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Beneficial effects of soybean lecithin and vitamin C combination in fingerlings gilthead seabream (*Sparus aurata*) diets on; fish performance, oxidation status and genes expression responses

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ABSTRACT

Soybean lecithin (SBL) individually or fortified with ascorbic acid (C) was supplied in fingerlings gilthead seabream *(Sparus aurata)* diets for 60 days and at the end of the experiment the impacts on fish growth performance, feed utilization, body composition, fatty acids profile and oxidation status were evaluated. The six experimental diets contained three levels of SBL; 0, 20 and 40 g/Kg diet with and without C supplementation (500 mg/Kg diet). Four hundred fifty juvenile gilthead seabream of 0.49 ± 0.02 g initial body weight were randomly housed into 18 tanks (120*L*) with a stocking density of 25 fish per tank. Results indicated that SBL and C blend supplementation improved fish growth performance and feed utilization compared to SBL alone. A significant increase ($P < 0.05$) in fish protein content when fish fed on 40 g SBL/Kg supplemented diets regardless of the C fortification (18.84% & 18.99%) . Furthermore, fish lipid content was decreased when SBL was added to the fish diets at 20 g/Kg level with C supplementation (3.81%) and at 40 g/Kg with (3.70%) or without C fortification (3.80%). SBL supplementation significantly increased ($P < 0.05$) both linoleic and linolenic fatty acid and in contrast, decreased EPA and HUFA contents and also n-3/n-6 ratios. Hepatic and muscular Malondialdehyde (MDA) contents were significantly decreased with increasing the concentration SBL from 20 to 40 g/Kg with C fortification (29.51 to 26.74 and 4.77 to 3.92 nmol/g tissue respectively) and in contrast, Glutathione GSH, reduced GSH levels increased (2.57 to 2.75 and 2.25 to 2.38 umol/mg protein respectively). Glutathione disulfide (GSSG) values were significantly decreased in all groups fed additives-supplemented diets relative to additives-free diet. CAT, NRF2, IGF1 genes expression showed a statistically significant ($P < 0.05$) up-regulation with increasing SBL supplementation level and the highest expression was in fish fed on 40 g SBL and 500 mg C/Kg supplemented diet. In conclusion, combined supplementation of 40 g SBL and 500 mg C/Kg diet induces better growth rate, modifies fatty acids profile enhances fish physiological competence and supports a potential antioxidant status.

1. Introduction

In the aquaculture, feed formulation may cost about 65% of the production cost (Ekmekçi and Gül, 2017). Therefore, enhancing feed utilization efficacy by improving the nutrients metabolism has the priority in the contemporaneous fish production. Any saving in the fish feed cost has a direct positive impact on the profitability of the aquaculture sector (Ibrahem et al., 2010; Saleh, 2020).

Soybean lecithin (SBL) is more available in a reasonable price compared to marine PLs so it has been used as a source of PLs in aquafeeds on the commercial scale (Tocher et al., 2008). SBL is considered as a feed attractant and a provider of essential fatty acids and some vitamins that are vital for fish growth (reviewed by Tocher et al., 2008; Cahu et al., 2009). Several studies on different fish species have indicated optimistic effects of dietary SBL supplementation on fish growth (Kumar et al., 2012; Davies et al., 2019; Taylor et al., 2015; El-Sayed et al., 2021), feed utilization (Adel et al., 2017; Balito-Liboon et al., 2018; Goda et al., 2018; Hassaan et al., 2019; Haghparast et al.,

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2019) and antioxidantion status (Gao et al., 2014; Adel et al., 2017; Haghparast et al., 2019), and disease resistance (Adel et al., 2017; Moghaddam et al., 2021).

Ascorbic acid (C) is an essential nutrient in fish feeds formulation and has a vital role in fish growth and maintaining physiological homeostasis. C supplementation in fish diet improves hematological and plasma biochemical functions, shares in protein metabolism (Tewary and Patra, 2008) and in the intestine C increases Fe absorption by converting its salts into ferrous form, which improves hemoglobin function (Zafar and Khan, 2020). Furthermore, C acts as a co-factor enzyme in matrix protein modification, and has a role in collagen, bone matrix, connective tissue, blood vessels synthesis (Harsij et al., 2020). Furthermore, C is an important antioxidant agent to scavenge reactive oxygen species (ROS) such as hydrogen peroxide, superoxide, and hydroxyl radical, and maintains antioxidant and ROS equilibrium (Yadav et al., 2015) where excess ROS induces oxidative stress such as DNA, protein and lipid oxidation (Chowdhury and Saikia, 2020). Deficiency of C induced negative symptoms such as impaired collagen formation, anemia and growth retardation (Zehra and Khan, 2012). Fish have no ability to synthesize sufficient C where they don't have plenty of L-gulonolactone oxidase which is a fundamental enzyme to transform L-gulonic acid to C (Dabrowski, 2001). Furthermore, C is a water-soluble vitamin that is not stored in the body but is excreted in the urine. Consequently, lack of adding C in fish diets may cause negative effects such as reduced growth rate, internal and external hemorrhage, spinal column deformation, fin erosion, exophthalmia and dark skin coloration (Tewary and Patra, 2008) and therefore, continuous C supplementation is needed.

On molecular basis, Jafari et al. (2021) registered that SBL supplementation improved antioxidative responses and *igf1* gene expression in juvenile *Stellate sturgeon (A. stellatus)*. Saleh et al. (2021) studied the expression of the insulin-like growth factors (*igf-1*and *igf-2*) in gilthead seabream and mentioned that their expression was increased by supplementing inosine to fish diet, when compared to the control. Furthermore, Chen et al. (2015) concluded that exogenous phospholipids supplementation improves growth and modulates immune response and physical barrier referring to *Nrf2* signaling factor in juvenile grass carp (*Ctenopharyngodon idella*). Catalase gene expression as a molecular marker of antioxidative metabolism was studied by Saleh et al. (2015) who recorded up-regulation the of CAT gene after Gilthead seabream larvae were fed on SBL.

Gilthead seabream is recognized as one of the most candidate to wide spread the mariculture activities (LIorente et al., 2020) . Thus in orded to carry on improving the diets formulation for this specis, there is a special interest to investigate the possibility of supplanting more than one nutrients to maximize the beneficial effects on fish performance where either of SBL or C, has multi-beneficial effects on fish performance and to the authors' knowledge, no previous work has studied the effects of dietary fortification of both in sea bream diets thus the current study was designed to assess the suitability and effects of dietary SBL supplementation with or without fortification of C on growth performance, feed utility, biochemical composition, fatty acids profile and ant oxidation status as well as genes expression of seabream fingerlings. The present study also aimed to determine which SBL level is recommended and answer the question concerning the significance of adding C in combination with SBL in gilthead seabream diet.

