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EFFECTS OF A COMMERCIAL FEED ADDITIVE (SANACORE® GM) ON IMMUNE-ANTIOXIDANT PROFILE AND RESISTANCE OF GILTHEAD SEABREAM (*SPARUS AURATA*) AGAINST *VIBRIO ALGINOLYTICUS* INFECTION

Mohsen Abdel-Tawwab[1♦], Talal A.M. Abo Selema[2], Mahmoud M. Abotaleb[3], Riad H. Khalil[2], Nader M. Sabry[4], Ashraf M. Soliman[5], Elsayed A.A. Eldessouki[6]

[1]Department of Fish Biology and Ecology, Central Laboratory for Aquaculture Research, Agriculture Research Center, Abbassa, Abo-Hammad, Sharqia 44662, Egypt
[2]Department of Poultry and Fish Diseases, Faculty of Veterinary Medicine, Alexandria University, Egypt
[3]Central Laboratory for Evaluation of Veterinary Biologics, Cairo, Egypt
[4]Fish Disease Laboratory, National Institute of Oceanography and Fisheries (NIOF), Egypt
[5]Limnology Department, Central Laboratory for Aquaculture Research Center, Agriculture Research Center, Abbassa, Abo-Hammad, Sharqia, Egypt
[6]Department of Fish Health and Disease, Faculty of Fish Resources, Suez University, Suez, Egypt
♦Corresponding author: mohsentawwab@arc.sci.eg, mohsentawwab@gmail.com

Abstract

The effects of a functional additive (Sanacore® GM; SAN) on immune and antioxidant indices, and the resistance of gilthead seabream (*Sparus aurata*) against *Vibrio alginolyticus* infection were investigated. For this, four diets containing 0% (the control), 0.1%, 0.2%, and 0.4% SAN were offered to triplicated groups of fish (20–23 g) for ten weeks. Subsequently, fish were injected intraperitoneally with *V. alginolyticus* and monitored for further ten days. Feeding the fish on SAN-supplemented diets showed positive effects on leukocyte counts and its differential percentages. Serum lysozyme activity and total immunoglobulin values, as well as blood phagocytic activity and indices, were linearly and quadratically higher in SAN-fed fish, especially at the 0.4% SAN diet. Similarly, linear and quadratic increases in catalase, superoxide dismutase, and total antioxidant capacity were observed in SAN-fed fish, particularly at the 0.4% SAN diet. Conversely, malondialdehyde values decreased in SAN-fed fish compared with the control group, which showed its highest value. The highest expression of the *IL-1β* gene coupled with the lowest *TNF-α* and *HSP70* genes expressions was found in the fish fed with the 0.4% SAN. On the other hand, fish fed on the control diet showed the lowest *IL-1β* gene coupled with the highest *TNF-α* and *HSP70* genes expressions. After bacterial infection, most of the control fish died with a relative percent of survival of 5.0%; meanwhile feeding gilthead seabream on SAN-enriched diets significantly enhanced their protection against *V. alginolyticus* infection fed on the 0.4% SAN diet showed 100% survival. The SAN administration to gilthead seabream especially at the 0.4% level led to significant promotions in antioxidative and immune responses and augmented the fish resistance to *V. alginolyticus* infection.

Key words: feed supplement, gilthead seabream, antioxidative status, immune response, *Vibrio alginolyticus* infection

Gilthead seabream (*Sparus aurata*) is one of the most commercially valuable marine fish species in Mediterranean aquaculture, with a worldwide production of 252,406 metric tons in 2019 (Leon-Bernabeu et al., 2021). Presently, the consumption of this species is well consolidated in Egypt; nevertheless, the aquaculture sector of this fish species is still far from viable, owing to a drop in fish performance and its susceptibility to bacterial infections. Fish intensification is a common practice in aquaculture systems but it may undergo overcrowding anxiety due to their high density leading to growth reduction (Yousefi et al., 2016; Abdel-Tawwab et al., 2018) and oxidative stress (Taheri Mirghaed et al., 2018; Yousefi et al., 2020). Furthermore, crowding stress conditions expose the fish to disease risk, resulting in annual massive mortality. In recent years, there has been an upsurge in concern about the adverse impacts of bacterial infections in aquaculture. Commonly, antibiotics are often used in aquaculture to control and manage several bacterial infections but the antibiotics utilization is prohibited in many countries worldwide due to the environmental pollution and the emergence of novel antibiotic-resistant microorganisms (Alderman and Hastings, 1998; Maron et al., 2013). As a result, aquaculturists are looking for eco-friendly alternative substances to improve fish growth, welfare, and immune systems.

