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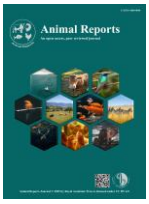

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The potential role of photobiotic in enhancing liver antioxidant status as determined by enzyme scavenging activity and molecular mechanisms in Gilthead seabream (*Sparus aurata*) teleost

Abdallah Tageldein Mansour [1, *], Cristóbal Espinosa [2,3], Sabrin Abdalrahman Morshedy [4], M. Ángeles Esteban [5]

1. Department of Aquaculture and Animal Production, College of Agriculture and food Sciences, King Faisal University, Al Hofuf, Kingdom of Saudi Arabia
2. Biotechnology Department, Institute of Agricultural and Environmental Research and Development of Murcia (IMIDA), 30150, Murcia, Spain
3. Aquaculture and Animal Production Technology Department. Institute of Agricultural and Environmental Research and Development of Murcia (IMIDA), 30150, Murcia, Spain
4. Fish and Animal Production Department, Faculty of Agriculture (Saba Basha), Alexandria University, Alexandria, 21531, Egypt
5. Fish Innate Immune System Group, Department of Cell Biology and Histology, Faculty of Biology, Regional Campus of International Excellence "Campus Mare Nostrum", University of Murcia, 30100 Murcia, Spain

Corresponding author: Mansour, A. T.: amansour@kfu.edu.sa; a_taag@alexu.edu.eg

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Abstract: Oxidative stress is one of the intensive aquaculture implications, especially in fish fed high dietary lipids. The use of natural plants as a source of antioxidant substances in the aquatic diet is an alternative natural-based solution to overcome. The present study aims to investigate the potential effects of *Moringa olifera* leave meal (MLM) on antioxidant status in the liver of Gilthead seabream (*Sparus aurata*) as determined by antioxidant enzyme activities (CAT, SOD and GR), antioxidant gene expression (CAT, CuZn-SOD, and GR), and regulatory pathway genes (nrf2, nkef-A, and nkef-B). *S. aurata* specimens (138.75 g) were divided into 4 groups in duplicate and fed MLM at increasing levels of 5, 10, and 15% at a daily feeding rate of 1.5%. Liver samples were collected after 15 and 30 days of intervention. The obtained findings revealed that dietary MLM significantly enhanced the liver activities of SOD and GR enzymes compared to the control group after 15 days of treatment, and the effect continued after 30 days. However, the catalase enzyme remains unaffected throughout the experiment. The gene expression of CuZn-SOD was significantly upregulated after 15 and 30 days of MOL intervention. However, the significant increase of GR gene expression was reported after 15 days only. CAT gene expression tended to increase with dietary MOL. The regulatory gene expression, including

nrf2, nkef-A, and nkef-B were significantly upregulated with dietary MOL at levels of 5-10%. In conclusion, the dietary intervention of MOL could enhance liver antioxidant status at both biochemical and molecular levels, and the possible mechanism is enhancing nrf2 pathways as antioxidant regulatory genes.

Keywords: Moringa leave; liver homogenate; oxidative stress; seabream; antioxidant enzyme; *nrf2* pathways

1 Introduction:

The bioactive substances in feed are responsible for nutritive as well as non-nutritive functions, such as reduction of oxidative stress, pathophysiological disorders, and immune suppression. Antioxidants is one of the most studied agents in aquaculture research such as vitamins (Tocher et al., 2003), microbial metabolites (Abdelaziz et al., 2024; El-Houseiny et al., 2025), algae and seaweeds (Alwaleed et al., 2024; Zahran et al., 2024), nanomaterials (Ahmed et al., 2024; Zahran et al., 2025), medicinal plants (Ibrahim et al., 2024; Mansour et al., 2024), and extracts (Oh et al., 2022; Almarri et al., 2023) to compete the dietary oxidized ingredients and improve antioxidant systems, immunity status, and growth (Ahmadifar et al., 2021). While synthetic antioxidants have been widely used, there is growing interest in natural alternatives due to concerns over potential toxicity and consumer demand for cleaner, sustainable products (Yu et al., 2021). Moreover, the use of antioxidants as phytogenic substances in the aquaculture feeds represent a safe alternative solution to antibiotic and improving fish resistance, and disease control (Jian & Wu, 2003).

