



Short communication

Supplementation with imuno-2865[®] in gilthead sea bream (*Sparus aurata* Linnaeus, 1758): Effects on hematological and antioxidant parameters



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ABSTRACT

The aim of this study was to evaluate the effects of IMUNO-2865[®] on hematological and antioxidative parameters in sea bream. Total of 640 sea bream were fed with diets containing 0 (Group 1), 1 (Group 2), 10 (Group 3) and 25 (Group 4) g of IMUNO-2865[®] kg⁻¹ feed during 90 days. Samples were taken each month and three months after the supplementation. A significant heterophils increase was observed in group 4 compared to group 1 after two months, and an increase in monocytes number was observed in group 4 compared to the other groups after one month. Glutathione peroxidase (GSH-Px) and paraoxonase-1 (PON1) were significantly increased in groups 3 and 4 compared to the control group three months into the experiment. Superoxide dismutase (SOD) was increased in group 4 compared to the control group from day 60 until the end of the experiment, and in groups 2 and 3 compared to the control after three months. Based on the differences in the cellular immunity and oxidative stress parameters, with an overall absence of mortality, the results of this study suggest that the use of IMUNO-2865[®] in aquaculture is safe and possess a cumulative immunostimulatory effect on sea bream.

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There is a strong push to replace the use of conventional chemical products in commercial aquaculture with naturally derived additives, which could be cost-effectively incorporated into the fish diet, and would significantly eliminate the environmental impact and other negative side-effects [4,9]. So far, numerous non-specific immunostimulants have been tested under aquaculture conditions, like medicinal plants [11], probiotics [3], prebiotics [6], beeproducts (Cuesta et al. 2004; [31], yeast [2,28], microalgae [35], Arabic gum [13], and other). However, only few are considered suitable for use in commercial aquaculture, such as products based on β-glucans, commonly derived from yeast or mushrooms (fungal

mycelia). β-glucans supplements seem to be promising in aquaculture, although its effects on the fish immune system varies depending of the fish species, route of application, dose and duration of administration, and its biochemical interaction with other immunostimulants [2,10,23,25,32].

IMUNO-2865[®] is a naturally obtained hemicellulose (high molecular weight polysaccharide complex) mixture extracted by a protected proprietary process from natural sources (*Gramineae*, *Poaceae* family of plants and mushrooms, grapeseed extracts) which contains a beneficial amino acids, oligosaccharides, glycoproteins, polyphenols and fatty acid mixture. Although well documented as healthy and natural supplement which enhances immune system function in humans [33,34] and marine mammals [26,30], its effect on the immune system of teleost fish is unknown. Thus, the aim of this study was to evaluate the effects of IMUNO-2865[®] on hematological and oxidative stress response in sea bream,

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the most important species in the Mediterranean aquaculture, as a basis for the introduction of this supplement for its commercial use in aquaculture.

The study was conducted over a period of 6 months at a sea bream farm in the Central Adriatic. All fish handling procedures during the sampling period followed the established standards for the humane care and use of animals, approved by the Ethical Committee for Animal Welfare of University of Zadar, Croatia. A total of 640 sea bream specimens from the same generation and origin (18 month old, weighing 277.8 ± 38.0 g) were divided into four groups, placed in 8 m^3 net cages, and were conditioned for two weeks. Fish were fed with commercial pelleted food (Skretting AS, Address: Sjøhagen 15, 4016 Stavanger/P.b. 319 Sentrum, 4002 Stavanger, Norway Skretting, Norway) at a rate of 10 g kg^{-1} dry diet biomass a day. Four experimental diets containing 0 g (control Group 1), 1 g (Group 2), 10 g (Group 3) and 25 g (Group 4) of IMUNO-2865[®] kg^{-1} feed were prepared 2 weeks in advance and stored at 4°C . In order to prepare the diets, an allocated amount of the commercial pellets was crushed, mixed with the appropriate IMUNO-2865[®] concentration and water, and made again into pellets, which were allowed to dry and were stored at 4°C until further use. Control diets were prepared in the same manner, but adding water only. Blood samples were collected on days 0, 30, 60, and 90, after which the supplementation ceased, and regular feeding regime was resumed at $15 \text{ g dry diet kg}^{-1}$ biomass a day. A final sampling was performed on day 180 (90 days after consumption of the last experimental diet). Temperature and dissolved oxygen were measured daily at the center of each cage at the depth of 1 m using Oxyguard handy alpha (OxyGuard, International A/S, Blokken 59, 3460, Birkeroed, Denmark). The sea temperature fluctuated among periods and averaged $19.1 \pm 0.44^\circ\text{C}$ (October), $17.3 \pm 0.39^\circ\text{C}$ (November), $14.2 \pm 0.33^\circ\text{C}$ (December), $12.1 \pm 0.41^\circ\text{C}$ (January), and $19 \pm 0.79^\circ\text{C}$ (March) during the experiment periods, respectively. Throughout the experiment, dissolved oxygen saturation remained between 90% and 100%, indicating that fish were not oxygen-stressed. Salinity was measured on the sampling days and was stable at 38.0 g L^{-1} throughout the experiment. Body weight (BW) and standard length (SL) were recorded for growth evaluation at each sampling time. The condition factor (CF) was calculated as $\text{BW (g)}/\text{SL}^3 \text{ (cm)} \times 100$. The specific growth rate (SGR) was calculated as the increase of weight (g) in respect to a given time interval (days). The fish was not fed the day before sampling and on sampling days.

