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SYNERGISTIC BENEFITS OF DIETARY SILYMARIN AND SELENIUM ON GROWTH, IMMUNE FUNCTIONS, ANTIOXIDANTS, AND GUT/LIVER HEALTH OF THINLIP MULLET (*LIZA RAMADA*) JUVENILES

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Abstract

This study investigates the synergistic impact of silymarin (SI) levels combined with inorganic selenium (sodium selenite: Se) on growth, feed utilization, biochemical parameters, antioxidants, innate immunity, intestinal and liver histology, and gene expression of thinlip mullet (*Liza ramada*) juveniles. The experimental design involved thinlip mullets initially weighing 3.5±0.13 g, distributed in a completely randomized design with 30 fish per hapa (0.5 × 0.5 × 1 m), and conducted in triplicate over 60 days. Seven experimental diets were employed, including a control (without SI and Se supplementation), a negative control (with only Se supplementation), and four treatments with varying levels of silymarin (250, 450, 650, 850 mg/kg) alongside selenium (0.5 mg/kg diet). The growth performance results highlighted significant enhancements in final body weight, weight gain, and specific growth rate, particularly in the SI 850 mg/kg + Se treatment. Survival rates, feed intake, and feed conversion ratios showed positive trends across the SI-Se supplemented groups. Biochemical profiles of serum exhibited that the control diet induced elevated concentrations of glucose, cholesterol, alanine aminotransferase, aspartate aminotransferase, and urea, while Se or SI supplementation significantly mitigated these levels, with the lowest concentrations observed in the SI-Se supplemented groups. Moreover, SI supplementation increased serum protein content. Antioxidant enzyme activities, represented by superoxide dismutase (SOD), catalase (CAT), and catalase (GPx), demonstrated notable improvements in the SI-Se fortified groups, with significantly elevated GPx activity compared to the Se-supplemented and control groups. Immune system responses, including lysozyme, bactericidal, nitro-blue tetrazolium (NBT%), and serum alternative complement pathway (ACH50) activities, were highest in the SI-Se augmented groups. SI and Se in *L. ramada* reduce liver pro-inflammatory gene expression (*IL-1β, hepcidin*) vs. control group. Histological examinations of the intestine and liver depicted structural enhancements, especially at moderate and high levels of SI with Se supplementation. The results indicate improved intestinal villi morphology and hepatic architecture, supporting the positive influence of dietary treatments on the health of thinlip mullet juveniles. In conclusion, the combined supplementation of SI at 850 mg/kg diet and Se at 0.5 mg/kg diet positively influenced the growth, biochemical profiles, antioxidant status, immune responses, gene expression, and histological integrity of thinlip mullet juveniles, providing valuable insights for optimizing aquafeed formulations.

Key words: bioactive supplementation, growth augmentation, immunity, *hepcidin* gene, *interleukin 1-β* gene, histological brilliance, *Liza ramada*

The crucial role of the aquaculture business in meeting the increasing global needs for seafood cannot be overstated. Recently, there has been a growing focus on improving the performance and health status of farmed fish. This shift aims to enhance overall productivity and promote sustainable practices within the aquaculture sector (Ahmed et al., 2019). The thinlip mullet (*Liza ramada*) is an economically important fish species widely farmed in various regions. The successful rearing of thinlip mullet juveniles is crucial for sustainable aquaculture production (Toutou et al., 2023). However, challenges such as suboptimal growth, disease susceptibility, and poor feed utilization have been observed, impacting the overall productivity and profitability of mullet farming (Ali et al., 2020; Dickson et al., 2016). The cultivation and maintenance of fish populations in aquaculture systems rely on various factors, including optimal nutrition and management practices. Exploring natural additives

to enhance the performance and health of fish species has garnered increasing interest in recent years (Dawood et al., 2018; Reverter et al., 2021). A growing fascination with bioactive compound-based dietary supplements has emerged in recent years, driven by their promising potential to enhance various aspects of fish health, including growth, immune response, and overall well-being (Pulido-Rodriguez et al., 2021).

One such compound that has gained attention is silymarin (SI; St. Mary's thistle, milk thistle: *Silybum marianum*), a natural flavonoid derived from the seeds of the milk thistle plant (Javeed et al., 2022). Silymarin has been recognized for its antioxidant, anti-inflammatory, and hepatoprotective properties in various animal species (Abdel-Moneim et al., 2015; Akbari et al., 2022). Studies have previously documented various positive effects of administering silymarin in diverse aquatic organisms (Abdel-Latif et al., 2023; Al-Shawi et al., 2022; El-Houseiny et al., 2022). Research shows that adding silymarin to fish diets improves growth, antioxidant defenses, and liver health in various species like Prussian carp (Yi et al., 2012), turbot (Wang et al., 2019 a), and large yellow croaker (Yao et al., 2020). It also benefits other fish by boosting lipid metabolism in grass carp and strengthening the immune system in rainbow trout (Ahmadi et al., 2012; Xiao et al., 2017). Moreover, impacts of silymarin on seabass larvae at the critical weaning stage were positive on growth, antioxidants, and survival rate (Shahin et al., 2023).

In conjunction with silymarin, selenium (Se), an essential trace mineral, has also shown potential health benefits in fish (Lu et al., 2024). Playing a crucial role in antioxidant defense mechanisms, immune system regulation, and thyroid hormone metabolism has been attributed to Se (Ralston et al., 2016). Investigations have demonstrated its ability to improve growth, reproductive development, and disease resistance in various aquatic organisms (Cusack et al., 2017; Li et al., 2023; Wischhusen et al., 2020). Dietary selenium requirements have been documented in several studies for various fish species. For example, *Salmo gairdneri* (1.3 g) exhibited optimal outcomes with a selenium dosage ranging from 0.15 to 0.38 mg/kg (Hilton et al., 1980). Juvenile *Epinephelus malabaricus* (12.2 g) exhibited favorable outcomes with a diet comprising 0.7 mg Se/kg (Lin and Shiau, 2005), while juvenile *Cyprinus carpio* L. (10.4 g) thrived with 0.434–0.517 mg Se/kg (Jin, 2007). *Haliotis discushannai* (0.68 g) demonstrated benefits at 1.408 mg Se/kg (Wang et al., 2012), and juvenile *Ctenopharyngodon idellus* (Liu et al., 2018) responded positively to a diet fortified with 0.83 mg Se/kg. These findings collectively underscore the diverse selenium requirements among different fish species.

The potential for silymarin to offer similar benefits in young thinlip mullet (*Liza ramada*), either on its own or when paired with selenium, remains unclear. Consequently, this study's goal is to investigate the potential enhancement of performance and health status in thinlip mullet (*L. ramada*) through the dietary intake of SI in combination with selenium (Se). Specifically, we have evaluated the influences of dietary supplementation with SI and Se on thinlip mullet growth performance, feed utilization, antioxidant status, immune response, gene expression, and liver health.

By elucidating the potential synergistic effects of SI and Se in thinlip mullet, this research can contribute to the development of sustainable aquaculture practices that promote the well-being and productivity of this economically valuable fish species. Moreover, this could provide insights into the wider utilization of natural additives in aquaculture for enhancing the performance and health of various fish species.

Material and methods

Ethical approval

Ethical clearance for this study was authorized from the College of Agriculture Committee for Animal Care at Alexandria University, Egypt, under reference number 19/23/07/24/3/35. Furthermore, all research procedures adhered to the ARRIVE guidelines v2.0 (Percie du Sert et al., 2020), ensuring the research protocol aligns with established ethical standards and safeguards the well-being of the fish subjects.

Experimental design and fish husbandry

The experimental thinlip mullet (*Liza ramada*) were sourced from a private farm in Kafr Elsheikh City, Egypt, and transported in a healthy state to the Baltim Research Station of Egypt's National Institute of Oceanography and Fisheries, where the feeding experiments took place. Prior to the initiation of the feeding studies, a seven-day acclimatization period was provided for all thinlip mullets, during which they were fed a basal diet (Table 1). The thinlip mullet population was then randomly and evenly distributed among 21 hapas (comprising 7 treatments), each initially weighing 3.5±0.13 g, with a stocking density of 30 fish per hapa (0.5 × 0.5 × 1 m). All treatments were conducted in triplicate and followed a completely randomized design, lasting for a duration of 60 days.