Lately, eco-friendly feed additives such as probiotics, prebiotics, phytobiotics, and organic acids/salts have acquired a great interest in the aquaculture sector due to their positive roles in enhancing the growth, health, and immunological response of several aquatic pathogens (Chakraborty and Hancz, 2011; Chakraborty et al., 2014; Van Hai, 2015; Reverter et al., 2017; Dawood et al., 2018; Alagawany et al., 2020) including vibriosis (Ghosh et al.,

2021; Yilmaz et al., 2022). Fish farmers and feed manufacturers are aware of the importance of functional feed additives to promote fish growth and health status and to strengthen the disease prevention strategy. Sanacore® GM (SAN) is a functional feed supplement has a health-promoting activity with broad-spectrum bacteriostatic and bactericidal activity and it can interrupt the communication system among pathogenic bacteria (Favero et al., 2020).

The production of gilthead seabream (*S. aurata*) is occasionally impeded by mass death because of vibriosis caused by Gram-negative bacteria from the genus *Vibrio*. *V. alginolyticus* is a Gram-negative bacterium that causes otitis and wound infection in many aquatic animals (Reilly et al., 2011) and is responsible for potent neurotoxin and tetrodotoxin formation (Noguchi et al., 1987). *V. alginolyticus* is repeatedly implicated in epidemics spreading in gilthead seabream cultured in the Mediterranean region, pushing fish death and consequential economic loss (Balebona et al., 1998 a, b). Thus, the current investigation illustrated the stimulatory effects of dietary SAN on the antioxidant status, immune response, and resistance of gilthead seabream to *V. alginolyticus* disease.

Material and methods

Diets formulation

SAN was bought from Adisseo Co., Cairo, Egypt, and was added to the control diet (45% crude protein; CP) at the rate of 0% (control), 0.1%, 0.2%, and 0.4%. The control diet was crushed and SAN levels were thoroughly mixed with the diets components for 30 minutes; then 200 mL water was added to each kg of the diet's ingredients. The diet paste was then pushed through a meat grinder, and the consequent threads were dried overnight before being pulverized to the required size (3 mm diameter). The feeds were stored in a fridge at –4°C till usage.

Experimental protocol

Healthy gilthead seabream fingerlings (20–23 g) were bought from a commercial fish farm (Borg-El-Arab, Alexandria, Egypt) and acclimatized with the experimental conditions for two weeks during which they were fed on the control diet (45% CP). Following that, the fish were stocked into 12 cages (30 × 10 × 3 m) at a rate of 8000 fish/cage to represent four treatments with three replicates. Fish were fed on either the control, 0.1%, 0.2%, or 0.4% SAN at 9:00, 13:00, and 17:00 h up to apparent satiation for ten weeks.

Samples collection

At the end of this experiment, ten fish per cage (30 fish/treatment) were taken by drop trap and anesthetized with 30 mg/L buffered tricaine methanesulfonate (MS222; Sigma-Aldrich, USA). The blood samples were then drawn from the caudal vein by a heparinized syringe in Eppendorf tubes. After that, the fish were dissected and liver and anterior kidney specimens were rapidly frozen in liquid nitrogen and stored in a –70°C freezer for subsequent gene transcription analysis.

The blood specimens were used instantly for counting white blood cell and doing the phagocytic assay. Other blood samples were taken without heparin in Eppendorf tubes and centrifuged at 4000 × g at room temperature (Centrifuge 5415 R®) for 10 min to obtain serum samples, which were kept at –20°C for further assays.

Haematological analyses

The leukocytes were quantified using an upgraded Neubauer hemocytometer according to Brown's procedures (Brown, 1988). For differential leukocyte count, blood smears were produced, air-dried, stained in methanol, and stained with May–Giemsa solution. Lymphocytes, monocytes, neutrophils, basophils, and eosinophils were identified in blood smears.

Immunological analyses

The phagocytic examination was performed using the hemocytometer to calculate the number of engulfed yeast (*Candida albicans*) cells by blood leucocytes following Kawahara et al. (1991). This required the determination of the total number of *Candida* cells engulfed by blood leucocytes. The number of *C. albicans* were adjusted to be 1×10^6 cells/mL and the number of blood leucocytes was adjusted to be 2.5×10^6 cells/mL. Afterwards, to each 1 ml of blood leucocytes, 1 ml of the yeast suspension was added, and this mixture was incubated under 5–10% CO_2 at 27°C for 1 h. Smears were prepared and stained with Giemsa stain. At least 100 cells were counted in different fields and phagocytic activity (PA), and phagocytic index (PI) were calculated accordingly as follows:

PA = % of phagocytic cells possessing yeast cells;

PI = Number of yeast cells phagocytized / Number of phagocytic cells.