Upon net retrieval from the cage, individual sea bream specimen deeped for 1 min in the anesthetic solution (0.1% 2-phenoxyethanol, Sigma). Once anaesthetized (within 1 min), the blood was collected from the caudal vein using a 5 ml syringe and a $0.8 \times 38 \text{ mm}$ hypodermic needle. All blood samples were collected within 3 min upon capture. For hematological parameters, 2–3 ml of blood was transferred in 4 ml lithium heparin tube and stored at 4°C until processing the same day within 8 h, while the remaining blood was divided into two 1.3 ml lithium heparin tubes (Sarstedt), centrifuged at 12,000 G for 90 s and resultant plasma was frozen at -85.0°C until further analysis. Total of 20 fish per group were sampled on each day of the sampling periods.

The hematological parameters (CBC and differential blood count: erythrocytes, total leukocytes, heterophils, basophils, eosinophils, monocytes, lymphocytes, and thrombocytes) were determined by a combined method that was standardized and cross-checked initially by the standard Natt and Herrick method for counting erythrocytes and leukocytes. The blood was diluted 200X w/Hayem's solution and the cells were manually counted in the Neubauer hemocytometer. Based on the cell count in the hemocytometer in 1 mm^3 of blood and ratio between different cells based on 3,000 cells, the cell number in 1 mm^3 was counted and

calculated for 1 L. Differential blood cell count and cellular morphology evaluation was performed on blood smears stained with May-Grünwald-Giemsa and evaluated microscopically at 1000X magnification (Immersion objective).

The serum activity of total *superoxide dismutase* (SOD-EC1.15.1.1) and glutathione peroxidase (GSH-Px-EC1.11.1.9) were determined by Randox commercial kits using the automated biochemistry analyzer (Olympus AU 640). The activity of paraoxonase-1 (PON1-EC3.1.8.1) was assayed by modified method of hydrolysis of paraoxon described by Charlton-Menys et al. [8] on the Olympus AU 640. Enzyme activities were presented in UL^{-1} ($1 \mu\text{mol p-nitrophenol formed}/\text{min}/\text{L}$).

All assays described were performed in three replicates in laboratory from 20 fish per group. Two-way ANOVA without replication was applied for testing differences in hematological and oxidative stress parameters, with ratio of supplement in feed (group) and sampling periods (month) as factors of interest, following normality test and Levene's test for homogeneity of variance. Holm-Sidak method was used for a post hoc comparison between groups and sampling periods. Differences were considered significant considered significant when $p \leq 0.05$.

No mortality was recorded throughout the experiment. No statistically significant differences were noted in BW increase between the experimental groups. Generally, BW and SL growth was ceased during the coldest period and did not vary among the groups throughout the trial, as well as CF, which decreased in all groups from November to January, and increased again in April.

Although blood parameters fluctuated among sampling periods (e.g. erythrocyte and leukocyte counts were highest at the end of the experiment), while lymphocytes were highest at the beginning, no significant differences were noted among treatment groups for total number of erythrocytes, leukocytes, basophils, lymphocytes and thrombocytes (Fig. 1). A significant increase in heterophils was observed in group 4 compared to the control group after two months (in December) ($P < 0.05$), and an increase in monocytes number was observed in group 4 compared to the other groups after one month (in November) of supplementation ($P < 0.05$). Number of eosinophils was highest prior to supplementation in group 3, while in groups 2 and 4 were higher compared to the control group ($P < 0.05$). A significant decrease of eosinophils was observed within group 2 after two months of supplementation to the end of the experiment ($P < 0.05$), and in group 3 after one month of supplementation to the end of the experiment ($P < 0.05$).