Aeration was consistently supplied using a compressed air pump at all experimental sites, and manual water exchange (two-thirds) was performed every second day. Throughout the 60-day period, the fish were maintained under a photoperiod regime of 12 hours of light and 12 hours of darkness. The study closely tracked various water parameters during the cultivation of *L. ramada*. These parameters included water temperature (maintained at 25.2±0.21°C), assessed with a thermometer, dissolved oxygen levels (6.78±0.54 mg/L), measured using a waterproof portable meter (Hanna waterproof IP67 model), pH levels (7.41±0.32), determined through a Martini Instruments Model 201/Portable digital, and total ammonia-nitrogen content (0.04±0.02 mg/L), analyzed calorimetrically with a Spectronic 601 (Milton Roy Company, USA).

Dietary treatments and feeding regimen

To evaluate the potential synergistic effects of silymarin (SI) and selenium (Se) supplementation in the aquafeed for *L. ramada*, a feeding study was undertaken. Seven practical diets, maintaining consistent protein (29%), lipid (7%), and energy content, were formulated. These diets varied in SI content (250, 450, 650, 850, 1050 mg/kg diet) (Shahin et al., 2023), while maintaining a constant dosage of Se (0.5 mg/kg diet) (Wang et al., 2007). The experimental diets included a control (without SI and Se supplementation), a negative control (with only Se supplementation), negative control (with only Se supplementation), $SI_{250\ mg/kg}$ + Se, $SI_{450\ mg/kg}$ + Se, $SI_{650\ mg/kg}$ + Se, $SI_{850\ mg/kg}$ + Se, and $SI_{1050\ mg/kg}$ + Se, all fed to *L. ramada* over a 60-day period. Formulation of these experimental diets incorporated high-quality protein sources such as fish meal (65% CP), soybean meal (48% CP), meat meal (55% CP), and DDGS, along with lipid sources like sunflower oil, composed to meet all the dietary needs of the fish (Table 1). Ingredients were carefully blended, including vitamin and mineral mixtures (according to Magouz et al., 2022), along with other essential compounds. Subsequently, pellets were manufactured using oils and water, resulting in 1–2 mm-sized pellets, employing a laboratory pelletizer. The formulated diets underwent air-drying at room heat and were subsequently stored at 4°C.

Throughout the study, the fish received a daily feed amount equal to 3% of their body weight. The fish received each diet manually three times daily at specific intervals (8:00, 14:00, and 20:00). For a detailed breakdown of the analysis of the experimental diets, including composition and nutritional content, please refer to Table 1.

Sources and administration of silymarin with Se

Silymarin (98% purity), sourced from Legalon silymarin® 140 mg, acquired from Madaus GmbH Co. in Köln, Nordrhein-Westfalen, Germany, was incorporated as a supplement in the experimental feed formulation. Inorganic selenium (sodium selenite: 99% purity), procured from Sigma-Aldrich Chemical in St. Louis, MO, USA, was employed as the supplementary element. To ensure homogeneity and optimal distribution, both silymarin and selenium supplements were meticulously blended into oil for a duration of 15 minutes. Subsequently, the thoroughly mixed supplements were carefully incorporated into the basal diet at their respective concentration levels. This comprehensive blending process was undertaken to guarantee uniformity in the distribution of SI and Se within the experimental diets, thereby facilitating accurate and consistent administration to the *L. ramada* during the feeding trial.

Table 1. Analysis of experimental diets: Composition and nutritional content (per dry matter)

Ingredients (g/kg)	Experimental diets						
	Control	Control + Se	$SI_{250\ mg/kg}$ + Se	$SI_{450\ mg/kg}$ + Se	$SI_{650\ mg/kg}$ + Se	$SI_{850\ mg/kg}$ + Se	$SI_{1050\ mg/kg}$ + Se
Fish meal (65% CP)	50	50	50	50	50	50	50
Soybean meal (48% CP)	365	365	365	365	365	365	365
Meat meal (55% CP)	100	100	100	100	100	100	100
DDGS	65	65	65	65	65	65	65
Wheat bran	55	55	55	55	55	55	55
Rice bran	200	200	200	200	200	200	200
Wheat flour	40	40	40	40	40	40	40
Sun flower oil	11	11	11	11	11	11	11
Broken rice	75	75	75	75	75	75	75
Mineral premix (Free Se)*	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Vitamin premix*	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Dicalcium phosphate	10	10	10	10	10	10	10
Methionine	6	6	6	6	6	6	6
Limestone	10	10	10	10	10	10	10
Salt	10	10	10	10	10	10	10
Total	1000	1000	1000	1000	1000	1000	1000
Selenium (mg/kg)	0	0.5	0.5	0.5	0.5	0.5	0.5
Silymarin levels (mg/kg)	0	0	250	450	650	850	1050
Percentage of nutrient content							
Dry matter (DM%)	89.70±1.04	89.83±0.39	89.97±0.21	89.97±0.09	89.89±0.23	89.80±0.41	89.98±0.56
Crude protein (CP, % DM basis)	29.09±0.80	29.09±1.01	28.98±0.29	29.02±0.61	28.92±0.38	28.96±0.48	29.04±0.23
Crude lipid (CL, % DM basis)	7.18±0.15	7.11±0.05	7.16±0.04	7.17±0.05	7.19±0.02	7.19±0.07	7.16±0.04
Ash	7.15±0.14	7.19±0.29	7.16±0.05	7.13±0.22	7.15±0.05	7.12±0.13	7.13±0.07
Crude fiber (CF, % DM basis)	2.86±0.16	2.91±0.32	2.96±0.42	2.86±0.31	2.81±0.16	2.92±0.40	2.88±0.52
NFE	53.70±1.21	53.69±1.65	53.73±0.27	53.81±1.19	53.91±0.31	53.80±0.97	53.77±0.77

*Vitamin and mineral mixture detailed by Magouz et al., 2022.

Tissue and blood collection, and homogenate preparation

Upon conclusion of the experiment, the thinlip mullet was carefully anesthetized using 50 µl/L of clove oil, and tissues including the intestine and liver were meticulously collected under aseptic conditions. Liver samples were homogenized using a VEVOR homogenizer (Model: FSH-2A) in a cold iced NaCl solution (0.86%). The homogenized mixture underwent centrifugation at 12,000 rpm for 10 minutes at 4°C, resulting in the collection of the supernatant, which was kept at −20°C for subsequent analysis. For RNA-related studies, liver samples were treated with RNA later (obtained from Sigma, USA), initially frozen at 4°C for 24 hours, and then stored at –18°C. This preservation method ensures the suitability of the samples for future gene expression studies. Intestine and liver samples were thoroughly rinsed with a sterile cold phosphate-buffered saline (PBS) solution with a pH approximately equal to 7.4. Post-rinsing, these samples were promptly immersed in a 10% buffered formalin solution for two days to facilitate optimal preparation for subsequent histological analysis. Blood collection was performed on the same fish selected for tissue collection in replicate, while an additional two fish were used for serum collection, contributing to a comprehensive dataset for further analyses.