2. Materials and methods

2.1. Experimental protocol and diet preparation

Sea bream were acquired from El-Anfoushy marine fish hatchery (NIOF) and transported to the fish nutrition Lab. Fish were acclimatized to the experimental conditions and they were fed on a commercial diet (Aller Aqua, Aller marine for seabream, Egypt) for one week. At the start of the experiment, 25 fish (average IW = 0.49 ± 0.02 g) were distributed in 18 tanks (120 L capacity / tank). The tanks were supplied with filtered sea water of 37 ppt salinity. Water temperature was 22.6 ± 1.2° C. Oxygen was supplied by aeration with a minimum level being 6.7 mg L^{-1}. Water quality parameters were registered according to the standard methods of APHA (1995) (pH 7.5 ± 0.21, nitrite 1.71 ± 0.06 mg/l and total ammonia: 0.02 ± 0.01).

Six diets were composed to fulfill fish dietary requirements (Saleh et al., 2018). Pellets were dried in an oven adjusted at 60 °C for 24 h and finally kept at −20 °C until further use. Experimental diets were formulated to contain three levels of soybean lecithin (0, 20 and 40 g SBL/Kg diet) and assigned as L0, L1 and L2 respectively which interacted with two levels of ascorbic acid which are 0 (C0) and 500 (C1) mg/Kg diet. Diets were assigned as: additives-free diet (L0C0), L0C1, L1C0, L1C1, L2C0 and L2C1 (Table 1). The supplementation levels were determined depending on previously reported data (Tocher et al., 2008; Dawood and Koshio, 2018) and the experimental diets were tested on triplicate basis and were slowly provided to the fish three times a day to apparent visual satiation.

At the end of the experiment, fish were harvested, counted, and weighed collectively (g). Parameters of growth performance were calculated as follows:

- Weight gain (WG, g) = $W_2 - W_1$;
- Specific growth rate (SGR; %/day) = 100 (Ln W_2 − Ln W_1)/T, where W_1 and W_2 are the initial and final weights (g), respectively, and T is the number of days of the feeding period.

Table 1
Formulation and proximate analysis (% dry weight) of the tested diets fed to seabream (*Sparus aurata*) for 60 days.

g/Kg	L0 C0	L0 C1	L1 C0	L1 C1	L2C0	L2C1
Fish meal[1] (FM)	480	480	480	480	480	480
Soybean meal[2] (SBM)	230	230	230	230	230	230
Wheat gluten[3] (WG)	50	50	50	50	50	50
Wheat bran	30	30	30	30	30	30
corn starch	100	99.5	92	91.5	88	87.5
Fish oil[4] (FO)	80	80	68	68	52	52
Vit. and Min. premix[5]	30	30	30	30	30	30
Vit c [6]	0	0.5	0	0.5	0	0.5
Soy. Lecithin[7]	0	0	20	20	40	40
Proximate composition (%)						
Dry matter (DM)	90.40	90.52	90.41	90.36	90.48	90.25
Crude protein (CP)	48.63	48.51	48.14	48.33	48.24	48.12
Total Lipids (L)	13.88	13.28	13.31	13.02	12.98	13.16
Ash	9.87	9.77	9.82	9.86	9.81	9.77
Fiber	1.69	1.68	1.63	1.63	1.62	1.62
Nitrogen Free Extract[8] (NFE)	25.93	25.93	26.98	26.98	27.19	27.19
Gross energy[9] (GE, MJ/Kg)	20.86	20.85	20.84	20.84	20.73	20.73
Total phospholipid (%)	3.49	3.48	4.69	4.67	5.85	5.85

[1] FM laboratory made (67% CP).
[2] SBM (44% CP) was supplied from Research Institute of Oil Crops, Agricultural Research Center Cairo.
[3] WG (77% CP, All Starch, Germany).
[4] FO, Iceland SR, produced from fresh capelin (*Mallotus villosus*), herring (*Clupea harengus*) + and/or blue whiting (*Micromesistius poutassou*).
[5] Local vitamin and mineral premix; (AGRE–VET, Co,) Each 1 kg contains: Vit A, 12,000,000 IU; Vit D, 2,500,000 IU; Vit E, 10,000 mg; Vit K3, 500 mg; Vit B1, 1000 mg; Vit B2, 5000 mg; Vit B6, 1500 mg; Vit B12, 50 mg; Biotin, 150; Folic acid, 1000 mg; Pantothenic acid, 10,000 mg; nicotinic acid, 30,000 mg; Magnesium, 60,000 mg; Copper, 4000 mg; Iron, 30,000 mg; Zinc, 4000 mg; Cobalt, 200 mg; and Iodine, 300 mg.
[6] Vit· C ascorbate-2-poly-phosphate, ROVIMIX® STAY-C® 35.
[7] Soybean lecithin (Aceitera Feneral Deheza S. A., Argentina).
[8] NFE = 100 − (crude protein + crude lipid + ash + Fiber).
[9] GE, calculated based on 23.64, 39.54 and 17.57 kJ/g for protein, lipids and carbohydrates, respectively.

- Protein Productive Value (PPV %) = 100 (protein gain/protein intake).
- HSI = 100× [liver weight (g)/ total body weight (g)
- VSI = 100 [viscera weight (g)/ total body weight (g)
- Condition factor (CF):=100× (TW/L^3), Where: TW = Total weight (g), L = Total fish length (cm)
- Fish survival (%) = 100 (Fish count at the end /Fish count at the beginning).

2.2. Proximate composition and fatty acids analyses

The proximate composition of the tested diets and whole fish were determined on triplicates according to AOAC (2005). The phospholipids content was determined according to Zhao et al. (2013) and the fatty acid profiles of the experimental fish were analyzed using the procedures described by Feng et al. (2017) and the extracted fatty acid methyl esters were detected by a gas chromatograph HP6890 (Agilent, United States).

2.3. Determination of antioxidant status and oxidative stress markers

At the end of the experiment, liver and muscle tissues were collected from six sampled fish per treatment. 0.2 g of each sample was ground in glass homogenizer tubes (pellet pestle motor) in cold PBS solution, pH 7.4. The homogenates were centrifuged at 13.000 x*g* for 15 min at 4 °C (Centrion H-401 centrifuge), and the supernatants were aliquoted and stored at −80 °C for use in measurement of oxidative stress markers and antioxidant enzyme assays.

2.3.1. Tissue content of total, reduced, and oxidized glutathione

Glutathione (GSH) and glutathione disulphide (GSSG) were assayed using the method of Griffith (1999) MERGEFIELD .wWw..wWw.QIQQA_CLUSTER.oOo.e602a1e26a504698bce4559863e1c7af.oOo.griffith1980determination.oOo.INTRANET_3DFDB0D8-9B13-44BD-9359-27564D730C22.xXx.SEPARATE_AUTHOR_DATE.xXx..oOo. * MERGEFORMATwhich depends on the oxidation of GSH by 5,5`-dithiobis-(2-nitrobenzoic acid) (DTNB) to yield GSSG and 5-thio-2-nitrobenzoic acid (TNB). Oxidized GSSG is reduced enzymatically by the action of glutathione reductase and NADPH to regenerate GSH. The rate of TNB formation is monitored at 412 nm and is proportional to the sum of GSH and GSSG present in the sample.