According to Ellis (1990), serum lysozyme (LYZ) was evaluated turbidimetrically with *Micrococcus luteus*. One unit of LYZ activity referred to the quantity of enzyme that causes a 0.001/min drop in absorbance in 1 mL serum. According to Siwicki and Anderson (1993), total serum immunoglobulin (total Ig) was calculated by precipitating Ig with polyethylene glycol and subtracting the initial and final total protein.

Evaluation of antioxidant capacity

Liver tissue (one gram) was homogenized with 10 mL of buffer (100 mM-Tris-HCl, 0.1 mM-EDTA, and 0.1 percent triton X-100 (v/v), pH 7.8) and centrifuged for half an hour at –4°C. Afterwards, the supernatants were used to measure catalase (CAT) and superoxide dismutase (SOD) using commercial fish-specific reagents (Biodiagnostic Co., Giza, Egypt). Malondialdehyde (MDA) levels were measured using the thiobarbituric acid technique (as a biomarker of lipid peroxidation), as

reported by Ohkawa et al. (1979). The activity of hepatic SOD was evaluated following McCord and Fridovich (1969) where one unit of SOD activity refers to the quantity of SOD necessary for a 50% reduction in cytochrome C reduction rate (i.e., to a rate of 0.0125 absorbance unit per min). According to Aebi (1984), the hepatic CAT activity was measured where one unit of CAT activity refers to the amount of enzyme necessary to break down one million moles of hydrogen peroxide per minute. According to Bartosz (2003), total antioxidant capacity (TAC) was determined using a colorimetric test on serum samples by the interaction of antioxidants with a specific quantity of exogenously given hydrogen peroxide.

Hepatic and renal genes expressions

Total RNA was isolated from (50 mg) liver and anterior kidney tissues (3 fish per aquarium, n = 9) using TRIzol reagent (Invitrogen, USA) following the manufacturer's instructions. The purity and volume of the collected RNA were determined using an ND-2000 spectrophotometer (NanoDrop 2000, Wilmington, DE, USA) (Zhang et al., 2018). Table 1 lists the primer sequences and NCBI Genbank database codes for genes used in quantitative real-time PCR (qRT-PCR). The PCR was programmed to maintain at 50°C for 2 min. Initial denaturation at 95°C for 3 min, followed by 45 rounds of 95°C for 20 s, 60°C for 20 s and an extension at 72°C for 60 s. The thermodynamic rounds were completed with a 10-minute extension at 72°C. The quantitative real-time of interleukin-1 beta (*IL-1β*), tumor necrosis factor-alpha (*TNF-α*), and heat shock protein 70 (*HSP70*) genes were adjusted to beta-actin (*B-actin*) gene used as a reference or housekeeping gene using the $2^{-\Delta\Delta CT}$ technique (Schmittgen and Livak, 2008).

Table 1. Primers used in RT-PCR assay in the present study

Target mRNA	Primer sequence 5′-›3′ (F: forward, and R: reverse)	GenBank Accession number
IL-1β	F: ATCTGGAGGTGGACAAAGC	AJ277166.2
	R: GCTGATGTTCAAACCGGAGTC	
TNF-α	F: ATGGTGGCGTACACAACT	AJ413189
	R: AACTCTCCGGTGAAGAGCTGT	
HSP70	F: AATGTTCTGCGCATCATCAA	EU805481
	R: GCCTCCACCAAGATCAAAGA	
β-actin	F: ATCGTGGGGCGCCCCAGGCACA	X89920
	R: CTCCTTAATGTCACGCACGATTTC	

IL-1β: Interleukin 1 beta, *TNF-α*: Tumor necrosis factor alpha, *HSP70*: Heat shock protein 70, *β-actin*: beta actin.