Growth performance and feed utilization study

Throughout the 60-day experimental trial, the thinlip mullet underwent systematic weight assessments at the initiation and subsequently at 15-day intervals. Individual fish were weighed at the conclusion of the study. The assessment of fish encompassed the examination of growth performance, feed utilization, and survival rate, considering parameters such as final body weight (FBW), weight gain (WG), average daily gain (ADG), specific growth rate (SGR), feed intake (FI), feed conversion ratio (FCR), protein efficiency ratio (PER), and survival rate (SR). The next formulae were employed for calculating various parameters:

$$WG\ (g) = FBW\ (g) - Initial\ Weight\ (g)$$

$$ADG\ (g/fish/d) = (FBW\ (g) - Initial\ Weight\ (g)) / Number\ of\ Days$$

$$SGR\ (\%/d) = [(\ln (FBW) - \ln (Initial\ Weight)) / Number\ of\ Days] \times 100$$

$$FI\ (g/fish) = Total\ Feed\ Supplied - Remaining\ Feed$$

$$FCR = Total\ Feed\ Consumed / Weight\ Gain$$

$$PER = Weight\ Gain / Protein\ Intake$$

$$SR\ (\%) = (Number\ of\ Survived\ Fish / Initial\ Number\ of\ Fish) \times 100$$

Chemical analysis

Nutrient analysis of diets and whole-body fish samples followed established Association of Official Analytical Chemists protocols (AOAC, 2000). Moisture content was determined by drying samples to constant weight at 105°C. Ash content was measured by incinerating samples at 550°C for 36 hours in a muffle furnace. Fat was extracted using an ether extractor (SoxROC, OPSIS, Sweden) for 6 hours. Protein content was quantified using the Kjeldahl method, which involves sample digestion with concentrated sulfuric acid and subsequent measurement in an automatic Kjeldahl apparatus (KD210, OPSIS, Sweden). Fiber content in diet samples was determined following the procedure described by Van Soest et al. (1991). Nitrogen-free extract was calculated by subtracting the sum of crude protein, crude fat, crude fiber, and ash content from 100.

Blood biochemistry

Biochemical parameters in the blood serum were evaluated using Bio-diagnostic® kits sourced from Bio-diagnostic®, Egypt. These kits encompassed a range of assessments, including total protein (g/dL, Cat. No. TP 20 20), albumin (g/dL, Cat. No. AB 10 10), glucose (mmol/L, Cat. No. GL 13 20), total cholesterol (mg/dL, Cat. No. TC 20 10), triglyceride (mg/dL, Cat. No. TG 20 11), ALT (glutamic pyruvic transaminase), and AST (glutamic oxaloacetic transaminase) (U/L, Cat. No. AT 10 34 and AT 10 45), urea (mg/dL, Cat. No. UR 21 10), and creatinine (mg/dL, Cat. No. CR 12 50). The analysis of these biochemical blood profiles adhered to the instructions provided in the respective kit packages, ensuring accuracy and consistency in the assessment of serum parameters.

Antioxidant enzyme activity

The assessment of superoxide dismutase (SOD) activity involved measuring the inhibition rate of autocatalytic adrenochrome production at 550 nm, following the method outlined by Misra and Fridovich (1972). Catalase (CAT) activity was assessed by monitoring the disintegration of hydrogen peroxide at 280 nm, as per the procedure described by Góth (1991). Glutathione peroxidase (GPx) level was quantified through the oxidation of NADPH, with absorbance readings taken at 412 nm, following the methodology outlined by Arun et al. (1999). All these analyses were conducted utilizing a microplate spectrophotometer, ensuring precision and consistency in the measurement of enzyme activities.

Innate immune indices

Serum lysozyme intensity was quantified using a 96-well microplate turbidimetric assay, adhering to the protocol established by Lygren et al. (1999). This method employed lyophilized *Micrococcus lysodeikticus* cells (Sigma-Aldrich, India) as the substrate. Briefly, 10 µl serum samples were dispensed into designated wells, followed by the addition of 190 µl of a substrate mixture containing 0.2 mg/mL *M. lysodeikticus* in phosphate-buffered saline (pH 7.4). The mixture was gently shaken at room temperature, and the subsequent decrease in turbidity was monitored at 450 nm after 1 and 5 minutes. Lysozyme activity was determined based on the amount of enzyme required to decrease absorbance by 0.001/min.

Serum bactericidal level was evaluated spectrophotometrically at 570 nm using a modified protocol based on the methods described by Gallage et al. (2016) and Wang et al. (2018). Briefly, 50 µl of serum samples were mixed with an equal volume of bacterial suspension (*Streptococcus agalactiae*, 1.4×10^8 CFU/mL) and incubated at 25°C for 2.5 hours. After incubation, the mixtures were transferred to 96-well microplates and treated with 15 µl of a 5 mg/mL MTT solution for 15 minutes at 25°C with gentle shaking. Formazan crystals formed during this reaction were dissolved with 50 µl of DMSO. Optical density at 570 nm (OD570) was measured in triplicate, and bactericidal activity was estimated as the percentage inhibition of *S. agalactiae* growth compared to a positive control.

$$\textit{S. agalactiae} \text{ inhibition } \% = \frac{OD_{Control} - OD_{Sample}}{OD_{Control}} \times 100$$

Respiratory burst activities in whole blood were measured at 630 nm using a nitroblue tetrazolium (NBT) assay, in accordance with the modified protocol by Secombes (1990). Additionally, serum samples were employed to determine alternative complement pathway activities (ACP), following the procedures outlined by Yano (1992).

Quantitative real-time PCR (qPCR)

Liver tissue from six fish per group (n = 6) was used to isolate total RNA using the Geneaid GenozolTri RNA Kit (Korea), following the manufacturer's protocol. The quality and concentration of the RNA were evaluated using a NanoDrop spectrophotometer (BioDrop, England). All RNA samples were then normalized to a concentration of 50 ng/µl. This study employed a one-step RT-qPCR kit with SYBR green dye for both reverse transcription and quantitative PCR reactions. The manufacturer's instructions were followed for expression analysis, utilizing *β-actin* as the reference gene. The reaction conditions involved: a 30-minute holding step at 50°C, followed by initial PCR activation at 95°C for 10 minutes. Subsequent cycles consisted of 95°C for 5 seconds and annealing at 60°C for 30 seconds, repeated for 45 cycles. Melting curve analysis confirmed the specificity of the PCR amplification, ensuring only one product was amplified at the expected size. Gene expression analysis was performed using the $2^{-\Delta\Delta Ct}$ method, where a fold change of 1 indicates no change (control), <1 represents down regulation, and >1 signifies upregulation. Expression of the investigated genes was normalized using the *β-actin* reference gene.

Intestinal and liver histoarchitecture

At the experiment's end, the animals were deeply anesthetized with 40% ethanol and their abdomens were opened. Samples of the intestine and liver were quickly removed and immersed in a 10% neutral buffered formalin solution for fixation over two days. After fixation, the tissues were processed through a series of steps: dehydration in increasing concentrations of ethanol, clearing in xylene, embedding in paraffin wax, and sectioning into 5-micrometer slices using a microtome (RM 20352035; Leica Microsystems, Wetzlar, Germany). The paraffin sections were then rehydrated and stained with hematoxylin and eosin (H&E) for general histomorphological analysis. Finally, representative photomicrographs were captured from the stained sections using a digital camera (Leica EC3, Leica, Germany) attached to a microscope (Leica DM500).

Data analysis

The results were presented as an average of three replicates, along with their standard errors (SE). Statistical analysis was completed using SPSS version 16 (IBM, Chicago, IL). Treatment effects were evaluated using a one-way ANOVA, followed by Duncan's multiple range tests to identify specific differences between individual group means. These comparisons were conducted at a significance level of 5%, providing a robust evaluation of the experimental findings.

Results

Growth performance and feed utilization

In a 60-day trial assessing the influence of silymarin and selenium dietary supplements on the growth performance and feed utilization of thinlip mullet (*L. ramada*), the data depicted in Table 3 highlights significant variations. The control group exhibited values of 8.25±0.14 g for FBW and 4.75±0.14 g for WG, resulting in an ADG of 0.08±0.00 g/fish/day and SGR of 1.43±0.03% per day. When Se was introduced alongside the control, FBW and WG increased to 11.50±0.29 g and 8.00±0.29 g, respectively, leading to an ADG of 0.13±0.01 g/fish/day and an SGR of 1.98±0.04%/day. Notably, the introduction of SI at 250 mg/kg, 450 mg/kg, 650 mg/kg, and 850 mg/kg alongside Se exhibited a progressive enhancement in growth parameters, with the highest values recorded at $SI_{850\ mg/kg}$ + Se. Survival rates fluctuated, while FI and FCR demonstrated improvements with increasing supplementation. PER also experienced a positive trend across the treatment groups.

Whole body chemical composition

The proximate composition of *L. ramada* whole body was evaluated at the conclusion of a 60-day feeding trial (Table 4). Across all treatments, the moisture, protein, fat, and ash content of the samples exhibited unremarkable variations.