2.3.2. Tissue content of malondialdehyde (MDA)

The tissues levels of MDA (lipid peroxidation end products) were assayed using thiobarbituric acid reactive substances assay (Draper and Hadley, 1990). In brief, the tissues homogenates were heated with thiobarbituric acid at a low pH to produce a pink chromogen, which was analyzed at a wavelength of 532 nm. In brief, 0.1 ml aliquots of the tissues homogenates were pipetted into a tube containing an equal volume of SDS solution (8.1%) followed by the addition of 0.75 ml acetic acid (20%), 0.75 ml, of TBA (0.8%) and 0.3 ml of distilled water. Then tubes were mixed with a vortex and incubated in a boiling water bath for 1 h then cooled to room temperature. An aliquot of 0.5 ml of distilled water was added to each tube followed by the addition of 2.5 ml of n-butanol. Then the tubes were vigorously mixed with a vortex then rotated in a centrifuge at 2500 ×*g* for 10 min. Absorbance of the organic layer was read at 532 nm in spectrophotometer against a blank containing phosphate buffer solution instead of the sample. The concentration (nmol/ml) of MDA in sample was obtained from a standard curve made by preparing serial dilutions of tetramethoxypropane (TMP) (Aldrich Chemical Co., USA) in ethanol and treating them like the sample. Results were expressed as nmol MDA/g tissue by dividing the concentration of MDA in the sample by the gm tissue in the same sample.

2.4. Gene expression analysis using qRT-PCR

Quantitative analysis of nuclear factor erythroid 2-like 2 (NFE2L2, also, known as NRF2), Insulin like growth factor1 (IGF1) and catalase (CAT) gene expression in the liver and muscle tissues was performed using quantitative real time-polymerase chain reaction (qRT-PCR). First, the total RNA was isolated from the 30 mg tissues using RNeasy kit (Qiagen, Germany) according to the manufacturer's instructions. The quality of total RNA was evaluated by 1% agarose gel electrophoresis. The purity and concentration were assessed by Nanodrop 2000 (Thermofisher, USA). One microgram of the isolated RNA was reverse transcribed using reverse transcriptase enzyme into complementary DNA (cDNA) using QuantiTect Reverse Transcription Kit (Qiagen, Germany) according to the manufacturer instructions. The QuantiTect Reverse Transcription procedure comprises 2 main steps: elimination of genomic DNA and reverse transcription into cDNA which then used for PCR quantification of the specific genes. Quantitative PCR using QuantiTect SYBR Green PCR Master Mix; was applied to determine the relative expression of NRF2, IGF1 and CAT genes use the specific primer sets for each gene (Table 2). The qPCR reactions were set up in a final 20 μL volume with 10 μL of SYBR Green Mix (Qiagen, Germany), 1 μL of total cDNA, and 200 nM of both forward and reverse primers. The PCR amplification was started with an initial denaturation for 10 min at 95 °C and then amplification by 40 cycles of PCR as follows: Denaturation at 95 °C for 15 s, annealing for 15 s and extension at 60 °C for 15 s. Each sample was run in duplicates and the specificity of reaction was verified by melting curve analyses. Data were normalized to β-actin (because of its abundance and Ct values consistency among tissues and treatments) using the Livak method (ΔΔCt) method and fold-changes were referred to L0C0 group.

2.5. Statistical analysis

Results expressed as percentages were transformed (arcsine square root) before statistical analysis. Data were checked for normal distribution (Shapiro–Wilk test) and homogeneity of variances (Levene test) before analysis. Then, differences between dietary groups for any given parameter are analyzed by two-ways analysis of variance followed by Duncan's multiple range tests. Data are presented as means ± SE, standard error ($n = 3$ per treatment, unless otherwise stated). Statistical significance between means was tested at the 0.05 probability level. All statistical tests were performed using GLM procedure according to the Standard Version of SAS 2009 software package for Windows.

3. Results

3.1. Growth and feed utilization efficiency

Results of fish growth and feed utility are summarized in Table 3. The values of WG, and SGR, as indicators of fish growth, indicate that the least fish growth was recorded in L0C0 group and these values were significantly ($P < 0.05$) increased when a blend of SBL (20 g/Kg diet) and C (500 mg/Kg diet) was used in L1C1 fish diet. Furthermore, results indicated that the highest fish growth was recorded in L2C1 fish group ($P < 0.05$). Moreover, the results pointed to the superiority of using a blend of SBL and C as a growth promotor rather than adding each of them separately. Results also showed that supplemented SBL alone at lower level (20 g/Kg diet) is better than using higher supplementation level (40 g/Kg diet).

Regarding the feed utilization efficiency, feed intake values were ($P > 0.05$) different among all dietary fish groups. Values of FCR, PER and PPV, in all experimental fish groups fed on diets supplemented with either one of the two supplements or a blend of both showed a significant improvement ($P < 0.05$) relative to L0C0 group and using of both SBL and C blend decreased FCR values significantly comparing with the other experimental groups ($P < 0.05$).

Table 2
Primer sequences of NRF2, IGF1, catalase, and β-actin.

Gene	Accession No.	Primer sequence		Melting Temp.	Annealing Temp.	Amplicon size (bp)	Efficiency
NRF2	XM_030427725	F:	CCCTTCACCAAAGACAAGCA	58.3	53	125	0.96
		R:	TTGAAGTCATCCACAGGCAG	57.8			
IGF1	XM_030440253	F:	TGTGTGGAGAGAGAGGCTTTTAT	59.1	52	156	0.95
		R:	AGCGAGCAGCCTTGCTAGTCT	63.8			
Catalase	XM_030425174	F:	TGGTCGAGAACTTGAAGGCTGTC	62.6	56	147	0.97
		R:	AGGACGCAGAAATGGCAGAGG	62.7			
B-Actin	XM_030406939	F:	CCATCCAGGCAGTGTTGT	57.1	52	63	0.99
		R:	CGGAGTCCATGACGATACC	57.1			

Table 3
Growth performance, feed utilization efficiency and somatic indices of gilthead seabream (*S. aurata*) fed experimental diets for 60 days (mean ± SE). Means in the same row with different letters are significantly different ($P < 0.05$).