Moringa oleifera, recognized for its rich nutrient profile and strong antioxidant qualities, has become a potential option for inclusion in aquaculture diets (Tabassum et al., 2021). This fast-growing tree, also referred to as the Horse radish tree or Drumstick tree, is a member of the Moringaceae family and was used by ancient civilizations including the Romans, Greeks, and Egyptians (Abdel-Latif et al., 2022). All parts of the Moringa tree as leaves, flowers, gums, roots and seeds are edible and have long been consumed by humans for many diseases treatment (Dzuvor et al., 2022). The leaves are exceptionally nutritious, offering proteins of high quality, and are noted to be rich in numerous phytochemicals such as carotenoids, vitamins, minerals, sterols, glycosides, alkaloids, flavonoids, moringine, moringinine, phytoestrogens, caffeoylquinic acids, and phenolic compounds found in the flowers, leaves, roots, fruits, and seeds (Fuglie, 1999; Guevara et al., 1999; Anwar et al., 2007). The moringa was used for treatment of many disease including inflammation, cardiovascular and liver diseases, (Iqbal & Bhanger, 2006; Chumark et al., 2008) with hypolipidaemic, antiatherosclerotic, antioxidant, immunostimulation and tumor suppressive effects (Murakami et al., 1998).

The gilthead seabream, *S. aurata*, is a subtropical Sparidae teleost, naturally grow up mainly in the Mediterranean Sea and rarely in the Black Sea, and in the Eastern Atlantic. Seabream is farmed extensively in lagoons, or intensively in tanks or cages (Sola et al., 2006). It is a highly valued teleost species, is renowned for its nutritional and economic importance and it consider one of the major farmed fish species in the Mediterranean region with an estimated production higher than 160,000 ton and the main producers are Greece, Turkey, Egypt and Spain (FAO, 2016). The increasing demand of consumers on high quality aquaculture products is the global concerns with maximizing species welfare and minimizing the environmental impact (Frewer et al., 2005).

However, like many farmed marine fish, it is susceptible to oxidative stress, particularly with using high lipid content in the diets to spare proteins, improve feed conversion and to decrease the amount of waste, which can compromise its health, growth, and overall quality (Thirunavukkarasar et al., 2022; J. Xu et al., 2022). Oxidative stress arises from an imbalance between the production of reactive oxygen species (ROS) and the organism's antioxidant defense mechanisms, leading to cellular damage compromise its health, growth, and reduced product quality (Mansour et al., 2022; El-Houseiny et al., 2023). Enhancing the antioxidant status of farmed fish is therefore critical for improving their health and the nutritional value of their edible tissues. Taking the previous consideration in to account the present study aims to explore the potential antioxidant effect of moringa leaves at different concentrations as a natural dietary supplement to improve the antioxidant status of Gilthead seabream at both enzymatic and molecular levels. By leveraging the bioactive properties of moringa, this research seeks to contribute to sustainable aquaculture practices while addressing the growing demand for healthier and more functional seafood products.

2 Materials and methods:

2.1 Moringa leaves and diets preparation

M. oleifera leaves (MOL) were harvested from the Botany Department's experimental farm at the Faculty of Agriculture (Saba Basha), Alexandria University, Alexandria, Egypt. The leaves were dried and grinded to fine powder before incorporating in the diets. Four MOL levels (0, 5%, 10%, 15%) were incorporated in seabream commercial diets (Skretting, Spain; protein percent adjusted to 45%) by excluding same portion of the diet or corn meal and the proximate composition were determined according to (AOAC, 2000) (Table 1). The commercial pellet was crushed and mixed with MOL powder alone or combined with yellow corn meal (used to adjust the final protein levels in all diets) (Dongmeza et al., 2006) and then tap water was added slowly to make clumped diets. The resulted pellets were processed at ambient temperature using a 2 mm die meat grinder, and the pellets formed were subsequently dried in a forced-air oven set at 37°C for 24 hours. After drying, they were packaged in polypropylene bags and stored at 4°C until needed.

2.2 Experimental fish

From a local farm at Murcia, Spain, 138.75 g Gilthead seabream (*S. aurata*) were maintained for one month at the Marine Fish Facility at the University of Murcia as a quarantine period. The fish were reared in RAS tanks (400 L) with a flow rate of 900 L h^{-1}, temperature (22 ± 1 °C) and 28 ‰ salinity, and a photoperiod cycle of 12 h light:12 h dark. A forty-eight apparently healthy and homogenies fish were acclimatized to the experimental conditions for 15 days and feed the control diets at 1.5% of their body weight. The Ethical Committee of the University of Murcia approved the fish handling and experimental procedures.