Biochemical profiles

Table 5 presents the biochemical profiles of *L. ramada* serum after a 60-day feeding trial. The control diet induced significantly elevated serum glucose, cholesterol,

ALT, AST, and urea concentrations compared to other dietary groups. Selenium or silymarin supplementation significantly reduced these elevated levels, with the lowest concentrations observed in the silymarin-selenium supplemented groups. Furthermore, silymarin supplementation significantly increased serum protein content compared to the control diet, particularly at supplementation levels of 250, 650, and 850 mg/kg feed. However, serum albumin, globulin, triglyceride, and creatinine concentrations remained unaltered (P>0.05) with experimental diets.

Antioxidant enzymes activity

Figure 1 depicts the impact of dietary supplementation with silymarin and selenium on the antioxidant status of *L. ramada* after a 60-day feeding trial. Among the groups, fish receiving the basal diet displayed the lowest activities of SOD, CAT, and GPx. The groups supplemented with silymarin and selenium demonstrated notably increased GPx activity in comparison to the selenium-supplemented and control diet groups. However, SOD and CAT activities showed no significant changes in the selenium-supplemented group compared to the silymarin-selenium supplemented groups.

Immune system responses

Table 6 demonstrates the influence of selenium and/or silymarin dietary supplementation on the immune response of *L. ramada* following a 60-day feeding trial. The silymarin-selenium supplemented groups exhibited the highest lysozyme, bactericidal, NBT%, and ACH50 activities compared to the selenium-supplemented group and the control diet group, which exhibited the lowest activities of these parameters.

Table 2. Primer sequences used for the RT-qPCR analysis

Primers name	Sequences of forward and reverse primers (5'-3')	Amplicon size (bp)	References
Hep-F	GCAATGCTGAATGCCTTCAT	221	(Mahrous et al., 2021)
Hep-R	GCTTCTGCTGCAAGTTCTGA		
il1β-F	GAGGAGCTTGGTGCAGAACA	190	(Abdel-Mageid et al., 2020)
il1β-R	CTTTGTTCGTCACCTCCTCCA		
β-actin-F	CCACGAGACCACCTACAACA	270	(Byadgi et al., 2016)
β-actin-R	CTCTGGTGGGGCAATGAT		

Hep: *Hepcidin*; *il1β*: *Interleukin 1-β*; F: Forward; R: Reverse.

Table 3. Impact of adding silymarin and selenium dietary supplements on the growth performance and feed utilization of thinlip mullet (*Liza ramada*) after a 60-day trial. (mean ± SE, n = 3)

Variables	FBW (g)	WG (g)	ADG (g/fish/d)	SGR (%/d)	SR (%)	FI (g/fish)	FCR	PER
Control	8.25±0.14 e	4.75±0.14 e	0.08±0.00 f	1.43±0.03 e	100±0.00 a	9.15±0.09 e	1.93±0.04 a	1.71±0.03 e
Control + Se	11.50±0.29 d	8.00±0.29 d	0.13±0.01 e	1.98±0.04 d	100±0.00 a	11.10±0.17 d	1.39±0.03 b	2.37±0.05 d
SI $_{250\ mg/kg}$ + Se	13.52±0.19 b	10.02±0.19 b	0.16±0.03 c	2.25±0.02 b	90±0.00 b	12.31±0.11 b	1.23±0.01 cd	2.68±0.03 b
SI $_{450\ mg/kg}$ + Se	12.47±0.07 c	8.97±0.07 c	0.15±0.01 cd	2.12±0.01 c	100±0.00 a	11.68±0.04 c	1.30±0.01 c	2.53±0.01 c
SI $_{650\ mg/kg}$ + Se	14.18±0.13 b	10.68±0.13 b	0.18±0.02 b	2.33±0.02 b	93.33±0.00 b	12.71±0.08 b	1.19±0.01 de	2.77±0.02 b
SI $_{850\ mg/kg}$ + Se	15.67±0.44 a	12.17±0.44 a	0.20±0.01 a	2.49±0.05 a	93.33±3.33 b	13.61±0.26 a	1.12±0.02 e	2.94±0.05 a
SI $_{1050\ mg/kg}$ + Se	11.56±0.41 d	8.06±0.41 d	0.14±0.01 de	1.99±0.06 d	95.24±3.33 b	11.14±0.25 d	1.38±0.04 b	2.38±0.07 d

SI: Silymarin; Se: Selenium; FBW: Final body weight; WG: Weight gain; ADG: Average daily gain; SGR: Specific growth rate; SR: Survival rate; FI: Feed intake; FCR: Feed conversion ratio; PER: Protein efficiency ratio. The data presented here show the means ± SE (standard error) of three replicates. Values in the same column with different letters indicate significant differences (P≤0.05) between them.

Table 4. Impact of adding silymarin and selenium dietary supplements on the whole body composition of thinlip mullet (*Liza ramada*) after a 60-day trial (mean ± SE, n = 3)

Variables	Moisture (%)	Protein (%)	Fat (%)	Ash (%)
Control	72.59±1.00	19.19±0.40	5.94±0.14	2.34±0.55
Control + Se	72.50±0.33	19.27±0.46	5.99±0.03	2.21±0.36
SI $_{250\ mg/kg}$ + Se	72.39±0.16	19.29±0.18	6.02±0.09	2.28±0.24
SI $_{450\ mg/kg}$ + Se	72.49±0.23	19.23±0.27	6.03±0.11	2.24±0.60
SI $_{650\ mg/kg}$ + Se	72.47±0.29	19.24±0.13	6.02±0.04	2.26±0.33
SI $_{850\ mg/kg}$ + Se	72.42±0.46	19.26±0.15	6.05±0.07	2.26±0.67
SI $_{1050\ mg/kg}$ + Se	72.45±0.53	19.21±0.16	6.02±0.04	2.31±0.67

SI: Silymarin; Se: Selenium. The data presented here show the means ± SE (standard error) of three replicates.

Table 5. Impact of adding silymarin and selenium dietary supplements on the biochemical profiles of thinlip mullet (*Liza ramada*) after a 60-day trial (mean ± SE, n = 3)