	L0 C0	L0 C1	L1 C0	L1 C1	L2C0	L2C1	*P value*
IW	0.48 ± 0.01	0.49 ± 0.01	0.48 ± 0.01	0.49 ± 0.01	0.50 ± 0.01	0.50 ± 0.01	0.13
FW	2.73 ± 0.12b	2.87 ± 0.08ab	2.94 ± 0.17ab	3.02 ± 0.21a	2.86 ± 0.09ab	3.41 ± 0.06a	0.01
WG	2.25 ± 0.12c	2.38 ± 0.08bc	2.46 ± 0.18b	2.52 ± 0.42b	2.35 ± 0.08bc	2.91 ± 0.07a	0.01
SGR	2.90 ± 0.12c	2.95 ± 0.02bc	3.00 ± 0.07b	3.03 ± 0.10b	2.91 ± 0.06bc	3.20 ± 0.06a	0.02
FI	4.15 ± 0.07	4.06 ± 0.03	4.30 ± 0.09	4.14 ± 0.25	4.05 ± 0.09	4.86 ± 0.42	0.64
FCR	1.85 ± 0.10a	1.70 ± 0.04b	1.75 ± 0.54b	1.64 ± 0.32c	1.72 ± 0.10b	1.67 ± 0.11c	0.01
PER	1.26 ± 0.36c	1.36 ± 0.17b	1.33 ± 0.37b	1.42 ± 0.19a	1.35 ± 0.40b	1.39 ± 0.57a	0.01
PPV	25.49 ± 0.17b	27.50 ± 1.31a	26.76 ± 0.97a	28.57 ± 1.37a	28.19 ± 0.37a	27.83 ± 2.39a	0.04
S (%)	94.50 ± 1.56	96.84 ± 1.96	98.08 ± 7.06	97.51 ± 12.85	98.24 ± 4.96	98.51 ± 4.06	0.40
K	1.64 ± 0.13 b	1.65 ± 0.13b	1.62 ± 0.09b	1.71 ± 0.15a	1.74 ± 0.17a	1.75 ± 0.06a	0.04
HSI	1.90 ± 0.16 b	2.06 ± 0.27 ab	1.98 ± 0.39 b	1.92 ± 1.58 b	2.27 ± 0.37 a	2.31 ± 0.52 a	0.03
VSI	10.47 ± 1.27a	9.85 ± 1.21b	9.05 ± 1.07bc	9.49 ± 1.45bc	8.77 ± 1.7c	9.42 ± 1.95bc	0.02

IW = Initial weight (g), FW = Final weight (g), WG = Weight gain (g), SGR = Specific growth rate (%/day), FI = Feed intake (g), FCR = Food conversion ratio, PER = Protein efficiency ratio, PPV = Protein productive value, S% = Survival rate, K = Condition factor, HSI = Hepatosomatic index, VSI = Viscerosomatic index.

Morphometric indices measured in the present experiment are also registered in Table (3). Results indicated an increment in K values in L1C1, L2C0 and L2C1 groups while for HSI values the increment was register in L2C0 and L2C1 groups only. VSI values indicated a depletion in their values in all experimental groups compared with L0C0 group ($P < 0.05$) and the least VSI value was register in L2C0 group (8.77)

3.2. Fish body composition and fatty acids analysis

Carcass biochemical composition of fish was significantly affected by the experimental diets (Table 4). Crude protein concentration was significantly higher ($p < 0.05$) in L2C0 and L2C1 groups relative to L0C0. The carcass lipid concentration was significantly lower in fish consumed L1C1 and L2C0 diets relative to fish fed L0C0, L0C1 and L1C0 diets. Significantly the lowest fish lipid content was recorded in L2C1 group relative to all other dietary groups ($p < 0\ 0.05$).

Fatty acid profile of seabream fed the test diets is provided in Table 5. Total SFA were not significantly affected by either of the two dietary feed additives however, MUFA content was significantly decreased in fish fed L2C0 and L2C1diets. On the other hand, linoleic acid (LA, C18: 2 n-6) and linolenic acid (LNA, C18: 3 n-3) levels are significantly increased in fish fed 40 g SBL/Kg regardless of C supplementation. Eicosapentaenoic (EPA, C20:5 n-3), Docosahexaenoic (DHA, C22:6 n-3) acids and the n-3/n-6 ratios were levelled off with the increase in dietary SBL supplementation ($p < 0.05$) and lowest ratios were in L2C0 and L2C1 groups. DHA/EPA ratio was also significantly affected by SBL and C supplementation ($P < 0.05$).

3.3. Hepatic and muscular glutathione system

As indicated in Fig. 1, all fish groups supplemented with SBL and C have significantly higher hepatic contents of total and reduced GSH compared with the L0C0 group except for the fish group fed on L1C0 diet which showed no significant changes in GSH content. However, all the supplemented groups have significantly lower hepatic contents of oxidized glutathione (GSSG) compared with the L0C0 group. Increasing the concentration of SBL from 20 to 40 g/Kg diet significantly increased the hepatic content of total and reduced GSH and the highest contents of total and reduced GSH were observed in the group L2C1 ($P < 0.05$).

As illustrated in Fig. 1, all groups supplemented with SBL and C have mild but significant higher muscle contents of total and reduced GSH compared with the L0C0 group. In contrast, all fish groups fed on SBL and C supplemented diets have significantly lower muscle contents of oxidized glutathione (GSSG) compared with the L0C0 group (Fig. 2).

3.4. Malondialdehyde (MDA) as an index of lipid peroxidation

The data presented in Fig. 3 indicated a statistically significant reduction ($p < 0.05$) in both hepatic and muscle MDA levels in all experimental groups compared with the L0C0 group. The least MDA level was observed in the hepatic and muscle tissues of fish fed on L2C1 diet. Furthermore, supplementing 500 mg C/Kg diet, with increasing the

Table 4
Carcass biochemical composition of gilthead seabream (*S. aurata*) fed experimental diets for 60 days (mean ± SE). Means in the same row with different letters are significantly different ($P < 0.05$).

Biochemical Parameter (%)	L0 C0	L0 C1	L1 C0	L1 C1	L2C0	L2C1	*P value*
Moisture	72.47 ± 2.23	72.79 ± 2.44	72.57 ± 3.11	72.32 ± 3.49	73.32 ± 2.49	74.30 ± 3.43	0.65
Protein	17.47 ± 0.19b	17.95 ± 0.04ab	17.99 ± 0.42ab	18.06 ± 0.82ab	18.84 ± 0.82a	18.99 ± 0.05a	0.03
Lipid	4.66 ± 0.20a	4.14 ± 0.09a	4.50 ± 0.34a	3.81 ± 0.33b	3.80 ± 0.33b	3.70 ± 0.55c	0.02
Ash	4.39 ± 013	4.63 ± 0.49	4.13 ± 0.34	4.71 ± 0.01	4.11 ± 0.09	4.10 ± 0.05	0.21

Table 5
Carcass fatty acid profile of gilthead seabream (*Sparus aurata)*) fed on experimental diets for 60 days (mean ± SE). Means in the same row with different letters are significantly different (% of total fatty acids, $n = 3$) ($p < 0.05$).

