (Figure 3b), while the renal cortex of G4 showed perivascular edema and fibrosis with vascular congestion (Figure 3c). Mild to moderate changes were exhibited in the renal medulla of G3, represented by moderate inter tubular mononuclear inflammatory cell infiltration and mild cloudy swelling of some renal tubules (Figure 3d) in some cases, while other cases were normal. The mid intestine of G5 was the most affected among the other experimental groups and showed focal to diffuse hyperplasia of submucosal cells, which sometimes led to discontinuity of the layers muscularis mucosa and serosa (Figure 3e). Other cases in the same group demonstrated the mid intestine with hypoplasia of submucosal tissue (Figure 3f). The G4 fish mid intestine showed mucinous degeneration or metaplasia of enterocytes to goblet cells in addition to focal submucosal hypoplasia (Figure 4a). The mid intestine of G3 revealed multibranched villi formation (Figure 4b), while G2 had fusion of some intestinal villi and mild congestion of submucosal blood vessels (Figure 4c).

Discussion

Growth Performance and Feed Utilization Indices

Fish meal is an excellent source of protein but with high cost so, the economic plant protein is used, especially SBM & CSM. However, there are antinutritional factors in SBM, such as phytic acid, which lowers zinc availability and protein digestibility. On the

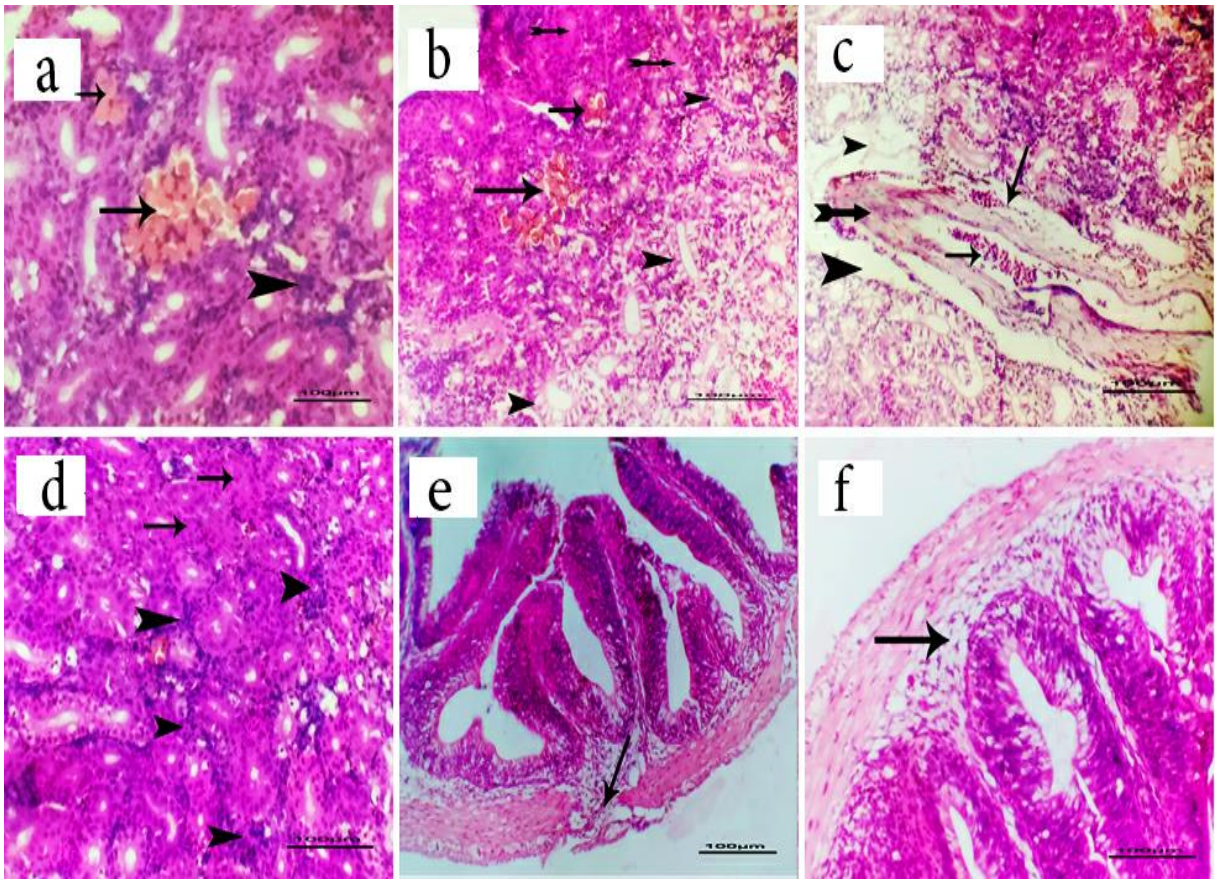

Figure 3. Photomicrograph of H&E stained kidney and mid intestine sections of different experimental G s at the last day of feeding trial showing. Renal medulla of G 5 with intratubular esinophilic casts formation (arrows) and intratubular mononuclear cells infiltration (arrow head). b) Renal cortex of G 5 with focal degeneration of renal tubules (arrows head) and intertubular eosinophilic casts (arrows) in addition to cloudy swelling of some renal tubules (tailed arrows). c) Renal cortex of G 4 with perivascular edema (arrows head) and fibrosis (tailed arrow) in addition to vascular congestion (arrows). d) Renal medulla of G 3 with moderate intertubular mononuclear inflammatory cells infiltration (arrows head) and mild cloudy swelling of some renal tubules (arrows). e) mid intestine of G 5 with focal hyperplasia of submucosal cells leading to discontinuity of layers muscularis mucosa and serosa (arrow) f) mid intestine of G 5 with hypoplasia of submucosal tissue (scale bar=100µm).