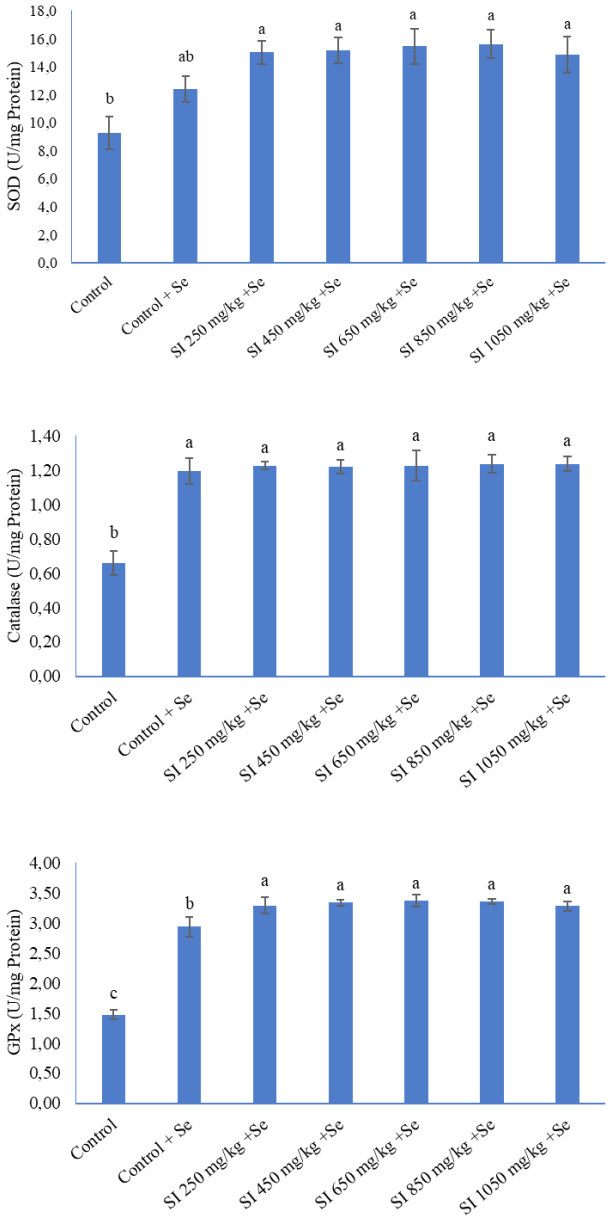
Variables	Total protein (g/dL)	Albumin (g/dL)	Globulin (g/dL)	Glucose (mmol/L)	Total cholesterol (mg/dL)	Triglyceride (mg/dL)	ALT (U/L)	AST (U/L)	Urea (mg/dL)	Creatinine (mg/dL)
Control	4.69±0.07 c	2.52±0.34	2.17±0.36	8.22±0.09 a	18.33±0.88 a	61.00±3.46	22.28±0.75 a	34.92±0.75 a	27.67±0.88 a	0.48±0.10
Control + Se	4.93±0.04 bc	2.88±0.51	2.05±0.55	6.07±0.05 b	16.67±0.88 bc	62.67±5.21	16.26±0.74 b	28.83±0.73 b	24.00±0.58 b	0.39±0.01
SI $_{250\ mg/kg}$ + Se	5.44±0.15 ab	2.52±0.30	2.92±0.18	4.99±0.01 c	13.67±1.45 c	63.67±4.91	10.98±0.47 c	23.49±0.53 c	21.33±0.88 c	0.48±0.08
SI $_{450\ mg/kg}$ + Se	5.04±0.45 bc	2.58±0.13	2.46±0.38	4.98±0.05 c	14.67±1.20 bc	64.33±4.10	11.79±0.33 c	24.35±0.37 c	19.67±1.20 c	0.38±0.02
SI $_{650\ mg/kg}$ + Se	5.50±0.08 a	2.59±0.18	2.91±0.20	4.97±0.07 c	13.00±1.15 c	64.67±6.48	12.53±1.19 c	24.97±1.02 c	19.33±0.33 c	0.41±0.06
SI $_{850\ mg/kg}$ + Se	5.75±0.12 ab	2.59±0.14	3.16±0.25	4.81±0.08 c	13.33±1.33 c	65.33±3.38	11.92±0.34 c	24.43±0.32 c	18.67±1.20 c	0.45±0.04
SI $_{1050\ mg/kg}$ + Se	4.96±0.05 bc	2.48±0.27	2.47±0.24	4.98±0.02 c	14.67±1.33 bc	66.33±3.84	11.68±0.49 c	24.25±0.52 c	19.00±0.58 c	0.44±0.05

SI: Silymarin; Se: Selenium; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase. The data presented here show the means ± SE (standard error) of three replicates. Values in the same column with different letters indicate significant differences (P≤0.05) between them.

Table 6. Impact of adding silymarin and selenium dietary supplements on immune system responses of thinlip mullet (*Liza ramada*) after a 60-day trial (mean±SE, n = 3)

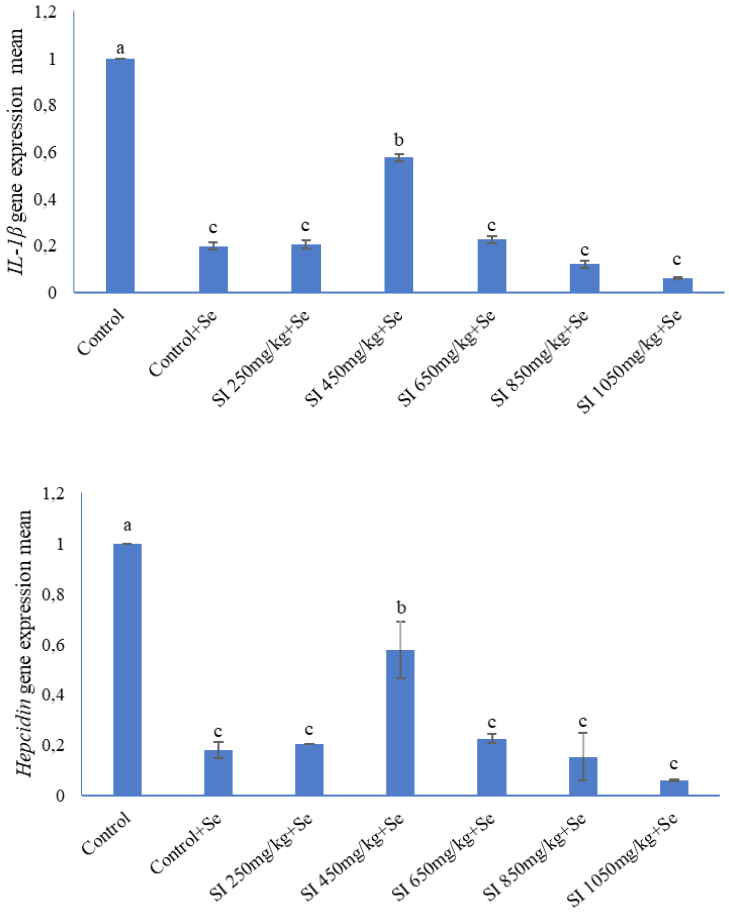
Variables	Lysozyme activity (U/ml)	Bactericidal activity %	NBT%	ACH50 (U/ml)
Control	209.57±3.40 d	3.92±0.12 c	0.20±0.005 c	39.12±1.42 c
Control + Se	307.25±4.52 c	7.74±0.13 b	0.28±0.005 b	43.73±0.68 b
SI $_{250\ mg/kg}$ + Se	340.44±2.04 b	9.94±0.14 a	0.32±0.004 a	57.83±0.68 a
SI $_{450\ mg/kg}$ + Se	343.73±1.58 b	10.07±0.20 a	0.32±0.007 a	58.20±1.03 a
SI $_{650\ mg/kg}$ + Se	350.98±2.43 b	10.16±0.10 a	0.32±0.009 a	59.13±1.60 a
SI $_{850\ mg/kg}$ + Se	372.87±3.03 a	10.23±0.23 a	0.33±0.004 a	59.38±1.27 a
SI $_{1050\ mg/kg}$ + Se	340.18±5.35 b	10.18±0.23 a	0.32±0.002 a	58.46±1.92 a

SI: Silymarin; Se: Selenium; NBT: Nitro-blue tetrazolium; ACH50: Serum alternative complement pathway. The data presented here show the means ± SE (standard error) of three replicates. Values in the same column with different letters indicate significant differences (P≤0.05) between them.



SI, Silymarin; Se, Selenium; SOD, Superoxide dismutase; CAT, Catalase; GPX, Glutathione peroxidase. Data are presented as the mean ± standard error (SE) of three replicates. Bars with different letters indicate statistically significant differences (P≤0.05) between groups.

Figure 1. Impact of dietary silymarin and/or selenium supplementation on the activities of antioxidant enzymes in thinlip mullet (*Liza ramada*) juveniles after a 60-day feeding trial



SI, Silymarin; Se, Selenium; *IL-1β, Interleukin-1β*. Values are expressed as means ± standard error (SE).