Bacterial infection test

After the feeding trial, fish resistance to *V. alginolyticus* infection was tested. A preliminary experiment was conducted before the challenge test to establish the pathogenic bacteria's LD_{50} (lethal dosage). Isolated strains were cultured in nutrient broth with yeast extract (NBYE) for 24 h before being incubated at 35°C. Then, bacterial cells were pelletized by centrifugation at 3,000 × g for 30 min and resuspended in 1.0 mL of 0.1% peptone water. However, fish were intraperitoneally (IP) injected with different levels of 24-h live bacteria; then, the mortality of the infected fish was recorded after 3 days of the infection. The LD_{50} which resulted in 50% fish mortality was found to be 1×10^6 CFU/mL and a sublethal dose was used for the bacterial challenge.

Ten fish from each cage were taken and moved into a new 100-L aquarium (n = 3 aquaria) for doing the challenge test. Following Schäperclaus (1992), fish were IP injected with 0.1 mL of *V. alginolyticus* ($\times 10^5$ CFU/mL) and then returned to the experimental aquaria. In addition, purified saline solution (0.85%) was IP injected in three fish used as a negative control. After 12 hours of bacterial infection, the fish were fed on the corresponding diets subsequently. Fish in all treatments were maintained for ten days to record any abnormal clinical signs and fish death. According to Amend (1981), the relative percentage of survival (RPS) of fish was determined after the bacterial challenge as follows:

RPS (%) = 100 [1 (% death in treated fish / % death in control fish)].

Data analysis

Before statistical analysis, the collected data were examined for normality of distribution using the Kolmogorov–Smirnov test and homogeneity of variances among different treatments using Bartlett's test. The data were then subjected to a one-way ANOVA with a Tukey post-hoc test to identify the impacts of dietary SAN amounts at $P<0.05$. The data were statistically analyzed using SPSS v.22 as described by Dytham (2011).

Results

Hematological assay

Leukocyte counts and their differentiation in gilthead seabream fed on SAN-containing diets are presented in Table 2. It is noted that feeding fish on SAN-containing diets linearly and quadratically increased the leukocyte counts, lymphocytes %, and monocytes %, where their highest values were significantly ($P<0.05$) observed in the treatment of 0.4% SAN. On the other hand, linear and quadratic decreases ($P<0.05$) in basophils % and eosinophils % were recorded in the treatments of 0.4% SAN compared with the control group (Table 2).

Antioxidative capacity

Dietary SAN significantly affected SOD, CAT, TAC, and MDA levels (Table 3). However, SOD, CAT, and TAC values showed linear and quadratic increases coupled with lower values of MDA levels along with increasing the levels of dietary supplementation of SAN, especially at the treatment of 0.4% SAN. However, fish fed with the control diet displayed the highest MDA values parallel with the lowest values of SOD, CAT, and TAC.

Table 2. Counts and differentiation of white blood cells (WBC) of gilthead sea bream (*Sparus aurata*) fed with different levels of Sanacore (SAN) for ten weeks

	SAN levels (%)				P value	
	0.0 (control)	0.1	0.2	0.4	linear	quadratic
WBC (cells $\times 10^3$ μL)	15.49±0.505 d	17.49±0.576 c	21.42±0.655 b	27.99±0.303 a	<0.001	<0.001
Lymphocytes (%)	39.3±0.88 d	44.0±0.58 c	50.0±0.58 b	57.3±0.33 a	<0.001	<0.001
Neutrophils (%)	1.3±0.33	1.4±0.33	1.3±0.33	2.0±0.00	0.092	0.166
Basophils (%)	18.0±0.58 a	15.3±0.33 b	12.0±0.00 c	7.7±0.33 d	<0.001	<0.001
Eosinophils (%)	23.7±0.88 a	20.0±0.00 b	15.3±0.33 c	8.7±0.33 d	<0.001	<0.001
Monocytes (%)	17.7±0.33 d	19.3±0.67 c	21.4±0.33 b	24.3±0.33 a	<0.001	<0.001

Means having the same letter in the same row are not significantly different at P≤0.05.

Table 3. Antioxidant parameters of gilthead sea bream (*Sparus aurata*) fed with different levels of Sanacore (SAN) for ten weeks

	SAN levels (%)				P value	
	0.0 (control)	0.1	0.2	0.4	linear	quadratic
MDA (nmol/mL)	26.2±0.52 a	23.3±1.10 b	19.2±0.77 c	16.9±0.84 d	<0.001	00.002
SOD (U/mg protein)	2.1±0.15 d	3.4±0.26 c	4.5±0.56 b	5.7±0.75 a	<0.001	0.001
CAT (U/mg protein)	1.89±0.073 d	2.32±0.077 c	2.86±0.053 b	3.16±0.029 a	<0.001	0.001
TAC (mM/L)	0.38±0.021 d	0.46±0.028 c	0.55±0.018 b	0.64±0.022 a	<0.001	0.001

Means having the same letter in the same row are not significantly different at P≤0.05.
MDA, malondialdehyde; SOD, superoxide dismutase; CAT, catalase; TAC, total antioxidant capacity.