other hand, CSM contains gossypol, which is considered the main determinant of the concentration of CSM that can be applied to the tilapia diet (El-Saidy and Gaber, 2004) as the gossypol could lower the available iron and lysine for the normal physiological function (Gadelha et al., 2014). We suggested that the combined use of SBM with the CSM in Nile tilapia diet could lower the anti-nutritional substances (phytic acid and gossypol) in the diet and thus increase the availability of zinc, iron, and lysine and improve the protein digestability. Inconsistency, our results of tilapia fed CSM at a level of 15% (20% of total protein) showed better performance than control G1, while the higher level of dietary CSM (50% of total protein) significantly elevated FCR and depressed WG and SGR. These results were in agreement with those obtained by (Zheng et al., 2012) in grass carp and (Li et al., 2023) in red-tailed catfish. Furthermore, the ε-amino group of lysine combines with the reactive aldehyde group of gossypol to form a Schiff base, which lowers lysine bioavailability (Rinchard et al., 2003). Noteworthy, lysine availability support the digestive enzyme activities, enhance protein synthesis, suppress protein degradation, and thereby improve the growth parameters of Juvenile Leopard Coral Grouper (Dou et al., 2023).

In this study, the incorporation of FS with CSM in G3 elevated the immune status and growth performance of Nile tilapia. It has been suggested that the ability of FS to bind to gossypol results in the failure of gossypol to pass through the intestinal membrane (Oguz et al., 2006), thus increase the bioavailability of lysine and iron. Noteworthy, the inclusion of CSM with lysine in diet, significantly, decreases the ammonia discharge, increases oxygen consumption, and elevates the O:N atomic ratio in the aquaculture environment as observed in Chinese mitten crab (Jiang et al., 2012). The elevation in O:N atomic ratio is a good indicator that the nutrients have been used by the fish (Mayzaud and Conover, 1988). Consequently, as the fish utilize the nutrient efficiently, this would be reflected as an improvement in FCR, which come in agreement with our results especially in presence of FS. From economic point of view, the cost effectiveness of CSM (Ojewola et al., 2006) and the improvement in FCR and immune status of Nile tilapia suggests that the inclusion of CSM in tilapia diets makes them more profitable.