Figure 2. Gene expression of *IL-1β* and *hepcidin* genes in thinlip mullet (*Liza ramada*) juveniles after a 60-day feeding trial

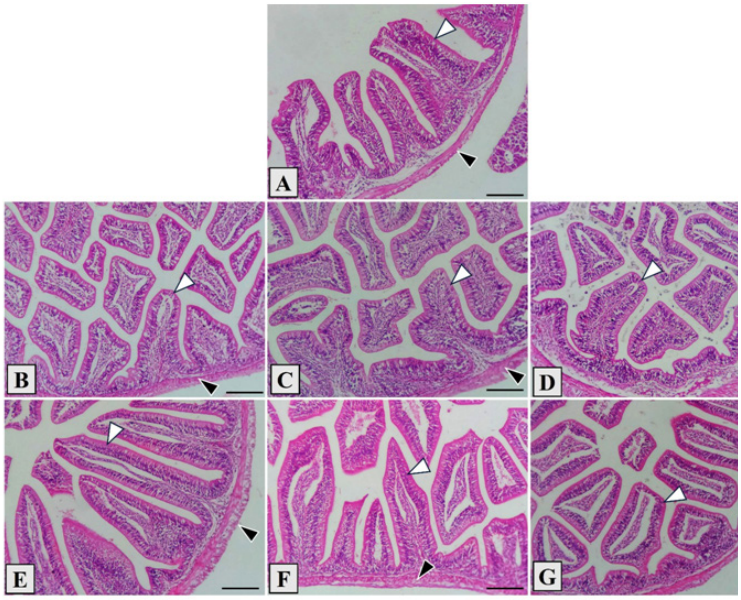


Figure 3. Histomicrograph showing the histological structure of middle segment of *Liza ramada* juveniles' intestine in the control group (A), selenium-supplemented group (B) as well as other groups supplemented by selenium and ascending levels of silymarin (C; 250, D; 450, E; 650, F; 850, G; 1050 mg/kg). The intestinal morphology exposed normal structure of both villi and wall with little improvement in only selenium-treated fish (B), moderate improvement at low levels of silymarin with selenium (C, D) and significant improvement at moderate and high levels of silymarin with selenium (E–G). Stain H and E. Bar = 100 µm

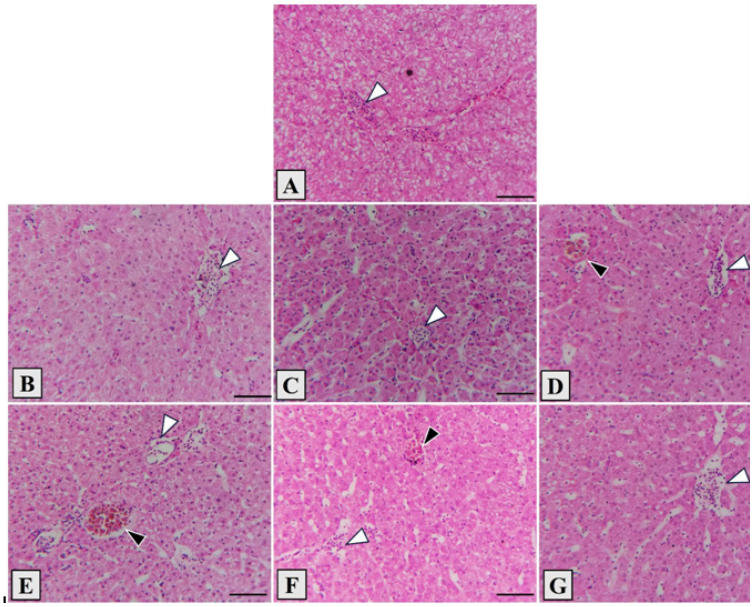


Figure 4. Histomicrograph showing the histological structure of *Liza ramada* juveniles' liver in the control group (A), selenium-supplemented group (B) as well as other groups supplemented by selenium and ascending levels of silymarin (C; 250, D; 450, E; 650, F; 850, G; 1050 mg/kg). A presented spongy appearance of hepatocytes with irregular fat vacuoles around hepatic central vein (white arrowhead). B represented intact hepatocytes with centrally located vesicular nuclei. C–G revealed improved hepatic architecture with increased glycogen evidence and melanomacrophage (black arrowhead). Stain H and E. Bar =50 µm

The relative mRNA expression of immune-related genes

Figure 2 depicts the gene expression of *interleukin-1β* (*IL-1β*) and *hepcidin*, two key immune genes, in *L. ramada* fish after a 60-day feeding trial. Relative to the control group, all studied genes exhibited downregulation in the treated groups. The expression levels decreased by approximately 0.8, 0.8, 0.5, 0.8, 0.88 and 0.95, and 0.92, 0.8, 0.5, 0.8, 0.85 and 0.95-fold for *IL-1β* and *hepcidin*, respectively.

Intestinal and liver histology

In all examined groups, the intestine of *L. ramada* revealed intact structures of the intestinal wall and intestinal villi (Figure 3 A–F). In the control group, the intestine exhibited a tunica mucosa composed of regularly arranged enterocytes, propria submucosa, tunica muscularis, and outer serosa. The histological findings revealed little improvement in only selenium-treated fish (Figure 3 B), moderate improvement at low levels of silymarin with selenium (Figure 3 C, D), and significant improvement at moderate and high levels of silymarin with selenium (Figure 3 E–G). This enhancement appeared in the form of increased villous height and villous area.

The histopathological examination of the liver in the control group (Figure 4 A) presented spongy appearance of hepatocytes with irregular fat vacuoles around hepatic central vein (white arrowhead). The selenium-subjected fish (Figure 4 B) represented intact hepatocytes with centrally located vesicular nuclei. Other fish groups (Figure 4 C–G) revealed improved hepatic architecture with increased glycogen evidence and melanomacrophage centers in some of them.

Discussion

Embarking on a journey into the intricate interplay of health and nutrition, the collaborative potential of silymarin and selenium takes center stage. Silymarin, sourced from milk thistle, boasts hepatoprotective prowess, complemented by selenium's indispensable role in antioxidant defense. Nevertheless, there is a dearth of information regarding the combined effects of dietary silymarin and selenium in fish. Consequently, the current study was devised based on the hypothesis that the concurrent use of dietary silymarin and selenium in the same fish feed might exhibit robust synergistic interactions. The findings demonstrated that the simultaneous provision of these two vital micronutrients resulted in pronounced synergistic effects, positively enhancing the growth performance, feed utilization, antioxidant status, liver health, immune response, and gene expression of *L. ramada*.

Growth emerges as a cumulative outcome of pivotal processes like assimilation, respiration, and excretion. Consequently, it stands out as a primary benchmark for evaluating the impacts of biological processes on fish in response to nutritional and immunostimulatory factors (Bertucci et al., 2019; Wang et al., 2022). In the current investigation, the inclusion of dietary silymarin in combination with selenium resulted in elevated FBW, WG, ADG, and SGR. These findings suggest that the addition of silymarin alongside selenium contributed to an enhancement in growth performance. Notably, a substantial growth-promoting effect was observed at silymarin levels of 250, 450, 650 and 850 mg/kg in conjunction with

selenium, with the most pronounced effects recorded at the 850 mg/kg silymarin + selenium combination. The remarkable impact observed can be ascribed to the elevated concentration of flavonolignans (total silymarin), which function as antioxidants (92.25 and 123 mg/kg dry weight, respectively). These antioxidants are presumed to have stimulated protein synthesis through enzymatic processes, as previously proposed by Banaee et al. (2011).

These outcomes are in accordance with the outcomes presented by Hassaan et al. (2019), who observed a notable improvement in the growth and feed intake of *Oreochromis niloticus* fingerlings fed diets augmented with silymarin at doses of 7.5 g or 10 g per kg of diet. Similarly, Al-Jubouri and Al-Obaydi (2021) noted improved growth in *Cyprinus carpio* L. when a diet fortified with 15% silymarin leaf meal was administered. A recent study also highlighted the impact of supplementing diets with dried silymarin, derived from the entire plant, on the growth of *Clarias gariepinus* (El-Houseiny et al., 2022). Significantly, dietary silymarin demonstrated growth-promoting effects in various fish species, including *Ctenopharyngodon idella* (Wei et al., 2020), *Pangasianodon hypophthalmus* (Abdel-Latif et al., 2023), *Scophthalmus maximus* L. (Wang et al., 2019 a), and *Larimichthys crocea* (Yao et al., 2020).

The use of functional bioactive constituents, such as flavonoids and phenolic compounds in plant extracts like silymarin, has been proposed to have a positive influence on feed digestibility and nutrient bioavailability. This, consequently, improves feed utilization and fosters increased protein synthesis (Ahmadifar et al., 2021; Citarasu, 2010). The enhanced growth performance observed in *L. ramada* in this study can be ascribed to the bioactive functional phytochemicals found in the utilized silymarin, which positively impact feeding intake, feed efficiency, and protein retention (Hassaan et al., 2019). Furthermore, the silymarin content may stimulate protein synthesis through the enzymatic system (Banaee et al., 2011). Earlier research has also suggested that dietary silymarin has the potential to modulate the expression of the growth hormone gene, thereby contributing to the growth of muscle fibers (Hassaan et al., 2019). Another hypothesis put forth by Wei et al. (2020) proposes that dietary silymarin fosters intestinal growth and enhances the intestinal physical barrier in fish, thus positively contributing to overall growth.