Table 1. Mixture and proximate chemical composition of experimental diets.

Ingredients (%)	Experimental diets			
	0%	5%	10%	15%
Commercial diet[1]	92.5	90	87.5	85
Moringa leaves	-	5	10	15
Corn flour	7.5	5	2.5	-
Proximate composition (%; dry mater bases)				
Ether extract	16.95	16.63	16.3	15.98
Crude protein	45.02	44.83	44.63	44.43
Ash	5.92	5.92	5.92	5.93
Crude fiber	5.92	5.92	5.92	5.93
Nitrogen-free extract (NFE)[2]	286.0	284.1	282.1	280.2

[1]Commercial pellets diet: D-2 Optibream AE 1P (Skretting, Spain).
NFE: nitrogen-free extract calculated using the following equation: NFE = 100- (crude protein + ether extract + crude fiber + ash) (NRC, 1993).

2.3 Experimental design and sample collection

At the beginning of the experiment, homogenous fish were randomly divided into four groups in duplicate at initial stocking density of 12 tank^{-1}. The daily feeding rate was adjusted to 1.5% and introduced two times per day. Fish samples were obtained after 15 and 30 days of feeding, six sample were randomly selected from each aquarium.

The sampling technique was as follow: fish were starved for 24 h and anesthetized using clove oil (50 mg ml^{-1} water). Before dissection, blood was extracted to avoid interference, then liver sample was obtained and divided into two portions. The first part was stored frozen at – 80 ºC for biochemical analysis. The second were stored in TRIzol Reagent (Invitrogen) at -80 ºC and used for gene expression analysis.

2.4 Liver homogenate

Liver samples (1 g) were homogenized using a T10 basic Ultra-Turrax homogenizer (IKA, Staufen, Germany) in 1 M sodium phosphate buffer in a sample: buffer ratio of 1:4 (w/v). Then the homogenate was centrifuged at 5000 rpm for 15 min (González-Silvera et al., 2021). The pellets were discarded, and the supernatant was used in the further antioxidant enzyme activities analysis.

2.5 Gene expression analysis by real-time qPCR

Relative expression of antioxidant genes, including Glutathione reductase (*GR*), Superoxide dismutase (*SOD*), Catalase (*CAT*), Nuclear factor erythroid 2-related factor 2 (*Nrf*- 2), Natural killer cell enhancing factor A (nkef A), and Natural killer cell enhancing factor B (*nkef* B) after 15 and 30 days of experiment (Table 2).

RNA was extracted from *S. aurata* liver (0.5 g) using TRIzol Reagent (Chomczynski, 1993). Subsequently, its quantity was measured, and its purity was evaluated via Nano drop spectrophotometry; the 260:280 ratios were between 1.8 and 2.0. Afterwards, the RNA treated with DNase I (Promega) to eliminate genomic DNA contamination. Complementary DNA

(cDNA) was generated from 1 μg of total RNA utilizing the SuperScript III reverse transcriptase (Invitrogen) along with an oligo-dT18 primer. Gene expression analysis was conducted using real-time qPCR on an ABI-PRISM 7500 (Applied Biosystems) with SYBR Green PCR Core Reagents (Applied Biosystems). The reaction mixtures, composed of 10 μL of 2× SYBR Green supermix, 5 μL of primers (each at a concentration of 0.6 μM), and 5 μL of cDNA template, were initially incubated for 10 minutes at 95ºC. This was succeeded by 40 cycles, each consisting of 15 seconds at 95ºC, 1 minute at 60ºC, followed by another 15 seconds at 95ºC, 1 minute at 60ºC, and a final 15 seconds at 95ºC. The mean expression levels of the *18s* and *ef1-α* genes served as a reference point for the normalization of cDNA loading, although the data from our preliminary experiment are unavailable. The $2^{-\triangle\triangle CT}$ method was applied to ascertain the expression outcomes following the verification of primer amplification with near-perfect efficiency, as described by Livak and Schmittgen (2001).