FA	L0 C0	L0 C1	L1 C0	L1 C1	L2C0	L2C1	*P value*
∑SFAs	24.25 ± 2.11	23.88 ± 2.99	25.97 ± 4.01	25.63 ± 2.51	26.88 ± 3.06	26.53 ± 2.44	0.22
∑MUFAs	33.21 ± 2.44a	33.0 ± 0.77a	32.45 ± 1.45a	31.58 ± 2.33ab	29.91 ± 1.04b	30.03 ± 1.43b	0.03
18:2 n-6 (LA)	8.51 ± 0.67b	8.55 ± 0.46ab	9.01 ± 1.03ab	9.32 ± 0.62ab	10.48 ± 1.56a	10.41 ± 1.74a	0.03
18:3 n-3 (LNA)	0.80 ± 0.4b	0.83 ± 0.12b	0.89 ± 0.08ab	0.87 ± 0.01ab	0.95 ± 0.01a	0.97 ± 0.02a	0.02
20:2 n-6	0.12 ± 0.01	0.11 ± 0.01	0.13 ± 0.02	0.16 ± 0.01	0.09 ± 0.01	0.20 ± 0.01	0.16
20:3 n-3	2.99 ± 0.43	3.0 ± 0.54	3.0 ± 0.23	3.20 ± 0.89	3.41 ± 1.01	3.22 ± 0.54	0.11
20:4 n-6 (ArA)	3.70 ± 1.11	3.72 ± 0.47	3.65 ± 0.95	2.99 ± 1.02	3.10 ± 0.84	3.12 ± 0.98	0.23
20:5 n-3 (EPA)	6.41 ± 1.43a	6.40 ± 2.01a	6.31 ± 1.97ab	6.29 ± .95ab	6.03 ± 1.53b	6.11 ± 1.29b	0.03
22:2 n-6	2.36 ± 0 .76a	2.35 ± 0.78a	2.33 ± 0.59a	2.21 ± 0.85b	2.25 ± 0.49b	2.30 ± 0.73ab	0.02
22:5 n-3	4.81 ± 1.00a	4.83 ± 1.02a	4.69 ± 0.98b	4.73 ± 0.89ab	4.65 ± 1.20b	4.60 ± 0.78b	0.01
22:6 n-3 (DHA)	11.81 ± 2.11a	11.80 ± 1.76a	11.44 ± 1.79a	10.98 ± 2.01ab	10.79 ± 1.06b	10.83 ± 1.56b	0.02
Σ PUFA	41.51 ± 3.67	41.59 ± 4.12	41.45 ± 3.56	40.75 ± 2.89	41.75 ± 4.00	41.76 ± 3.74	0.07
Σ HUFA	26.02 ± 1.34a	26.03 ± 2.01a	25.44 ± 1.19a	25.20 ± 1.99a	24.08 ± 1.06b	24.00 ± 1.12b	0.04
∑n-3	26.82 ± 2.01	26.86 ± 3.22	26.33 ± 2.22	26.07 ± 3.53	25.83 ± 3.11	25.73 ± 1.89	0.10
∑n-6	14.69 ± 2.12b	14.73 ± 1.59b	15.12 ± 2.05b	14.68 ± 1.11b	15.92 ± 2.04a	16.03 ± 1.06a	0.03
n-3/n-6 ratio	1.83 ± 0.05a	1.82 ± 0.11a	1.74 ± 0.29ab	1.78 ± 0.76ab	1.62 ± 0.06b	1.60 ± 0.87b	0.01
DHA:EPA	1.84 ± 0.05a	1.83 ± 0.03a	1.81 ± 0.01a	1.75 ± 0.02b	1.79 ± 0.34ab	1.77 ± 0.22ab	0.03

SFAs = saturated fatty acids, MUFA = monounsaturated fatty acids, LA = Linoleic acid, LNA = Linolenic acid, ArA = Arachidonic acid, EPA = Eicosapentaenoic acid, DHA = Docosahexaenoic acid.
PUFA = Polyunsaturated fatty acids, HUFA = Highly unsaturated fatty acids.

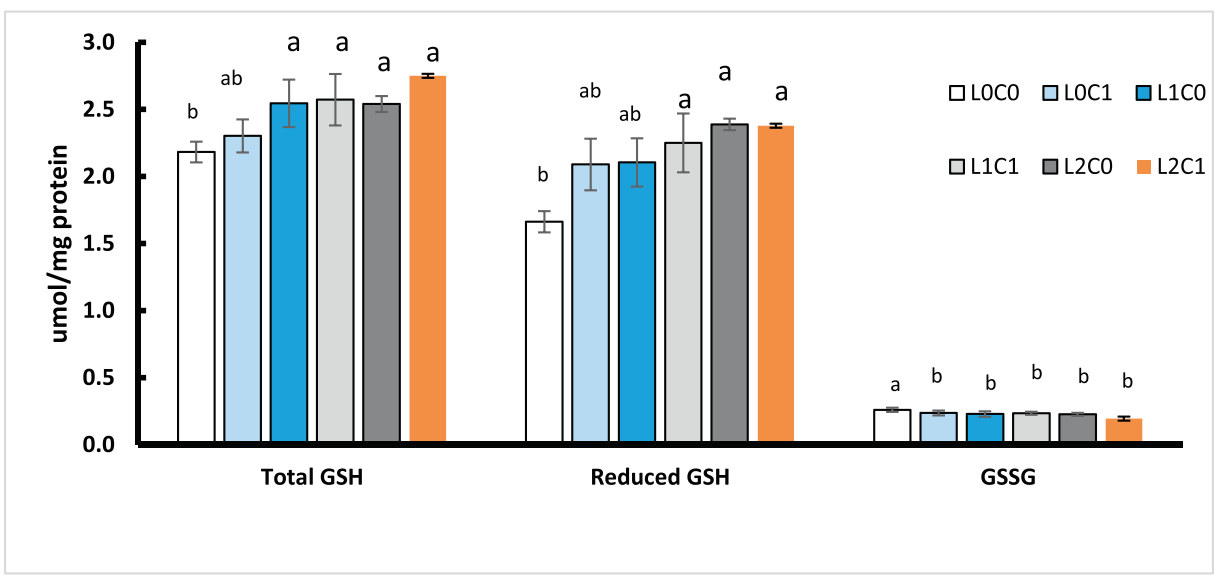

Fig. 1. Effect of fortification of SBL with or without vitamin C supplementation on the Total GSH, reduced GSH and GSSG contents in hepatic tissues of gilthead seabream ($n = 6$).
Effect of fortification of SBL with or without vitamin C supplementation on the Total GSH, reduced GSH and GSSG contents in muscular tissues of gilthead seabream ($n = 6$).