Table 4. Immunological parameters of gilthead sea bream (*Sparus aurata*) fed with different levels of Sanacore (SAN) for ten weeks

	SAN levels (%)				P value	
	0.0 (control)	0.1	0.2	0.4	linear	quadratic
Phagocytic activity (%)	20.3±0.33 c	22.3±0.88 c	26.3±0.89 b	31.0±0.58 a	<0.001	<0.001
Phagocytic index	2.04±0.015 c	2.11±0.021 c	2.49±0.012 b	3.28±0.044 a	<0.001	<0.001
LYZ activity (Unit/mL)	0.11±0.006 c	0.13±0.003 c	0.22±0.003 b	0.31±0.014 a	<0.001	0.001
Total Ig (mg/mL)	1.02±0.061 d	1.25±0.054 c	1.46±0.025 b	1.71±0.052 a	<0.001	0.001

Means having the same letter in the same row are not significantly different at P≤0.05.
LYZ, lysozyme; total Ig, total immunoglobulin.

Immunological biomarkers and bacterial challenge

Linear and quadratic increases in phagocytic activity, phagocytic index, LYZ activity, and total Ig levels were observed along with an increase in dietary SAN supplementation, especially at the 0.4% treatment, which exhibited their highest values (Table 4). Furthermore, the RSP of gilthead seabream significantly enhanced as SAN levels increased in diets, and the highest RSP value was recorded in fish fed with the 0.4% SAN (100.0%; Figure 1). On the other hand, fish fed on the control diet exhibited the lowest RSP value (5.0%; Figure 1).

Genes mRNA expressions

The dietary supplementation of SAN to gilthead seabream significantly affected the expression of hepatic and renal *IL-1β, TNF-α,* and *HSP70* genes (Figures 2 and 3). The lowest mRNA expression of the *IL-1β* gene was observed in the control treatment; while, feeding fish on the 0.4% SAN diet showed the highest *IL-1β* gene expression. Conversely, hepatic and renal *TNF-α* and *HSP70* genes expressions were linearly and quadratically downregulated along with the increase in dietary SAN levels and their lowest expressions were found in the treatment of 0.4% SAN. Meanwhile, their highest mRNA expressions were detected in fish fed on the control diet (Figures 2 and 3).

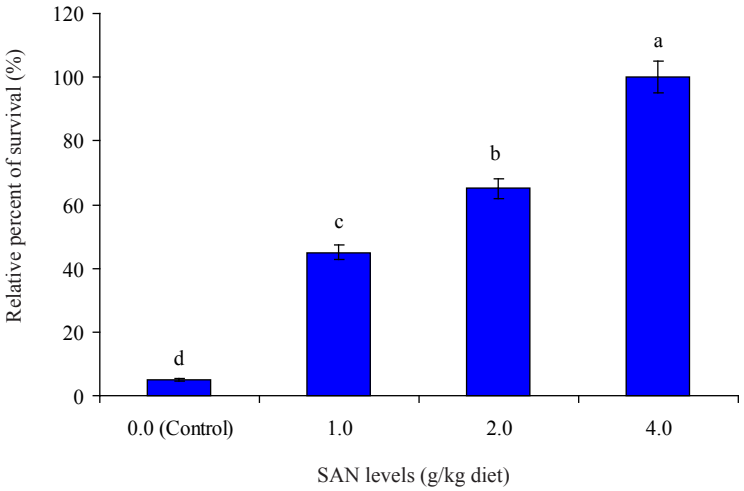


Figure 1. Changes in relative percent of survival of gilthead sea bream (*Sparus aurata*) fed with different levels of Sanacore (SAN) for ten weeks and post-challenged by *V. alginolyticus* infection for 10 days. Bars assigned by different letters are significantly different at P<0.05