Table 2 Real-time qPCR forward and reverse primers of *Sparus aurata*

Gene name	Abbreviation	Gene bank number	primer sequences (5'→3')
Ribosomal protein S18	*18s*	AM490061	F: GAAAGCATTTGCCAAGAAT R: AGTTGGCACCGTTTATGGTC
Elongation factor 1 α	*ef1-α*	AF184170	F: TGTCATCAAGGCTGTTGAGC R: GCACACTTCTTGTTGCTGGA
Glutathione reductase	*GR*	AJ937873	F: CAAAGCGCAGTGTGATTGTGG R: CCACTCCGGAGTTTTGCATTTC
Superoxide dismutase	*CuZn-SOD*	AJ937872	F: CCATGGTAAGAATCATGGCGG R: CGTGGATCACCATGGTTCTG
Catalase	*CAT*	FG264808	F: TTCCCGTCCTTCATTCACTC R: CTCCAGAAGTCCCACACCAT
Nuclear factor erythroid 2-like 2	*Nrf- 2*	XM_030427725.1	F: GTTCAGTCGGTGCTTTGACA R: CTCTGATGTGCGTCTCTCCA
Natural killer enhancing factor A (or *pdrx1*)	*nkef A*	GQ252679	F: CTCCAAGCAATAATAAGCCCAAAG R: TCACTCTACAGACAACAGAACAC
Natural killer enhancing factor B (or *pdrx2*)	*nkef B*	GQ252680	F: CAAGCAGTAAATGTGAAGGTC R: GATTGGACGCCATGAGATAC

2.6 Statistical analysis

Data are expressed as mean ± SE values. Before performing the statistical analysis, all data were checked for homogeneity and normality using Levene test and Shapiro-Wilk, respectively. One way analysis of variance (ANOVA) followed by tukey test were used to differentiate significant differences among means at $P < 0.05$. Statistical analyses were performed using IBM-SPSS for Windows (IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp).

3 Results:

3.1 Antioxidant enzyme activities

Fig. 1 showed the antioxidant enzyme activities in *S. aurata* fed *M. olifera* supplemented diets after 15 and 30 days. The results showed CAT activity was not affected with moringa supplementation even after 30 days of intervention. A significant increase in SOD activity in

groups fed 5 and 10% moringa supplemented diet than the control or the higher supplementation levels after 15 and 30 days of supplementation. Meanwhile, glutathione reductase increased significantly with 15% moringa supplementation compared to other groups along the treatment period.