The Hematological Studies

The hematological findings in our study showed normocytic normochromic anemia in G4, represented by significantly decreased ($p \leq 0.05$) total erythrocytic count, Hb content, and Ht value in addition to normal MCV and MCHC. This improvement in G3 and G5 may be due to the addition of FS to the diets. G2 showed nonsignificant changes in the hemogram, which indicates that 20% CSM in the fish diet had no adverse effect on the fish hemogram. Our results were in agreement with (Soltan et al., 2011) who replaced fish meal with different levels of CSM, and with (Gaber et al., 2012), who replaced fish meal with 100% CSM. Anemia has been attributed to gossypol's toxic effect on RBCs and suppression of Ht and Hb values, which are considered common markers of its toxicity in fish (Mbahinzireki et al., 2001). In complete accordance with the outcomes, Garcia-Abiado et al. (2004) reported that the Ht and Hb of tilapia fed diets where fish meal was replaced by CSM in an increasing amount (25, 50, 75, or 100%) were significantly lower than those of the controls. In contrast, Lee et al. (2006) documented that the substitution of fish meal by CSM at 25, 50, 75, and 100% had no polynomial impact on Ht and Hb values in rainbow trout. The cytotoxic effects of gossypol are commonly linked to lower Hb and Ht levels as well as increased red blood cell fragility, as the binding ability of gossypol molecules to iron and/or the amino acids

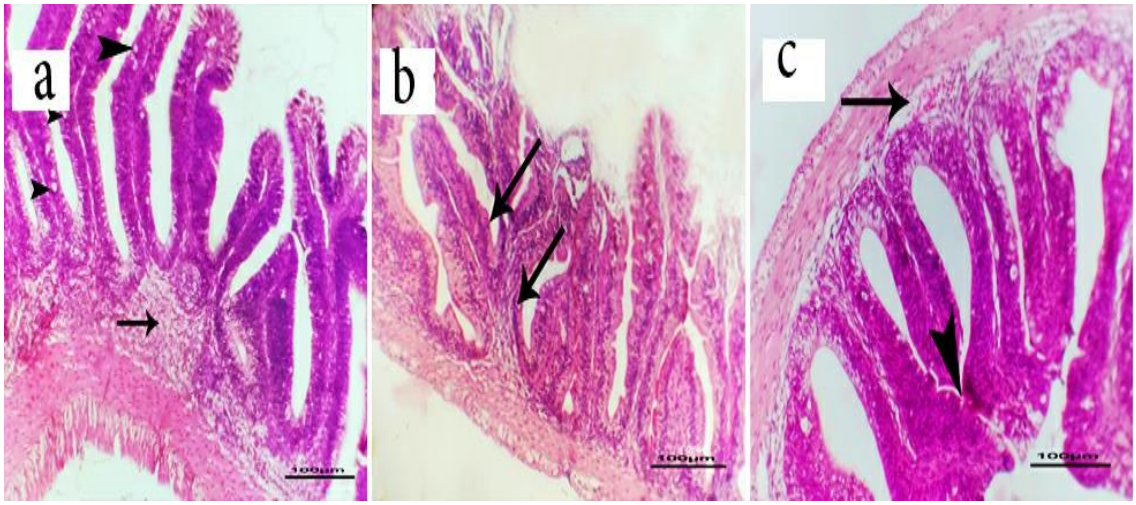

Figure 4. Photomicrograph of H&E stained mid intestine sections of different G s at the last day of feeding trial showing. a) mid intestine of G 4 with mucinous degeneration or metaplasia of enterocytes to goblet cells (arrowshead) in addition to focal submucosal hypoplasia (arrow) b) mid intestine of G 3 with multibranched villi formation (arrows). c) mid intestine of G 2 with fusion of some intestinal villi (arrowhead) and mild congestion of submucosal blood vessels(arrow).(scale bar=100µm).

limited their availability in the body and enhanced erythrocyte fragility (Makinde et al., 1997). The hematocrit levels of rainbow trout fish fed diets containing CSM were much lower (Lee et al., 2002), and they attributed this to the cumulative effect of gossypol and/or lowered iron content in CSM-containing diets, which enhanced the fragility of erythrocytes. Our findings were in agreement with (Barraza et al., 1991) who reported that free gossypol traps Fe, causing anemia and erythrocyte fragility. Moreover, gossypol enhance cytosolic Ca^{2+}-activity which, stimulate cell membrane contraction that initiate eryptosis (apoptosis-like suicidal death of erythrocyte) resulting also, in anemia (Zbidah et al., 2012).