Glutathione peroxidase (GSH-Px) (U L^{-1}) and PON1 were significantly increased in groups 3 and 4 compared to the control group three months (in January) into the experiment ($P < 0.05$). Superoxide dismutase (SOD) (U L^{-1}) was increased in group 4 compared to the control group from December until the end of the experiment (April). Groups 2 and 3 also demonstrated increased SOD compared to the control after three months of supplementation ($P < 0.05$). Surprisingly, serum paraoxonase (PON1 in U L^{-1}) was elevated in the control group after two months compared to the treatment groups ($P < 0.05$) (Fig. 2).

In our study, intake of different doses of IMUNO-2865[®] did not significantly alter the total number of erythrocytes, leukocytes, basophils, lymphocytes and thrombocytes. However, the supplementation with 25 g of IMUNO-2865[®] kg^{-1} of regular fish diet resulted with a statistically significant increase in the number of heterophils after 60, and number of monocytes after 30 days of the experiment, compared to the control and other treatment groups. These cells are the main phagocytic cells with a primary role in the removal of microorganisms (e.g. bacteria) and small particulate matter [21]. In this experiment, an overall increase in the number of circulating neutrophils and monocytes may be attributed to the increase in the non-specific immune response to external stress

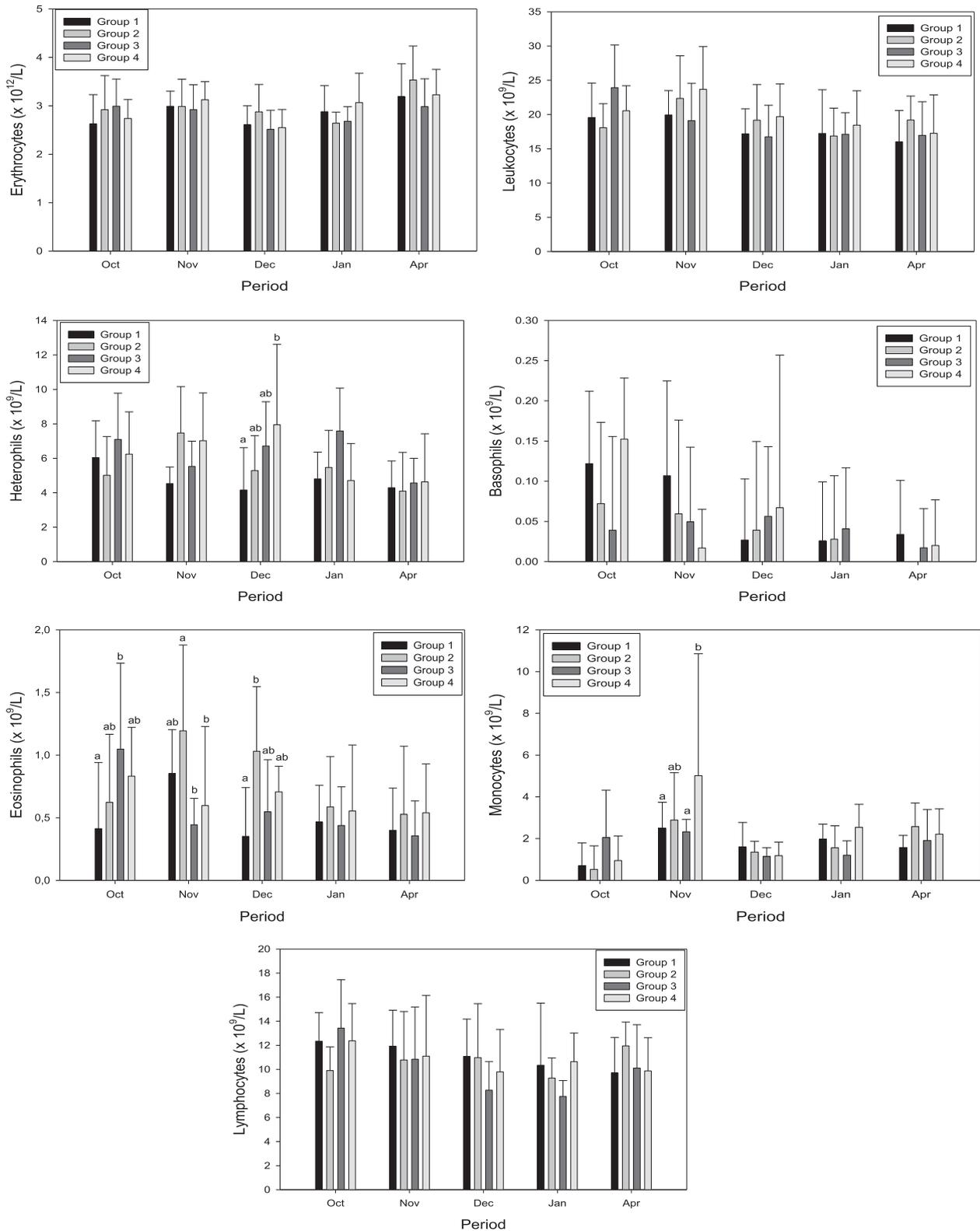


Fig. 1. Variations (Mean \pm SD) in hematological parameters in the control group (Group 1) and treatment groups (Groups 2, 3 and 4) supplemented with 0, 1.0, 10.0 and 25.0 g of IMUNO-2865[®] kg⁻¹ of feed, respectively. Different letters indicate statistical significant differences between groups ($P < 0.05$). Two-way ANOVA was used to test differences among groups and periods ($P < 0.05$) followed by Holm-Sidak post hoc test ($N = 20$).

[29], since no mortality and no pathology was noted in any of fish to suggest the occurrence of clinical disease. Therefore, the highest dose of supplementation used in this experiment may had the

strongest influence on the first line of the sea bream immune system under aquaculture conditions. Although the reason of elevated eosinophile numbers at the beginning was unknown, we