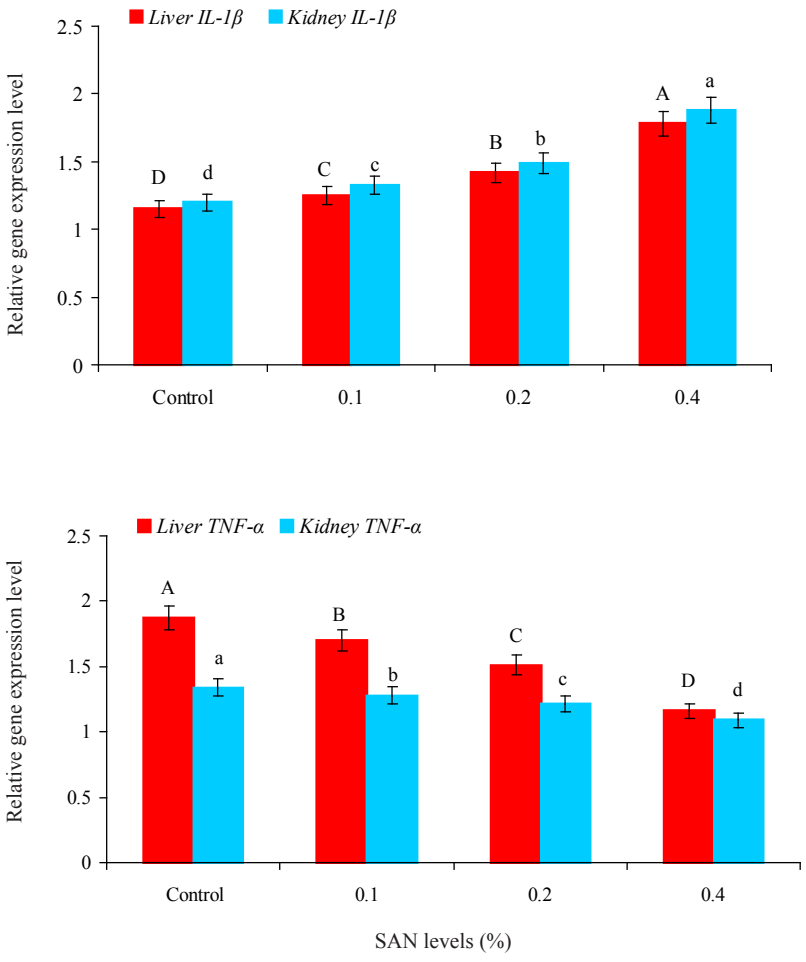


Figure 2. Relative gene expression levels of IL-1β and TNF-α in the liver and kidney of gilthead sea bream (*Sparus aurata*) fed with different levels of Sanacore (SAN) for ten weeks compared with the control group. Bars having different letters (capital letters for liver and small letters for kidney) are significantly different at P<0.05 (Tukey HSD test)

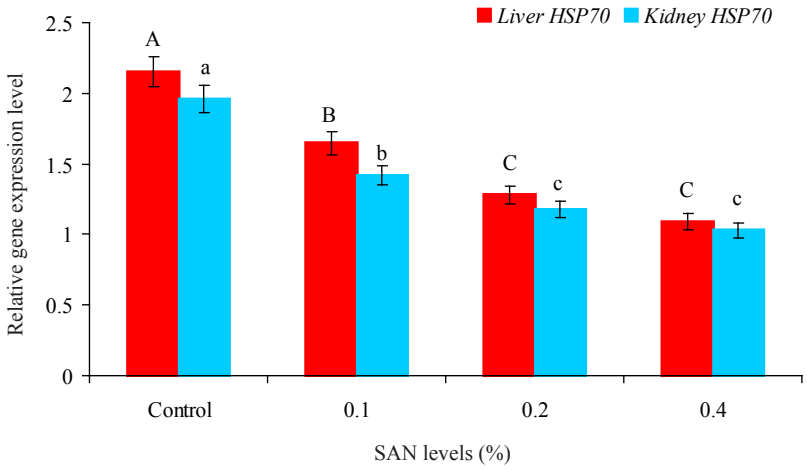


Figure 3. Relative change expression levels of HSP70 in liver and kidney of gilthead sea bream (*Sparus aurata*) fed with different levels of Sanacore (SAN) for ten weeks compared with the control group. Bars having different letters (capital letters for liver and small letters for kidney) are significantly different at $P<0.05$ (Tukey HSD test)