Additionally, (Gaber et al., 2012) observed that Ht, Hb and RBC values were significantly higher in fish fed fish meal (FM) substituted with CSM (40%) with supplementation of 580 and 870 Fe kg $diet^{-1}$ than in fish fed a control diet. Our study's hematological values are in contrast to (Barros et al., 2002), who documented that channel catfish fed 50% CSM plus 671 mg Fe kg-treated $diet^{-1}$ had no progress in growth or in hematological parameters, which may be due to the inclusion of CSM in compounds that lead to the suppression of iron absorption. Our findings were consistent with (Thakur et al., 2019), who found that the addition of 20% nondehulled cottonseed cake to broilers' diet did not alter the complete blood count.

Leukogram

Leukogram findings revealed significantly decreased ($p \leq 0.05$) total leukocytic, heterophilic and lymphocytic counts in the G4, giving the image of immunosuppression as a consequence of gossypol toxicity. These findings agreed with (Ivana et al., 2014), who discovered that gossypol interferes with animal immune function, leading to reduced resistance to infections and impaired vaccine efficacy. Gossypol may cause leukopenia, mainly lymphopenia, resulting in immunocompetence (Braga et al., 2012). Gossypol has an immunosuppressive effect by suppressing lymphocyte proliferation and initiating apoptosis (Xu et al., 2009; Quintana et al., 2000). In our experiment, fish fed graded proportions of CSM and supplemented with FS performed better than fish that were fed comparable diets but did not receive FS. According to El-Saidy and Gaber (2004), the amount of gossypol and readily available lysine in CSM affects its suitability as a protein source in the Nile tilapia diet.

The Biochemical Parameters

AST and ALT activities are suggestive of general health conditions, goodness of vascular system and hepatic performance (Kumar et al., 2011). Biochemical markers are important for assessing fish general performance and physiological state in response to stress. Fish with elevated blood AST and ALT levels may have enzyme leakage through damaged plasma membranes or elevated liver enzyme production Yang and Chen (2003). In this trial, an elevated replacement level of SBM by CSM without FS supplementation led to increased activity of AST and ALT compared with those that had not. These findings suggest that FS could ameliorate the function of the liver and kidney of fish fed high levels of CSM. Our results are in accordance with (Hassaan et al., 2019) and (Liu et al., 2022). In contrast to our findings, Cai et al. (2011) found that the addition of CSM above 400 g/kg to the crucian carp diet has no effect on ALT and AST activities. In the present study, fish received CSM in the meal at a high concentration (50%) without provision of FS supplementation, and the blood AST and ALT levels increased, which could have negative consequences on the health of the liver. Gossypol's. The detrimental effects of gossypol on the liver and its role in releasing free radicals that change the redox state of the liver may be responsible for this deleterious effect on the liver (Kovacie, 2003). Moreover, the increment in CSM inclusion rate in juvenile grass carps diet enhances the expression of genes associated with ferroptosis and inflammation, whereas ferrous ion supplementation suppress their expression (Liu et al., 2023).

Concerning urea and creatinine levels in fish that received high levels of CSM without FS (G4), fish revealed a significant elevation in the aforementioned parameters, and our results were consistent with (Zhu et al., 2017) and (Yu et al., 2022). In our study, G4 showed a significant drop in serum total protein and albumen, which was attributed to the fact that gossypol is delivered in the blood by binding to serum albumen Royes and Vander Jagt (1983). Moreover, a decrease in serum protein was consequent to the disorder of protein synthesis and metabolism (Yu et al., 2022). Our findings are in accordance with (Zeng et al., 2015) who reported that total protein and albumin were decreased with high dietary CSM at 35 d of age in duck meal.