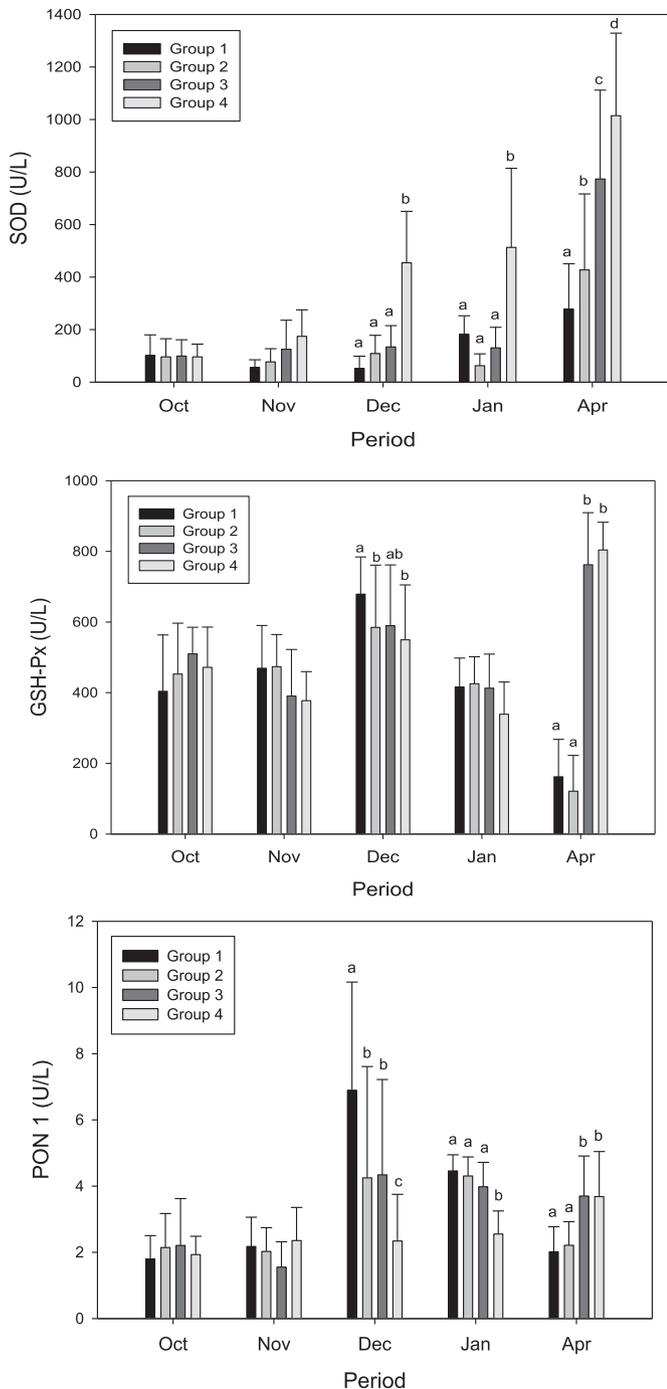


Fig. 2. Variations (Mean \pm SD) of antioxidative enzymes (PON 1, GSH-Px, SOD) in the control group (Group 1) and treatment groups (Groups 2, 3 and 4) supplemented with 0, 1.0, 10.0 and 25.0 g of IMUNO-2865[®] kg⁻¹ of feed, respectively. Different letters indicate statistically significant differences between groups ($P < 0.05$). Two-way ANOVA was used to test differences among groups and periods ($P < 0.05$) followed by Holm-Sidak post hoc test ($N = 20$).

speculate that the supplementation with minimum (group 2) and medium (group 3) dosage of IMUNO-2865[®] may have some stimulating effect on the release of eosinophils into the blood, as a part of the overall immune stimulation and the reaction to the presence of a novel substance being resorbed from the intestine.

Oxidative stress has become an important area of interest in aquatic toxicology [24]. Reactive oxygen species (ROS), such as superoxide anion radicals, hydrogen peroxide, and hydroxyl

radicals, are continuously formed in fish through many physiological and pathological biochemical processes, as in other living organisms. The cellular damage from the oxidative stress is counteracted through antioxidant defense system, which is comprised of antioxidant molecules (vitamin C and E, carotenoids, selenium, glutathione) and several antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), serum paraoxonase (PON), and others that either efficiently scavenge free radicals or inhibit the activity of oxidizing enzymes [1]. Since the free radical-antioxidants enzyme interactions play integral role in different aspects of disease pathogenesis and outcome of the cellular injury, an increase in the activities of some key antioxidant enzymes reflects the efficient elimination of ROS from the cell [20]. Supplementation with 10 and 25 g of IMUNO-2865[®] kg⁻¹ resulted with a significant increase in PON and GSH-Px compared to the control and the low-level treated group. The predominant positive supplementation effect in our study was based on the serum level of the SOD, the primary antioxidant protection system against continuously generated superoxide radicals (O_2^-) and hydrogen peroxide (H_2O_2), which contribute to the maintenance of a relatively low level of the hydroxyl radical (OH) especially harmful to