Similar to silymarin, selenium serves as another crucial micronutrient that contributes to the growth and physiological well-being of fish. Previous research studies have underscored the importance of an adequate dietary selenium supply for optimal body growth in fish and other animal species (Hoffmann and Berry, 2008; Jamil, 2013; Khan et al., 2016; Li et al., 2023). In a previous investigation, the addition of vitamins C, E, and selenium to the diet was observed to significantly boost WG in *O. niloticus* (Kim and Mahan, 2003). Furthermore, selenium has been acknowledged for its potential to enhance the growth performance of various aquatic animals, including *Acanthopagrus schlegelii* (Wang et al., 2019 b), *C. idellus* (Liu et al., 2018), *O. niloticus* (Lee et al., 2016), among others. Selenium supplementation has also been shown to elevate selenium concentrations in the muscle tissue of specific fish species (Buckley, 2000).

Research by Fonseca et al. (2013) exposed a significant increase in both WG and feed conversion efficiency (FCE%) in tilapia when their diet was fortified with 400 mg/kg of vitamins C and E, respectively, alongside 0.4 mg/kg of selenium oxide. This enhancement in growth performance compared to the control group can be attributed to the essential roles played by these micronutrients in aquatic animals (Khan et al., 2016; Wang et al., 2003). Individual supplementation with these nutrients has been linked to several benefits, including enhanced erythrocyte production and reduced fat accumulation in liver tissue (Góth, 1991; Khan et al., 2015).

Moreover, the fish receiving a diet enriched with both silymarin and selenium exhibited the lowest FCR and the highest PER compared to those on the control diet without supplements or the negative control containing only selenium. These findings can be ascribed to the presence of bioactive compounds in silymarin and selenium, which not only enhance feed efficiency but also impact protein retention. In support of this, Citarasu (2010) and Khan et al. (2016) have previously documented that the active components in plant extracts and micronutrients from selenium can enhance nutrient digestibility and availability. This, in turn, results in improved feed utilization and ultimately leads to increased protein synthesis.

Similar positive outcomes regarding growth development and feed utilization efficiency were observed with silymarin extract supplementation in studies on *C. idellus* by Jia et al. (2013) and on *C. carpio* by Xiao et al. (2017). However, Yi et al. (2012) found no remarkable impacts on the growth performance or feed utilization of *Carassius auratus gibelio* when supplementing with silymarin extract or flavomycin. These discrepancies in results might be explained by variations in fish species, rearing environments, or the differing compositions of the diets employed.

Taken together, the current investigation unveils a robust synergistic interplay between dietary silymarin and selenium. The concurrent supplementation of these micronutrients in *L. ramada* feed exhibited a notable enhancement in growth and feed utilization parameters, including FBW, WG, ADG, SGR, and PER, accompanied by a significant reduction in FCR.

While our results demonstrate a noticeable improvement in the growth of fish and feed utilization with the inclusion of silymarin and selenium, the analysis of the proximate composition of *L. ramada* whole body revealed consistent values across all treatments for moisture, protein, fat, and ash content. This compositional stability aligns with prior research in other fish species conducted under diverse experimental conditions (Abdel-Latif et al., 2023; Lin et al., 2021). The lack of significant variations in these proximate components suggests

that the dietary interventions incorporating silymarin and selenium did not exert discernible effects on the fundamental composition of *L. ramada* whole-body tissues.

Dietary constituents in fish feed can influence organismal biochemical parameters by affecting metabolic processes. Alterations in ALT and AST activities in the liver, serum, and blood serve as indicators of liver health, damage, and cell membrane integrity in fish (Çiçek and Özoğul, 2021). Elevated enzyme levels often signify hepatic injury, indicating enzyme leakage from hepatocytes (Brusle and Anadon, 1996).

In the current experiment, the control diet led to substantially increased serum AST and ALT contents compared to other dietary groups. Selenium or silymarin supplementation effectively mitigated these elevated enzyme levels, with the lowest concentrations observed in groups supplemented with both silymarin and selenium. This aligns with previous findings showing that silymarin in *O. niloticus* diets reduced ALT and AST activities (Hassaan et al., 2019), and in *Oncorhynchus mykiss*, dietary silymarin controlled the activity of the AST and ALT enzymes (Banaee et al., 2011). However, contrasting results were reported by Al-Shawi et al. (2022) in *Cyprinus carpio*, where silymarin extract did not affect enzyme activities. These discrepancies may be attributed to differences in fish species, silymarin doses, or experimental designs across the studies.

The liver is the primary site of selenium accumulation in fish (Hodson and Hilton, 1983), and liver tissue degeneration has been observed in fish exposed to selenium (Sorensen and Bauer, 1984). Our results are consistent with Hao et al. (2014), who reported decreased ALT and AST in loach with selenium supplementation. Conversely, Abdel-Tawwab et al. (2007) found elevated AST and ALT activities in *Clarias gariepinus* fed organic selenium. Our findings suggest improved liver functions and hepatoprotective effects from dietary silymarin and selenium, which may be related to the hepatoprotective and antioxidant properties of silymarin (Abenavoli et al., 2018; Owatari et al., 2018) and hepatoprotective properties of flavonoids (Davila et al., 1989). The hepatoprotective effects could be linked to silybin, a component of silymarin known for its antioxidant activities against free radicals and metal ions. It inhibits lipid peroxidation and safeguards membrane permeability properties, thereby counteracting hepatic injury (Borsari et al., 2001).

Elevation in urea and creatinine levels is indicative of renal and gill dysfunction (Nelson et al., 1999). Based on our findings, no significant differences were observed in the levels of urea and creatinine between *L. ramada* fed diets supplemented with silymarin and selenium and those fed the reference diet. This implies that dietary supplementation with silymarin and selenium has beneficial effects on fish renal functions without posing a risk to their kidneys.

Fish supplemented with both 650 and 850 mg SI/kg and selenium showed increased total protein and globulin content, along with decreased albumin levels compared to other groups. This aligns with the findings of Bunglavan et al. (2014), who observed that elevated serum globulin and a reduced albumin: globulin ratio indicated enhanced immunity in laboratory animals. Therefore, incorporating dietary silymarin and selenium at 650 and 850 mg SI/kg alongside selenium appears to positively impact the immune status of *L. ramada*. Similar observations were made in rainbow trout, where diets enriched with 100 and 800 mg silymarin/kg resulted in significantly higher total plasma protein levels compared to the control group (Banaee et al., 2011). Additionally, Abdel-Tawwab et al. (2007) found that *C. gariepinus* fed a diet containing 0.5 g of organic Se/kg exhibited elevated levels of albumin, globulin, and total protein.

Assessing the antioxidative response is crucial for measuring fish health (Elumalai et al., 2020; Mahboub et al., 2022). Groups supplemented with both silymarin and selenium exhibited significantly higher glutathione peroxidase (GPx) activity compared to the selenium-only and control diet groups. However, superoxide dismutase (SOD) and catalase (CAT) activities remained unchanged in the selenium-supplemented group compared to the groups receiving both silymarin and selenium. Similar findings regarding dietary silymarin were reported in *O. niloticus* (Hassaan et al., 2019), *P. hypophthalmus* (Abdel-Latif et al., 2023), and *C. gariepinus* (El-Houseiny et al., 2022). Hao et al. (2014) observed significantly increased GPx and SOD activities in loach liver tissues fed a diet with 0.5 mg Se/kg, aligning with our selenium-related observations. Notably, the group with 0.48 mg Se/kg displayed the lowest levels of malondialdehyde (MDA). This suggests a possible synergistic antioxidative effect of combined silymarin and selenium supplementation, particularly on GPx activity.