Discussion

Fish hematology has a beneficial role in monitoring fish health status. In the current study, dietary supplementation with SAN at a level of 0.4% significantly increased leukocyte count, lymphocytes % and monocytes %, accompanied with significantly lower basophils % and eosinophils %, particularly in the fish fed on the 0.4% SAN diet. Lymphocytes are cells of adaptive immunity that can eliminate wounded somatic cells (tumors or virus-infected cells) and release immune modulation cytokines (Tizard, 2002; Raulet, 2004). Monocytes have phagocytosis and unspecific cytotoxic capabilities, and they are considered transitory blood cells since they travel across connective tissue and transform into macrophages throughout the inflammatory process (Meseguer et al., 1994; Witten et al., 1998; Cuesta et al., 1999). Hence, the obtained results herein indicate the positive effects of dietary SAN on the immunological status of gilthead seabream. In a similar study, Župan et al. (2015) fed gilthead seabream on diets with IMUNO-2865®, which is a commercial product containing hemicellulose (high molecular weight polysaccharide complex), mixture extracted from *Gramineae, Poaceae,* mushrooms, grapeseed and containing beneficial amino acids, oligosaccharides, glycoproteins, polyphenols, and fatty acids mixture. They found that dietary IMUNO-2865® increased the number of circulating neutrophils and monocytes in fish and they attributed these results to the enhancement in the non-specific immune response.

Fish have a vital defense mechanism called the innate immune system (Secombes and Fletcher, 1992; Magnadottir, 2006; Saurabh and Sahoo, 2008). The innate immune system's main components are cellular (macrophages, monocytes, and granulocytes) and humoral (LYZ, complement C3, natural antibodies, cytokines, and antimicrobial peptides) (Magnadottir, 2006; Saurabh and Sahoo, 2008; Biller-Takahashi and Urbinati, 2014). Leukocytes (mostly monocytes and neutrophils) generate lysozyme, which attacks the pathogen's cell wall's peptidoglycan (Ellis, 1999, 2001; Magnadottir, 2006; Uribe et al., 2011). Various immune-stimulant products target the immune system, and blood leukocytes are vital parts of the fish's innate immunity activated by immune-stimulant administration (Chakraborty and Hancz, 2011; Uribe et al., 2011; Biller-Takahashi and Urbinati, 2014). Dietary SAN, in the current study, boosted soluble immune (total Ig and lysozyme) and cellular innate immune components (leukocytes), as well as phagocytic index and activity. These results are much more expected because dietary SAN contains several compounds with antibacterial activity and immunological functions. The rise in LYZ activity could be attributed to the increase of neutrophil numbers and/or LYZ production (Costas et al., 2011; Uribe et al., 2011). In a similar study that used commercial feed supplements, Župan et al. (2015) discovered that IMUNO-2865® supplementation significantly elevated leukocyte counts, SOD, and glutathione peroxidase (GPx) of sea bream. Magouz et al. (2020 a, b) found significant increases in lysozyme and phagocytic activities in Nile tilapia and common carp, respectively, fed with AQUAGEST®.

The dietary supplementation of SAN to gilthead seabream in the present study linearly and quadratically enhanced the phagocytosis activity. During phagocytosis, oxygen exhaustion increases via a molecular process known as leukocyte respiratory burst, resulting in oxygen drop with superoxide anion formation (Biller-Takahashi and Urbinati, 2014). The SOD reacts with the superoxide anion and produces hydrogen peroxide. The resulting hydrogen peroxide produced by SOD in turn is decomposed by CAT (Rzeuski et al., 1998). Furthermore,

granular leukocytes produce myeloperoxidase enzymes that react with hydrogen peroxide generating hypochlorite, which leads to the formation of chloramines (Biller-Takahashi and Urbinati, 2014). All these molecules are oxidative substances and can damage cells membranes (Verlhac and Gabaudan, 1997; Caipang et al., 2012). SOD and CAT enzymes are implicated in the endogenous enzymatic antioxidant mechanisms that help to counteract the negative effects of oxidative stress where their principal functions are to conserve the redox homeostasis and modulate any inequality in the antioxidant system (Livingstone, 2001; Abdel-Tawwab and Wafeek, 2017; Hoseinifar et al., 2021). Reduced activity of these enzymes causes lipid peroxidation and the increase in MDA production (Yousefi et al., 2018). The dietary SAN exhibited high antioxidant capabilities characterized by enhanced levels of SOD, CAT, and TAC, coinciding with the decrease in MDA levels in the fish. Studies on other commercial feed additives found similar antioxidant effects (Abdel-Tawwab et al., 2020). Župan et al. (2015) found that IMUNO-2865® supplementation promoted sea bream antioxidative stress response since SOD and GPx values increased in IMUNO-2865® treated fish groups. The use of multiple functional feed supplements is supposed to be more efficient than solitary supplements. In this regard, Abdel-Tawwab et al. (2020) fed common carp with multi-stimulants blend (AQUAMAX) and found that SOD, CAT, and GPx activities significantly raised, while lipid peroxidation, measured as MDA, significantly decreased with AQUAMAX supplementation up to 1.0–5.0 g/kg diet. Zhang et al. (2021) investigated the influence of complex Chinese herbal medicines (CCHM) on growth performance, digestive enzyme activity, antioxidant status, immunity, and *Vibrio parahaemolyticus* resistance in white leg shrimp (*Litopenaeus vannamei*). They discovered that SOD, CAT, and GPx activities were considerably higher in the hepatopancreas of shrimp fed a 10 g CCHM/kg diet than in the control group.