Oxidative stress occurs when ROS levels rise above the antioxidant system's capacity to scavenge them (Ray et al., 2012). MDA is lipid peroxidation byproducts, and its concentration frequently indicates the extent of lipid peroxidation and cell damage (Pille et al., 2005). GSH-Px can convert ROS and hydrogen peroxide into water (Dröge, 2002). This may be explained in our work by a drop in GSH-Px levels and elevated MDA serum levels in G4 in comparison to the control group, which is consistent with the findings of (Yu et al., 2022) and (Li et al., 2023). Gossypol has the ability to bind with iron, resulting in a state of oxidative stress that blocks the release of respiratory enzymes in addition to generating increased amounts of ROS in mitochondria Kim and Waller (1984), which explains why gossypol leads to the release of free radicals and alters the redox reaction (Kovacie, 2003). The ability of the liver to scavenge hydrogen peroxide diminished as a consequence of high dietary CSM

In the present study, CSM addition lowered plasma cholesterol levels. The alteration in lipid metabolism may be due to elevated arginine-to-lysine levels in CSM, which in turn leads to decreased total cholesterol and HDL-C values Park and Liepa (1982). In agreement, hypocholesteremia was by recorded Nwoha (1995) in rabbits and rats and (Liu et al., 2016) in grass carp after CSM supplementation in diet. This could be explained by the ability of FS to suppress lipoxygenase enzyme juvenile grass carps fed high CSM diet and thus reduces lipid peroxidation through reducing peroxides and hydroxyl radicals' generation (Liu et al., 2023). In contrast, Beynen and Liepa (1987) reported that CSM elevated the serum total cholesterol percentage in hamsters. This difference between the results could be attributed to dissimilarity of species.

Histopathological and Histomorphometric Findings

Our pathological results revealed complete harmony with other parts of our research as matching between histomorphometric measurements and body performance findings supporting each other and declared the positive role of 20% CSM protein replacement in increasing body WG as a result of increasing the ASA of the intestine. The same findings of 50% CSM protein replacement were not recorded, which was nearly similar to that obtained by (Liu et al., 2022) and could be due to the binding of gossypol with membrane proteins, lipid monolayers and bilayer membranes altering its electrochemical function with inhibition of many membrane transport systems Cuéllar and Ramírez (1993). The gossypol level increased with increasing percent of diet replacement, which could be explained by the pathological effect of high percent replacement on some tissues, such as the liver, kidney, and even intestine. Hepatic lesions may be a consequence of the impact of gossypol on hepatopancreatic metabolism (Haokun et al., 2016). The absorbed gossypol accumulates in both the liver and kidney with hepatotoxic and nephrotoxic effects (Ivana et al., 2014) leading to previously demonstrated liver and kidney lesions. Those lesions detected in iron-treated groups were variable with no preference, although adding FS exhibited good results in decreasing the negative effect of gossypol (Martin, 1990), while our results recorded organ effects, which may be attributed to the action of those combination molecules on these organs as a chemical substance. Both hepatic and renal lesions were reversible, as they did not affect the serum biochemistry of the investigated fish.

Conclusion

1- Replacement of SBM by 20 % CSM without supplementation of FS insignificantly ($p \leq 0.05$) improved growth indices, hematological, immunological parameters.

2- Incorporation of CSM at rate of 50% without FS supplementation, significantly, reduced growth performance and health status of Nile Tilapia

3- Addition of FS to diet contain 50% CSM alleviated the adverse effect of gossypol on growth performance, blood picture, organ functions, and histopathological effects on the liver, kidney, and intestine of Nile tilapia.

4- However, the results obtained from diets containing SBM replaced by 20% CSM protein with FS supplementation seem to be safer and more applicable.

More improvement could be made on this topic to be applied in large scale to obtain a better sample size and more reliable results.

Ethical Statement

This paper was reviewed and approved by the ZU-IACUC committee at the Faculty of Veterinary Medicine, Zagazig University, Egypt. Approval number: ZU-IACUC/2/F/344/2023.

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Author Contribution

All authors contributed to the study conception and design, material preparation, data collection and analysis. The experimental diet and growth performance were performed by **Mohamed A. Bakry**. The hematological and biochemical investigations was performed by **Sara A. Gad; Doaa I.A. Mostafa and Hend M. Megahed**. The histopathological studies and histomorphometric measurements were performed by **Rehab E. Mowafy. Dr Abd-Alla A. Mokhbatly** sharing in the design of the study, writing, and revision of the manuscript and in the approval of the final draft of it. The first draft of the manuscript was written by all authors who commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Conflict of Interest

The author(s) declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

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