fish due to their high cell content of polyunsaturated fatty acids [17]. SOD and GSH-Px are known to be the major antioxidant enzymes in erythrocytes, where ROS are continuously generated [22]. Increased values of GSH-Px and, especially, SOD could indicate that IMUNO-2865[®] supplementation promotes sea bream antioxidative stress response during the supplementation, and has a long-term and potentially cumulative effect, since SOD values remained increased in treatment groups even 90 days after the supplementation. Data about the effects of β -glucan preparations on the oxidative stress response in aquatic animals are scarce [22,23]. Immunostimulatory activity and increased SOD activity in hemocytes and muscle of the American white shrimp *Litopenaeus vannamei* was recorded by Campa-Córdova et al. [5] when juvenile shrimp were immersed with β -glucan and sulfated polysaccharide for 6 h. The pre-treatment of shrimp *Penaeus monodon* with β -glucan markedly increased SOD activity in hemocytes, and inhibited the reduction of SOD activity by white spot syndrome virus (WSSV) infection [7] [22]. reported higher SOD and catalase (CAT) activities in grass carp challenged with grass carp hemorrhage virus (GCHV) after injected with β -glucan for 15 days. To our knowledge, this is the first study on the effects of a natural arabinosylin and β -glucan based product on the antioxidative enzymes activity in *Sparus aurata*.

Based on the cellular and oxidative stress parameters in the blood, the results of this study suggest that the use of IMUNO-2865[®] in aquaculture is safe, reflecting with an overall positive and cumulative immunostimulatory effect on sea bream during the period of stress. In regard of the results, both medium and highest supplementation doses seem to be beneficial during the winter period, when this species feed intake is lowest. Although not investigated, we can speculate that during the warmer periods IMUNO-2865[®] could be effective with smaller ratio in feed, due to the higher feed intake of sea bream with an increase of temperature. However, future research will be beneficial in order to determine its full effectiveness through targeted bacterial/viral challenge studies and physical stress tests under controlled experimental conditions.

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