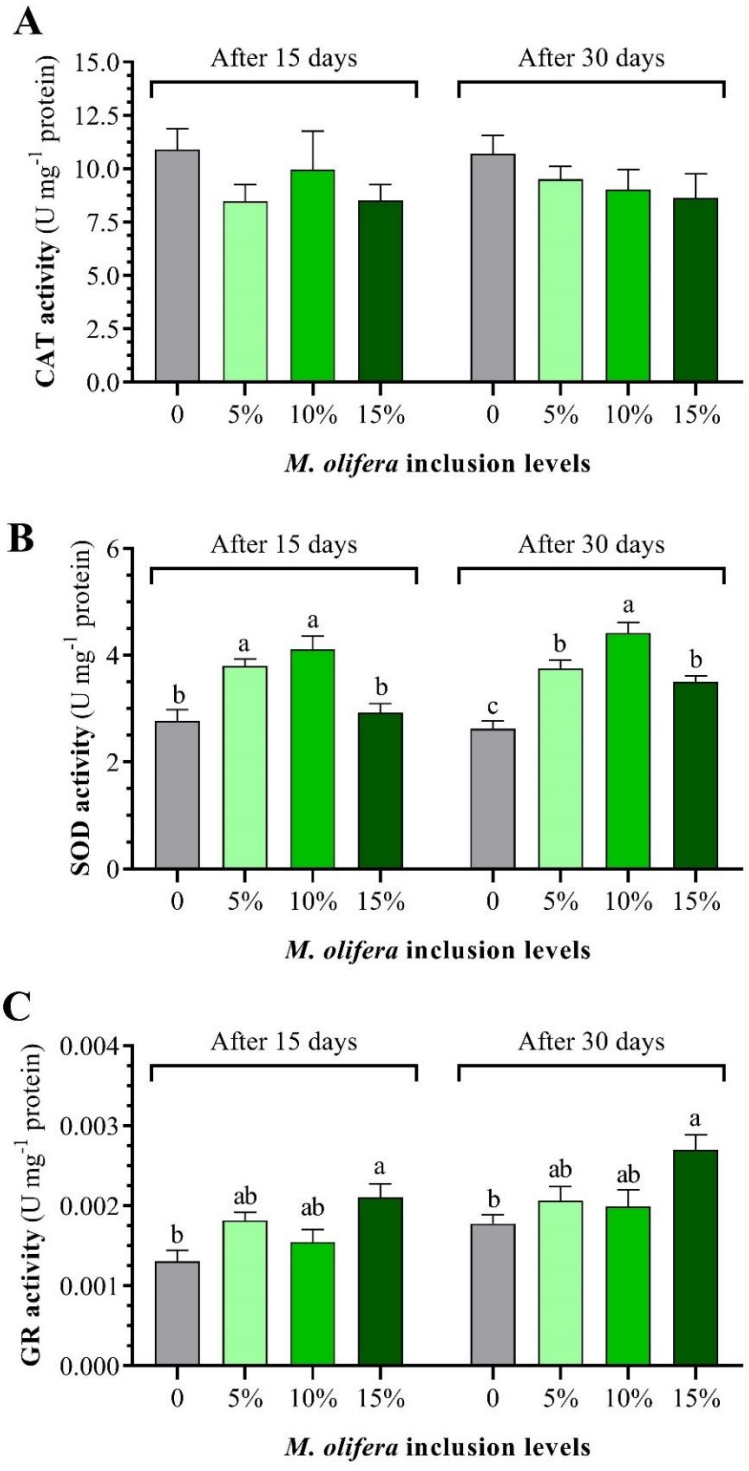

Fig. 1: Effect of dietary *Moringa olifera* leave meal on antioxidant enzymes activity in liver of Gilthead seabream (*Sparus aurata*). A: catalase; B: superoxide dismutase; C: glutathione reductase. Different letters indicate significant differences among groups at $P \leq 0.05$.

3.2 Antioxidant genes expression

The changes in expression levels of different antioxidant enzyme genes were reported in Fig. 2. The *CAT* expression tended to increase in groups fed 5 and 10% moringa than the control or the group fed 15% moringa supplemented diet. The transcriptomic of *CuZn-SOD* gene showed remarked increase with all moringa supplementation groups than the control. Furthermore, GR RNA expression was significantly increased with 5-10% moringa supplementation levels after 15 days of treatment. However, the changes in GR gene were lost after 30 days of experiment.

Fig. 3 showed the expression levels of different antioxidant regulatory genes in liver of *S. aurata* fed diet supplemented with moringa leave. The expression of nrf2 gene was markedly increase after 15 days of treatment with 5-10% supplementation levels. Meanwhile, this significant increase maintained only with 5% supplementation level after 30 days of treatment. The transcription of nkef-A gene was significantly reported with all moringa supplementation levels after 15 and 30 days of treatment. The same trend was observed with nkef-B gene expression after 15 days of treatment. However, after 30 days of treatment, the significant differences were observed only in group fed 5% moringa supplementation.

4 Discussion:

The dietary increasing levels of MOL induced significant differences on SOD and GR activities in liver homogenate of Gilthead seabream. Levels of 5-10% moringa supplementation reported the highest SOD activity. The transcriptional level of *CuZn-SOD* was significantly upregulated with all moringa treatments at both sampling times. Meanwhile, GR enzyme showed the highest significant activity at 15% moringa level after 15 and 30 days of treatment. In consistence, the expression of *GR* gene was upregulated after 15 days of treatment, and stabilized after 30 days, which could be due to oxidative stress adaptation. CAT activity could be less sensitive to dietary moringa, also the expression of *CAT* gene was slightly increased after 15 days of treatment and this effect was lost after 30 days. In the same manner, dietary supplementation with MOL significantly upregulated the expression of CAT and SOD genes in gills and skin of seabream in a dose dependent manner (up to 5% supplementation levels) (Mansour et al., 2020). Moreover, dietary MLM significantly decreased free radical levels and increased the activities of SOD, CAT, and reduced glutathione in the blood of grass carp, *Ctenopharyngodon idella* under normal condition or after thermal stress (Faheem et al., 2022). In liver of Nile tilapia, MOL supplementation significantly decreased MDA levels along with a significant rise in hepatic CAT, SOD and GPx enzymes in all MOL-supplemented groups. Furthermore, supplementation with MOL-nanoparticles restored the normal antioxidant status of Nile tilapia exposed to zinc oxide nanoparticles toxicity and Oxyfluorfen Toxicity (Hamed et al., 2022; Ibrahim et al., 2022).