Dietary strategies can modulate the immune response in fish by influencing immune cells through various pathways, including metabolic, neurological, and endocrine mechanisms (Pedersen and Hoffman-Goetz, 2000). In fish, phagocytosis, a critical defense mechanism against pathogens, involves lysozyme activity, bactericidal activity, respiratory burst activity (measured by nitroblue tetrazolium, NBT), and serum complement activity (ACH50) (Kumari and Sahoo, 2005). Lysozyme, known for its antimicrobial properties, plays a key role in the innate immune system and serves as a vital marker for evaluating the overall immune function across various fish species (Saurabh and Sahoo, 2008). Produced by leukocytes, lysozyme breaks down the cell walls of microorganisms and triggers the production of lysozyme, further activating the immune complement system (Cecchini et al., 2000). NBT activity represents a significant indicator of the innate immune defense mechanism in fish (Miyazaki, 1998), while bactericidal activity is essential for the host's ability to combat pathogens (Kawakami et al., 2000). Interestingly, in our study, groups supplemented with silymarin and selenium exhibited the highest lysozyme, bactericidal, NBT%, and ACH50 activities compared to the selenium-supplemented group and the

control diet group, which displayed the lowest activities of these parameters.

Several studies have reported increased serum lysozyme activity in fish species fed diets containing *S. marianum*, including *C. gariepinus* (El-Houseiny et al., 2022), *O. mykiss* (Ahmadi et al., 2012), and *C. carpio* (Alishahi et al., 2011). Similarly, *S. maximus* receiving silymarin-based diets exhibited higher serum lysozyme activities compared to those on non-silymarin diets (Wang et al., 2019 a). Moreover, Abdel-Latif et al. (2023) observed elevated levels of total immunoglobulin and lysozyme in the serum of *P. hypophthalmus* fed milk thistle extract (MTE; *Silybum marianum*) for 60 days. These findings suggest that silymarin possesses immune-modulating properties, likely due to its functional phytochemicals like flavonoids, phenolic acids, and polyphenols (Abenavoli et al., 2018; Ahmadifar et al., 2021). Additionally, selenium supplementation has been shown to enhance B-lymphocyte production, which significantly improves fish lysozyme activity and ultimately strengthens their immune system (Khan et al., 2015).

Nutrigenomics has emerged as a powerful tool for exploring the impact of nutrients on aquaculture development (Alhoshy et al., 2022; El Basuini et al., 2020; Shadrack et al., 2022; Shehata et al., 2022). Researchers are actively investigating the intricate relationship between functional genes and their expression profiles, uncovering fascinating connections between optimal feeding strategies, performance, and health in aquatic organisms. Compelling evidence suggests that various nutrients and formulated feeds can regulate gene expression within individual cells, leading to enhanced growth development and robust immunity in aquatic species (Alhoshy et al., 2022; El Basuini et al., 2020; Shadrack et al., 2022; Shehata et al., 2022).

As far as we know, no information has been acquired regarding the immune response-related genes in diets with silymarin and selenium for *L. ramada*. We assessed the mRNA abundance of crucial genes associated with immunity, marking the first inaugural report on this subject. The roles of *interleukin 1-β* (*IL-1β*) and *hepcidin* genes are pivotal in understanding various physiological processes. *IL-1β*, a pro-inflammatory cytokine, plays a crucial role in the regulation of immune responses and inflammation. It is involved in the activation of immune cells and the induction of fever, contributing to the body's defense mechanisms (Younis et al., 2021). On the other hand, hepcidin, a key regulator of iron homeostasis, plays a central role in controlling iron levels in the body. It regulates iron absorption in the intestines, iron release from macrophages, and iron transport in the bloodstream. The balance maintained by hepcidin is essential for preventing both iron deficiency and iron overload, ensuring optimal functioning of various physiological processes (Rodrigues et al., 2006).

Understanding the roles of *IL-1β* and *hepcidin* genes is vital in unraveling their contributions to immune response regulation and iron metabolism, shedding light on potential implications for health and disease. The current study revealed that feeding supplementation levels of silymarin alongside Se in *L. ramada* significantly downregulated the expression of liver pro-inflammatory genes, including *IL-1β* and *hepcidin* compared to the control group. Studies have shown that silymarin and selenium can prevent inflammation by reducing pro-inflammatory cytokines and boosting anti-inflammatory cytokines (Prabhu et al., 2020; Hassaan et al., 2019).

Examining the histomorphology of the intestine and liver is crucial for gaining insights into the normal and abnormal physiological conditions of fish (Abdel-Latif et al., 2023, 2024). In the present study, the intestinal histology of *L. ramada* displayed intact structures of the intestinal wall and villi across all groups. The control group exhibited a tunica mucosa composed of regularly arranged enterocytes, propria submucosa, tunica muscularis, and outer serosa. Histological observations indicated marginal improvement in fish treated with selenium alone, moderate improvement at low silymarin levels in combination with selenium, and significant enhancement at moderate and high silymarin levels alongside selenium. This enhancement manifested as increased villous height and villous area. Similar findings were reported in *S. maximus* diets supplemented with silymarin, preserving normal intestinal histology and enhancing histomorphometric parameters like microvilli and villi height (Wang et al., 2019 a). Additionally, silymarin supplementation led to improved intestinal histology in *P. hypophthalmus* and juvenile *C. idella* (Abdel-Latif et al., 2023; Wei et al., 2020). Comparable results were observed in juvenile *Lates calcarifer* (Ilham et al., 2016) and *O. niloticus* (Ghazi et al., 2022) when supplemented with selenium.

The histopathological examination of the liver in the control group revealed a spongy appearance of hepatocytes with irregular fat vacuoles around the hepatic central vein (white arrowhead). In contrast, selenium-treated fish exhibited intact hepatocytes with centrally located vesicular nuclei. Other groups displayed improved hepatic architecture with increased glycogen evidence and the presence of melanomacrophage centers in some cases. Common carp, when fed diets incorporating *S. marianum*, exhibited normal histological sections in their hepatopancreatic tissues (Jindal et al., 2019). Similarly, *C. gariepinus* were observed to maintain a normal histomorphological structure in their hepatopancreatic tissues (El-Houseiny et al., 2022) and *P. hypophthalmus* (Abdel-Latif et al., 2023). Selenium yielded similar results in *O. niloticus* (Iqbal et al., 2020) and juvenile *L. calcarifer* (Ilham et al., 2016). The authors elucidated these findings by highlighting the synergistic effects of silymarin and selenium in enhancing the physical barrier function of the fish intestine, subsequently contributing to the maintenance of histological integrity.

Conclusion

In conclusion, the present study sheds light on the synergistic benefits of dietary supplementation with si-

lymarin and selenium in enhancing the growth performance, immune response, gene expression, and histomorphological features of *Liza ramada*. The combined supplementation exhibited notable improvements in parameters such as fish body weight, growth rate, feed efficiency, and immune activities, as evidenced by elevated lysozyme, bactericidal, NBT%, and ACH50 activities. Histological examinations of the intestine and liver revealed significant enhancements in structural integrity, particularly in villous height, villous area, and hepatic architecture. These positive effects were more pronounced at moderate and high levels of silymarin (650 and 850 mg/kg, respectively) alongside selenium (0.5 mg/kg). Considering the observed benefits, it is recommended to incorporate silymarin at a concentration of 850 mg/kg in the diet, supplemented alongside selenium at 0.5 mg/kg, for optimal results in promoting the growth, health, gene expression and overall well-being of *L. ramada*. This tailored dietary approach has the potential to offer valuable advancements in aquaculture practices, contributing to the sustainable development of fish farming industries.

CRediT authorship contribution statement

Akram Ismael Shehata: conceptualization; methodology; data curation; data analysis; data interpretation; original draft preparation; writing review and editing; supervision. Shimaa A. Shahin: methodology; writing review and editing. Ayaat M. Elmaghraby: methodology; resources; data analysis; writing review and editing. Mayada Alhoshy: data curation; writing review and editing. Ali A. Soliman: methodology; resources; writing review and editing. Asem A. Amer: resources; methodology; writing review and editing. Yusuf Jibril Habib: writing review and editing. Mahmoud S. Gewaily: methodology; data analysis; writing review and editing. Mohammed F. El Basuini: methodology; formal analysis; data analysis; original draft preparation; writing review and editing.

Declaration of competing interest

The authors declare no conflict of interest.

Data availability statement

The data sets supporting and/or analyzed during the current study are available from the corresponding author upon request.

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