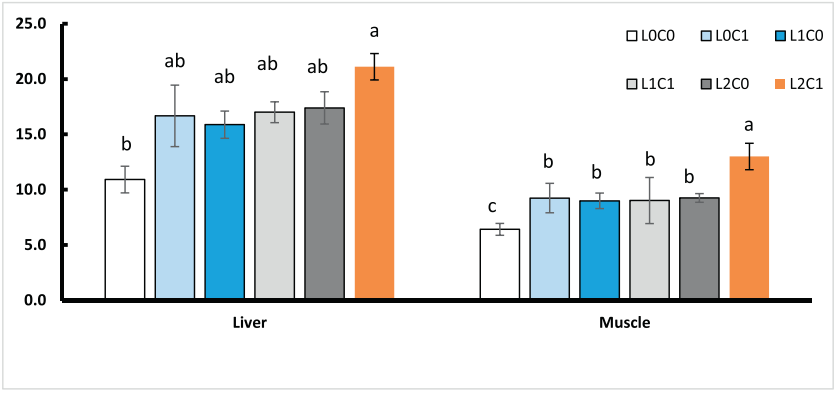

Fig. 2. Effect of fortification of SBL with or without vitamin C supplementation on the GSH/GSSG ratio in hepatic and muscle tissues of gilthead seabream.

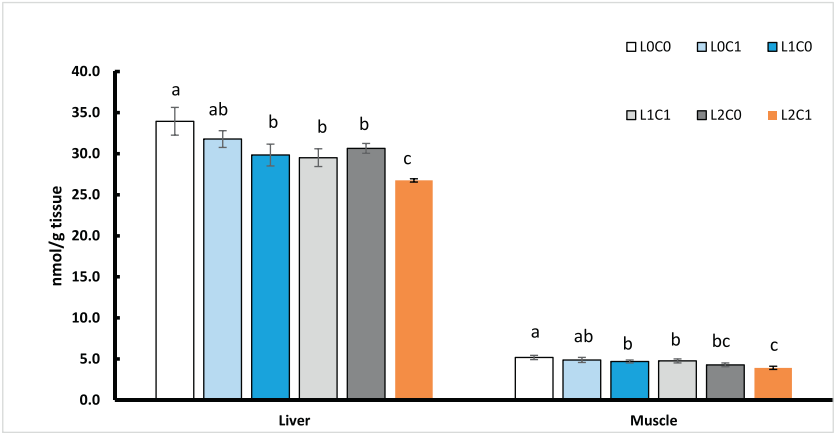

Fig. 3. Effect of fortification of SBL with or without vitamin C supplementation on the MDA content in hepatic and muscle tissues of gilthead seabream ($n = 6$).

concentration of SBL from 20 g/Kg to 40 g/Kg significantly reduce MDA levels ($p < 0$ 0.05).

3.5. Catalase gene expression

In hepatic tissues, fish fed on L1C1, L2C0 and L2C1 diets showed significantly enhanced expression of CAT gene compared with L0C0 (Fig. 4). In fish muscle, the diets supplementation with different doses of SBL alone or fortified with C did not significantly affect the expression of CAT gene in the muscular tissues (Fig. 4). The NRF2 gene expression in liver tissues showed a significant up-regulation in L1C1, L2C0 and L2C1 groups compared with L0C0 and L0C1 groups and the highest expression was observed in the fish fed on L2C1 diet (Fig. 5). In muscular tissues, only the two fish groups supplemented with SBL at level of 40 g/Kg (L2C0 and L2C1) have significantly higher expression relative to L0C0 and L0C1 fish groups (Fig. 5). In the liver and muscular tissues, L1C1, L2C0 and L2C1 fish groups have marked up-regulation in the IGF-1 compared to L0C0 group ($P < 0.05$) (Fig. 6).

4. Discussion

Fish feeds supplementation with functional additives such as phospholipids (PLs) not only enhances nutrients digestibility but also promotes growth performance, immune response and fish health (Wang

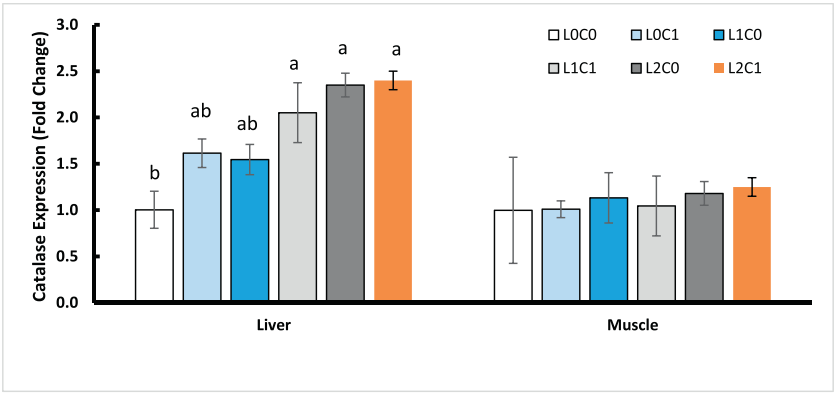

Fig. 4. Effect of fortification of SBL with or without vitamin C supplementation on the catalase gene expression of gilthead seabream.

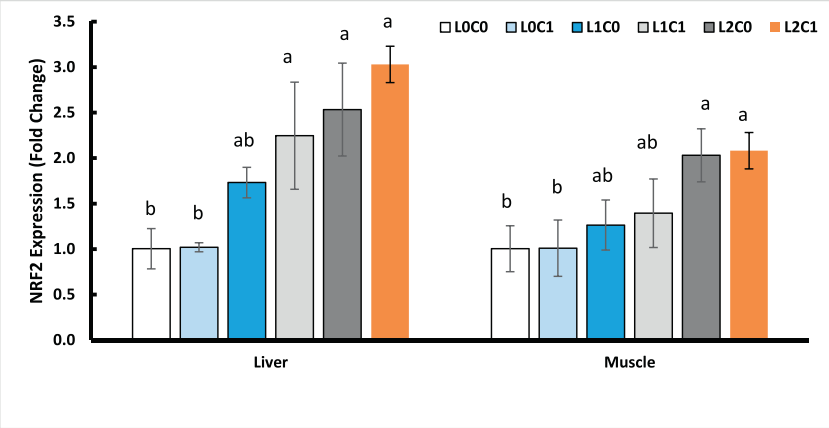

Fig. 5. Effect of fortification of SBL with or without vitamin C supplementation on the Nuclear Erythroid Related Factor2 (NRF2) gene expression of gilthead seabream.