The improvement of antioxidant status in the present study could be attributed bioactive compounds presents in MOL, such as flavonoids (quercetin, kaempferol), polyphenols, and vitamins (C, E, A) that have strong antioxidant properties (Kashyap et al., 2022; Momın & Memiş, 2023). MOL reported to has high content of flavonoids, phenolic compounds as the main active components, followed by saponin, tannins and cyanogenic glycosides (García-Beltrán et al., 2020). These compounds can directly scavenge reactive oxygen species (ROS) and reduce oxidative stress, leading to increased antioxidant enzyme activity. Moringa extracts (aqueous and ethanolic) showed strong dose dependent antioxidant capacity as revealed by ABTS assay (García-Beltrán et al., 2020).

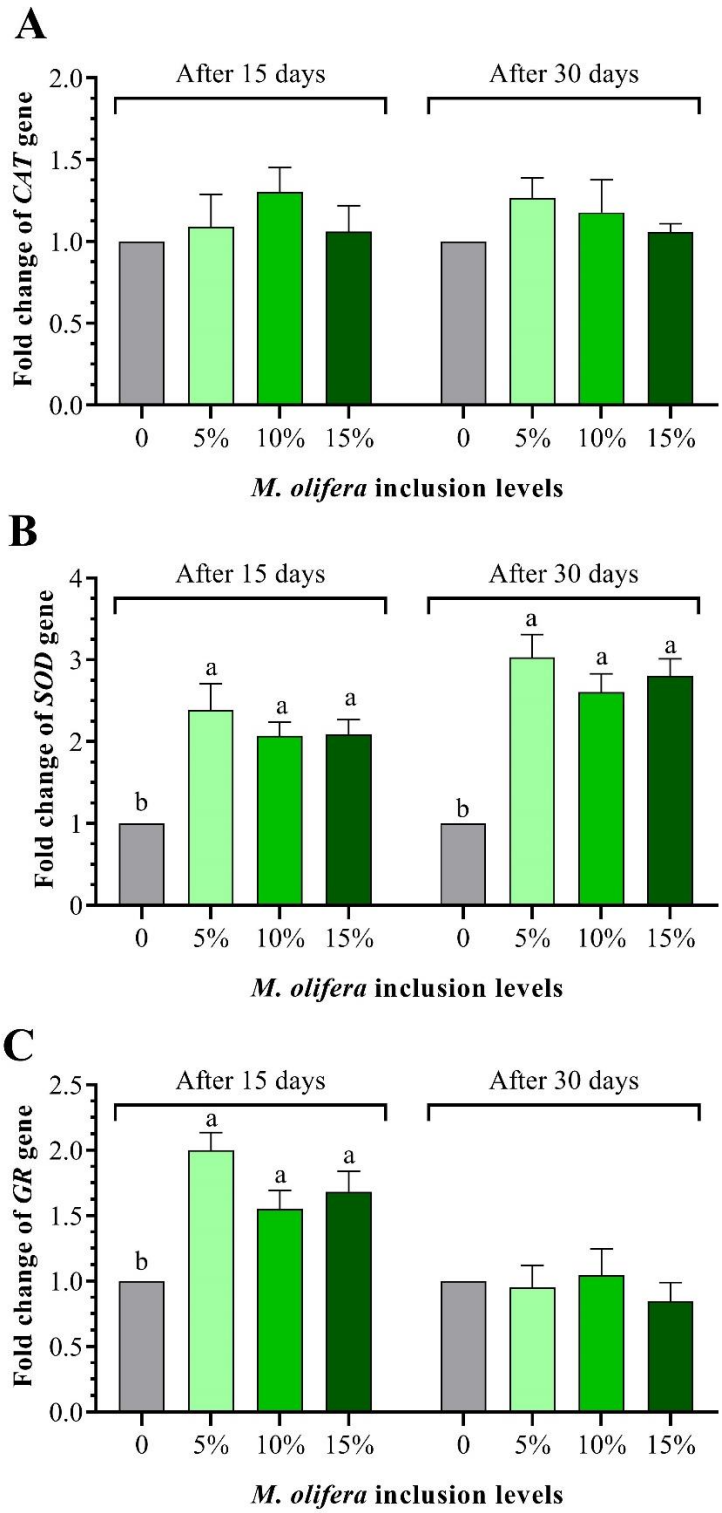

Fig. 2: Effect of dietary *Moringa olifera* leave meal on fold changes of antioxidant enzymes gene expression in liver of Gilthead seabream (*Sparus aurata*). A: catalase; B: superoxide dismutase; C: glutathione reductase. Different letters indicate significant differences among groups at $P \leq 0.05$.

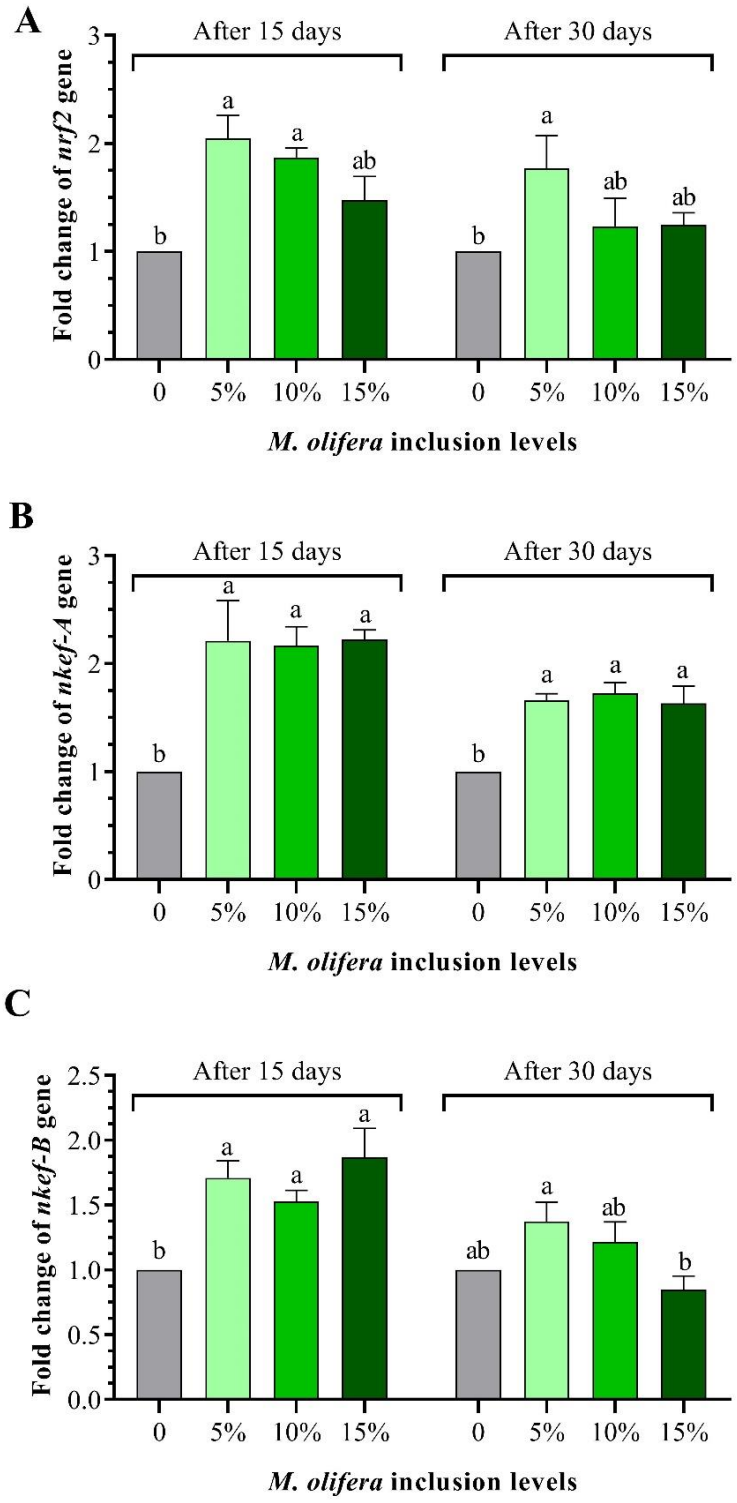

Fig. 3: Effect of dietary *Moringa olifera* leave meal on fold changes of antioxidant enzymes gene expression in liver of Gilthead seabream (*Sparus aurata*). A: *nrf2*; B: *nkef-A*; C: *nkef-B*. Different letters indicate significant differences among groups at $P \leq 0.05$.