Cytokines are inflammatory markers in aquatic animals, and stress condition leads to inflammation in fish (Yarahmadi et al., 2016; Hoseini et al., 2019). Interleukins are a subset of cytokines implicated in the regulation of the fish immune system (Secombes et al., 2011; Savan and Sakai, 2006). Furthermore, finding these genes in the fish body aids in the development of critical applications for the advancement of immune stimulants in the aquaculture industry. Moreover, the pro-inflammatory cytokines *IL-1β* and *TNF-α* have roles in fish hematopoiesis, inflammatory responses, and adaptive immunity (Secombes et al., 1996). *TNF-α* is also concerned with inflammation, apoptosis, and cell proliferation (Goetz et al., 2004). Because of its essential function in enhancing cellular immunity, especially under stress, *HSP70* is one of the primary genes necessary to maintain cellular proteins and defend cells against apoptotic changes (Ming et al., 2010). The expression of the *HSP70* gene is generally upregulated in response to various stressors to protect the organism against cellular damage (Padmini and Usha Rani, 2008). The current study revealed that SAN-fed fish showed higher mRNA expression levels of the *IL-1β* gene, whereas *TNF-α* and *HSP70* genes were downregulated. These findings suggest that dietary SAN improves immunological responses and has anti-inflammatory properties. As a result, the highest level of SAN supplementation (0.4%) in this experiment may have the immune stimulatory effects on the immune system's first line of sea bream.

The current study showed that the RSP of gilthead seabream against *V. alginolyticus* infection was significantly enhanced as SAN levels increased in diets and it was 100% at the 0.4% SAN treatment, whereas fish fed on the control diet showed the lowest RSP value (5.0%). In this regard, Favero et al. (2020) proved the antimicrobial activity of SAN where significant reductions of the cells count and alteration in the morphology of *Streptococcus agalactiae* and *Francisella noatunensis* subsp. *orientalis* cells were observed at different SAN concentrations. In a similar study, Favero et al. (2020) fed Nile tilapia with different SAN levels at 0.3% for 20 days and finally, were challenged with *F. noatunensis* supbsp. *orientalis* via water immersion, and with *S. agalactiae* via the intraperitoneal route. They found that the SAN-fed fish groups presented significantly lower mortality compared to the control fish group, of 32% and 17% for franciselosis and streptococcosis, respectively. Palenzuela et al. (2020) compared the effects of dietary SAN at two different levels to a basal diet through a laboratory-controlled challenge with parasite diseases transmitted by *Enteromyxum leei*. They found that SAN-enriched diets reduced anorexia and growth inhibition in parasite-challenged fish, particularly in the highest SAN dosage group, which did not vary substantially from the control group. They attributed these results to the benefits of the SAN inclusion in fish diets at the higher dose. Furthermore, different phytobiotics, prebiotic, and probiotic ingredients were shown to improve the immune status and enhance the fish resistance against the possible bacterial infection (Hoseinifar et al., 2015; Ringø and Song, 2016; Vallejos-Vidal et al., 2016; Banerjee and Ray, 2017; Piazzon et al., 2017; Abdel-Latif et al., 2020; Ghosh et al., 2021; Yilmaz et al., 2022).

Conclusion

Finally, dietary SAN exhibits anti-inflammatory, immunological, and antioxidant benefits in gilthead seabream, particularly at the level of 0.4%. Feeding fish at this SAN level reduces inflammation, oxidative stress boosts immunological responses and increases resistance to *V. alginolyticus* infection. Further work is needed to explore the effects of dietary SAN on intestinal microbiota and histopathological architecture.

Data availability statement

Data of the present article are available under request.

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of fish were followed by the authors.

Consent for publication

All authors reviewed and approved the manuscript for publication.

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