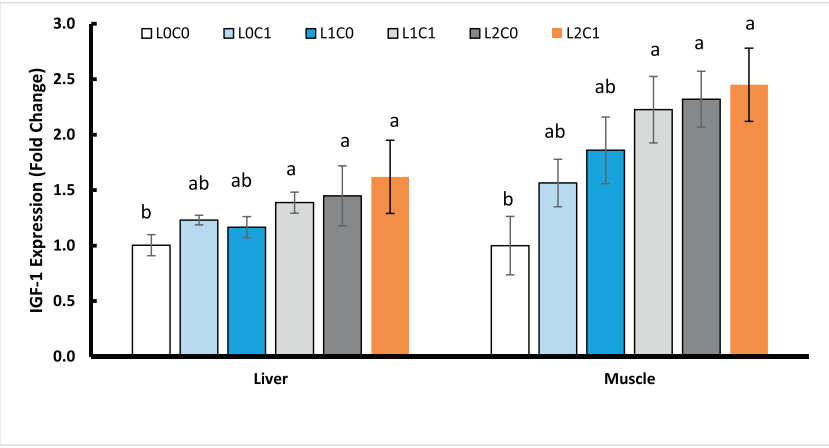

Fig. 6. Effect of fortification of SBL with or without vitamin C supplementation on the Insulin-like Growth Factor 1 (IGF1) gene expression of gilthead seabream.

et al., 2017, Pagheh et al., 2018). In the current study, no significant variations in survival rates were recorded among all dietary groups ($P > 0.05$). The maximum somatic growth was recorded in L2C1 group indicating the superiority of adding 40 g SBL and 500 mg C simultaneously in fish diet relative to supplement one of them alone in the diet. The present results are consistent with Aničić et al. (2013) who reported that brown bullhead *(Ameiurus nebulosus)* fed diets contained a mixture of encapsulated C and SBL improved FW, SGR, FCR and K. Several authors studied dietary SBL supplementation in different marine fish species as: Uyan et al. (2007, 2009), De Santis et al. (2015), La et al. (2018) and Pagheh et al. (2018) on Japanese flounder (*Paralichthys olivaceus*), amberjack (*Seriola dumerilli*), Atlantic salmon (*Salmo salar*), yellowtail (*Seriola quinqueradiata*), silvery-black porgy (*Sparidentex hasta*), respectively and also Rinchard et al. (2007) on rainbow trout (*Oncorhynchus mykiss*). All results reported an enhancement in growth performance, feed utility and general health status. Likewise, Gao et al. (2013) found that supplementing beyond 400 mg C/ kg diet improved growth and health of juvenile red sea bream (*Pagrus major*). The enhancement in fish somatic growth when fish fed on a mixture of SBL and C-supplemented diets may be explained by different reasons; i) Inclusion of PLs promote fish growth performance (Pagheh et al., 2018) as fish have a restricted synthesis capability (Tocher et al., 2008). ii) Dietary lecithin may be hydrolyzed in the fish digestive tract to the form lysophosphatidylcholine (an important precursor of PLs) which probably save energy for their biosynthesis (Liorente et al., 2020). The saved energy may be utilized in some metabolic processes, including somatic growth; iii) Dietary lecithin enhances the feed digestibility and stimulates the synthesis and secretion of lipoproteins, and consequently, the utilization of dietary lipids improves fish somatic growth (Seiliez et al., 2006). Moreover, iv) Vitamin C might be helpful for proper nutrient utilization, because C plays an important role in certain aspects of protein metabolism (Li and Schellhorn, 2007); v) Vitamin C is an essential molecule for improvement of overall health of animals (Dawood and Koshio, 2018).

Furthermore, the present results indicated that insulin-like Growth Factor 1 (IGF1) gene expression was significantly improved in fish fed on SBL at the two tested levels when fortified with C in fish diets, this result is coincided with the improved growth indices when fish fed on a diet supplemented with a mixture of SBL and C particularly in L2C1 group.

In the current study, FI values indicated that the improvement in fish growth was not attributed to the increase in fish appetite. In contrast to the present results, Koven et al. (2001) recorded an enhancement in sea bream microdiets feeding rate up to day 30 post-hatching when fed on SBL-supplemented diets. This contradiction may suggest that dietary PLs are age-dependent feed attractants. Significant improvement in values of FCR in fish fed SBL and C blend supplemented diets (L1C1 and L2C1) followed by diets supplemented with either SBL or C in comparison with additive free diet (L0C0) might have resulted in better growth performance and indicated the positive effect of using a mixture of SBL and C in maximizing feed utilization efficiency and consequently improving fish growth.

The present results showed a direct relationship between SBL supplementation level and HSI. In contrast, VSI values significantly decreased as the level of SBL incorporation in diets increased indicating

the dietary SBL efficiency to transport the lipids from fish gut to the rest of the body probably by inducing lipoprotein synthesis. The present data also showed that the highest K values were recorded in fish fed the higher SBL supplementation level. The increase in HSI values might be attributed to the lipid accumulation due to the high percentage of LA in SBL, which promoted lipid accumulation in the liver (Piedecausa et al., 2007). Pagheh et al. (2018) reported that HSI values were higher in silvery-black porgy fed SBL-free diet compared with fish fed SBL-supplemented diets; nevertheless, they recorded no significant differences in VSI and K values among all dietary groups. The contradictory between results may attributed to fish species, developmental stage, experimental conditions including interacting dietary nutrients in the experimental diets.

The fish protein content in the present study was the highest in fish fed on L2C1 followed by L2C0 diets indicating the beneficial effect of combing both SBL and C in fish diet. Parallel to the present results, Aničić et al. (2013) reported that the highest concentrations of muscle protein were in fish fed on a blend of SBL and C that indicated the complementary role of both feed additives in increasing fish protein content inducing better growth and health status. The increment in carcass protein may be explained by that the phospholipids supplied through the addition of the lecithin reduced the required energy to biosynthesis of phospholipids. Although these phospholipids might be hydrolyzed in the digestive tract during the absorption, some of them may remain intact in the form of lysophosphatidylcholine, an important precursor in the biosynthesis of phospholipids (Aničić et al., 2013). Furthermore, according to Li and Schellhorn (2007), C has an essential role in some aspects of protein metabolism.

It can be claimed that PLs also have a vital structural role in the lipids digestion as they are indispensable in developing intra-luminal mixed micelles and bile salts (Werner et al., 2013). The depletion of fish lipid content when SBL was added to fish diets at 20 g/ kg combined with C supplementation and at 40 g/Kg regardless of C supplementation was coincided with depletion in fish lipid content. This depletion in carcass lipid content may be attributed to that SBL improved lipid emulsification and aided intestinal digestion (Kasper and Brown, 2003), and also to the effect of C in preventing the fish lipid accumulation. The present findings are similar with Ji et al. (2003) who reported that dietary fortification with L-ascorbyl-2-monophosphate-Mg in red sea bream diets decreased lipid accumulation in the intraperitoneal and liver tissues. Furthermore, Koven (2003) reported that SBL–supplemented diet significantly increased the uptake of labelled dietary lipids by improving of emulsification process in gilthead sea bream larvae. However, this result was not proved in a consequent study on gilthead sea bream fed with PL-supplemented diets (Hadas et al., 2003). Dietary PL supplement also enhanced the absorption of dietary neutral lipids in European sea bass post-larvae, but without increased emulsification. Hachero-Cruzado et al. (2020) reported that larval diets deficient in PLs could induce accumulation of lipid vacuoles in the intestinal enterocytes. Several authors suggested that dietary PLs may be required for the efficient lipid exportation from the enterocytes (reviewed by Tocher et al., 2008). In the same context, Hadas et al. (2003) mentioned that in sea bream larvae, labelled oleic free fatty acid transportation from the intestine to different tissues depend upon supplementation with phosphatidylcholine.