Furthermore, oxidative stress is closely linked to inflammation response (Biswas, 2016; Mesa-Garcia et al., 2018). *M. oleifera* has anti-inflammatory properties that reduce the production of pro-inflammatory cytokines (e.g., TNF-α, IL-6, IL-1β) (Mansour et al., 2020). This reduction in inflammatory gene indirectly decreases oxidative stress, allowing antioxidant enzymes to function more efficiently (Hussain et al., 2016).

In the present study, *M. oleifera* reduced oxidative stress and inflammation, it may indirectly reduce oxidative stress-induced gene suppression, allowing *CuZn-SOD* and GR to be more actively transcribed. In the same line, MOL significantly upregulated antioxidants gene expression and modulated inflammatory response in Nile tilapia even after infection with *Eromonas hydrophila* (El-Kassas et al., 2022). However, in the current findings, CAT gene expression remains unchanged while SOD and GR increased suggests a selective regulatory effect of MOL on antioxidant genes. This might be because CAT is already expressed at optimal levels in this tissue, while SOD and GR are more responsive to oxidative stress. The Nrf2 pathway is a key regulator of antioxidant responses (Luo et al., 2023). In the present study the expression of key oxidative stress and transcriptional regulatory genes (*Nrf2, Nkef-A*, and *Nkef-B*) in response to different moringa inclusion levels after 15 and 30 days revealed moringa activated the cellular defense system against oxidative stress. *Nrf2 and Nkef-B* significantly upregulated at 5 and 10% moringa supplementation after 15 days, this effect maintained with 5% moringa after 30 days of treatment. Meanwhile, *Nkef-A* expression was maintaining its upregulation with all moringa supplementation levels along all sampling time.

The molecular effects of MOL could attributed to the high bioactive compounds which could activate Nrf2 and modulate antioxidant gene expression cascade. Flavonoids administration found to activate Nrf2 pathway and inhibited the NF-κB pathway, which in turn enhance the expression of antioxidant enzymes and reduced reactive oxygen species, and inflammatory biomarkers (W. Xu et al., 2022). In same trend, flavonoids reduced liver oxidative-stress by enhancing Nrf2 expression and mediated inflammation genes expression (T. Xu et al., 2022). In addition, several studies proved the effect of phenolic compounds in enhancing Nrf2 expression (Jovanović et al., 2021; He et al., 2022). These results indicated that moringa inclusion could activates and maintain the cellular defense system against oxidative stress.

5 Conclusion:

The obtained results revealed that dietary *M. oleifera* leave meal (MLM) as a reach source of polyphenols and flavonoids significantly enhanced the liver activities of SOD and GR enzymes compared to the control group. However, catalase enzyme remains not affected throughout the experiment. The gene expression of *CuZn-SOD* was significantly upregulating after 15 and 30 days of MOL intervention. However, the significant increase of GR gene expression was reported after 15 days only. CAT gene expression tended to increase with dietary MOL. The regulatory genes expression, including nrf2, nkef-A, and nkef-B were significantly upregulated with dietary MOL at levels of 5-10%. The possible antioxidant mechanism of MLM is influencing the nrf2 pathway that enhances antioxidant defense mechanisms.

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Authors Contribution:

Abdallah Tageldein Mansour: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Writing-Original Draft; Cristóbal Espinosa: Methodology, Investigation – Resources, Data Curation, Writing-Review & Editing; Sabrin Abdalrahman Morshedy: Visualization, Writing-Review & Editing; M. Ángeles Esteban: Conceptualization, Resources - Data Curation, Supervision.

Ethical approval:

The animal study protocol was approved by the Institutional Review Board Murcia University, Murcia, Spain.

Informed consent: not applicable**.**

Data availability statement:

The authors declare that data can be provided by corresponding author upon reasonable request.

Conflicts of interest

There is no conflict of interest to declare.

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