A direct relationship between the amount of LA and LNA acids in fish and the dietary SBL levels has been reported in many fish species such as; Caspian brown trout (Sotoudeh et al., 2011), rainbow trout (Azarm et al., 2013), gilthead seabream (Saleh et al., 2015) and juvenile barramundi (Salini et al., 2016). In the present study, supplemented SBL at 40 g/Kg level also elevated body n-6 PUFA content. Comparable results were also reported in several fish species fed diets contained graded levels of PLs (Tocher et al., 2008). El-Sayed et al. (2021) reported that Nile tilapia (*Oreochromous niloticus*) fed graded levels of SBL-supplemented diets had higher n-6 PUFA contents.

Fish oil (FO) is the primary source of highly unsaturated fatty acids (HUFA), including arachidonic (ARA, 20:4n-6), eicosapentaenoic (EPA, 20:5 n-3) and docosahexaenoic (DHA, 22:6 n-3) (Turchini et al., 2009). In the present study, the substitution of the dietary FO with SBL induced depletion in ARA, EPA and DHA fish contents. The same results were recorded previously in gilthead seabream (Benedito-Palos et al., 2008, Alves Martins et al., 2010) and other fishes like; barramundi (Salini et al., 2016), and silvery-black porgy (Pagheh et al., 2018). In contrast to the present results, El-Sayed et al. (2021) recorded higher n-3/n-6 ratios in fish fed on diets supplemented with SBL than SBL-free diet. The contradiction could be attributed to that freshwater fishes have the capability to transform LNA and LA fatty acids to the n-3 and n-6 HUFA by elongation and desaturation (Huang et al., 2008) and therefore, the increased levels of LNA and LA may have induced higher HUFA content in fish.

Oxidative stress is an increment in the free radicals' cell production, inducing cell and tissue damage. Reduced activity of the antioxidants with or without increased reactive oxygen species (ROS) levels is a risk factor for all organisms (Castro and Tafalla, 2015; Huang et al., 2015). Estimating antioxidant non-enzymatic concentrations (e.g. total GSH, reduced GSH and GSSG), redox status (e.g. GSH:GSSG ratio), as indicators for oxidative stress, and oxidative damage markers (e.g. MDA) has been used as tools for assessing the fish oxidative stress (Van der Oost et al., 2003). Total GSH is free and bound to proteins inside the living cells andthe enzyme glutathione reductase returns back the free GSH from its oxidized form (GSSG) depending upon the oxidative stress. Moreover, inside living cells, the ratio of reduced to oxidized glutathione is often used as an indicator of the cell toxicity (Zitka et al., 2012).

In the present study, GSH, reduced GSH contents and GSH/GSSG ratios were significantly higher in the SBL and C blend supplemented group in an increasing level-dependent way in both liver and muscle tissues compared to the free-supplemented group. Dietary supplementation of a mixture of SBL and C in sea bream diets caused a significant decrease in MDA contents, and this reduction was significantly obvious for the L2C1 group compared to the other fish dietary groups. These findings proof the potential antioxidant role of SBL and C blend in fish diets. The present results are supported by Haghparast et al. (2019) where supplemented SBL improved the antioxidant status of Caspian brown trout and also Ciji et al. (2021) mentioned that SBL could enhance the antioxidant status of golden mahseer fry (*Tor putitora*). Kumar et al. (2014) and Adel et al. (2017) have reported that dietary SBL supplementation increased CAT, SOD, glutathione- S-transferase and glutathione peroxidase activities in milkfish (*Chanos chanos*) and common carp (*Cyprinus carpio*). Furthermore, C is a major antioxidant additive that reduces the oxidative stress in animals (Gao et al., 2013) and acts as a reducing agent and plays a main role as a water soluble antioxidant scavenging the ROS and preventing cellular components from free radicals damage. Liang et al. (2017) mentioned that yellow catfish (*Pelteobagrus fulvidraco Richardson*) fed C-supplemented diets had higher CAT, SOD and GPX activities and lower MDA levels than those fed C-deficient diet.

On molecular base, nuclear factor erythroid 2-related factor 2 (*Nrf2*) is a main transcription agent in the redox homeostasis regulation, which has a vital role in metabolism processes and cell defense (Wang et al., 2018). In the present study, *Nrf2* and *CAT* genes expression levels were significantly improved in fish fed on the SBL at the two supplementation levels when blend with C and the highest expression was recorded in L2C1 group. To the author's knowledge, no previous studies have evaluated the effects of SBL on fish *Nrf2 and CAT* genes expression levels to compare the results with. However, Caxico Vieira et al. (2018) indicated that C supplemented diets boosted the expression of genes correlated to Nile tilapia (*Oreochromous niloticus*) antioxidantation capacity when subjected to higher salinity.

5. Conclusion

The present results mirrored the important role of combined

supplementation of soybean lecithin (SBL) with ascorbic acid (C) in improving fish growth performance and feed utilization compared to supplementation of one of them alone. The present results indicate that adding SBL fortified with C at levels of 40 g and 500 mg /Kg diet respectively, induced an enhancement in fish performance, increased carcass protein content and in contrast, reduced lipid content and altered the fish fatty acids profile. Furthermore, mixing of SBL with C has a potential antioxidant role. In the present study, gene expression data and growth performance results mirrored the important role of SBL and C supplementation in growth hormone regulation.However, more immunological analyses on molecular basis are needed to provide more detailed results illustrating the effects of dietary SBL and C on the fish immunity where both optimal growth and immunity should be taken into account to achieve maximal benefits.

Authorship statement

Category 1.

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Acquisition of data: Norhan Saleh, Elham Wassef, Maher Kamel, Rasha El-Tahan.

Analysis and/or interpretation of data: Norhan Saleh, Elham Wassef, Maher Kamel, Rasha El-Tahan, Ehab El-Haroun.

Category 2.

Drafting the manuscript: Norhan Saleh, Maher Kamel, Rasha El-Tahan.

Revising the manuscript: Norhan saleh, Elham Wassef, Maher kamel, Ehab El-Haroun.

Category 3.

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Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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