Spray-dried plasma promotes growth, modulates the activity of antioxidant defenses, and enhances the immune status of gilthead sea bream (*Sparus aurata*) fingerlings[1]

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ABSTRACT: Terrestrial animal byproduct meals, including nonruminant blood meal and blood products, represent the largest and largely untapped safe source of animal protein available within the international market for the aquafeed industry. Spray-dried blood and spray-dried plasma (SDP) proteins have long been recognized as high-quality feed ingredients for farmed animals. In this study, we evaluated the inclusion of SDP from porcine blood (SDPP) in growing diets for gilthead sea bream. Three isonitrogenous (CP = 51.2%) and isolipidic (fat = 12.4%) diets manufactured by cold extrusion (0.8 to 1.5 mm pellet size) were prepared by substituting high-quality fish meal with 0, 3, and 6% SDPP. The diets were tested for a period of 60 d at 22°C with 4 replicates each (400-L cylindroconical tanks, 150 fish per tank, and initial density = 0.5 kg/m^3). The SDPP inclusion in diets for gilthead sea bream fingerlings were evaluated in terms of growth performance, feed utilization, histological organization of the intestinal mucosa, activity of oxidative stress enzymes (catalase, glutathione S-transferase, glutathione peroxidase, and glutathione reductase) in the intestine, and nonspecific serum immune parameters (lysozyme and bactericidal activity). Results from this study indicated that dietary SDPP promoted fish growth in terms of BW and length; fish fed 3% SDPP were 10.5% heavier ($P < 0.05$) than those fed the control diet. Spray-dried plasma from porcine blood modulated the activity of the antioxidative defenses in the intestine ($P < 0.05$) and increased the density of goblet cells in the intestine ($P < 0.05$) and benefited the host by providing an effective immune barrier against gut pathogenic microbiota. The nonspecific serum immune response in fish fed diets with SDPP was greater ($P < 0.05$) than in fish fed the control diet. These results indicated that the inclusion of SDPP in gilthead sea bream feed could be beneficial for the fish by enhancing intestinal and serum innate immune function and the activity of antioxidative stress enzymes of the intestine and promoting growth performance.

Key words: fish, goblet cells, immunostimulant, oxidative stress, spray-dried plasma

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INTRODUCTION

Spray-dried blood and plasma proteins are recognized as safe, high-quality feed ingredients for farmed animals, including swine, cattle, and poultry (Campbell et al., 2003; Ferreira et al., 2009; Frugé et al., 2009; Henn et al., 2013). On a nutritional basis, blood products with the highest nutritional value are generally those that have been spray dried, because the proteins are subjected to less heat damage and denaturing during the spray drying process compared to traditional rendering processes (Luzier et al., 1995). Spray-dried plasma (**SDP**) has an excellent AA profile and close to 99% digestibility (Bureau et al., 1999) and, when included in pig diets, results in an improvement of feed intake, animal growth, and feed efficiency (Ferreira et al., 2009). In addition to use as a source of protein, SDP has also been recommended in animal diets as a source of immuno-

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logical support due to their high levels of globulin proteins, including immunoglobulins (Pérez-Bosque et al., 2006; Campbell et al., 2010; Gao et al., 2011). Although rendered blood byproducts have many good qualities (Breck et al., 2003; Tacon, 2005), there is limited literature on the effects of dietary SDP inclusion in aquatic feeds, especially fish species (Johnson and Summerfelt, 2000). In this context, there is a global recognition that terrestrial animal byproduct meals, including nonruminant blood meal and blood products, represent the largest and largely untapped safe source of animal protein and lipid available within the international market for the aquafeed industry (Bureau, 2000; Tacon, 2000). The objective of this study was to evaluate the inclusion of SDP from porcine blood (**SDPP**) as a protein source in feeds for gilthead sea bream (*Sparus aurata*) fingerlings by assessing its impact on fish growth, feed utilization, oxidative stress condition, and organization of the intestinal mucosa and nonspecific hematological immune parameters.

MATERIAL AND METHODS

All animal experimental procedures were conducted in compliance with the experimental research protocol approved by the Committee of Ethics and Animal Experimentation of the Institut de Recerca i Tecnologia Agroalimentàries (**IRTA**) and in accordance with the Guidelines of the European Union Council (Anonymous, 2010) for the use of laboratory animals and the recommendations of the Association of Animal Behavior (ASAB, 2003).

Experimental Diets

A control diet (0% SDPP) was formulated with high levels of marine-derived protein sources to contain 51% CP, 12% fat, and 20 MJ/kg GE and fulfill the known nutritional requirements of juvenile sea bream. Based on this basal formulation, 2 additional diets were produced, in which SDPP (AP820P; APC Europe, SA, Granollers, Spain) was incorporated at 3 and 6% levels, at the expense of high-quality fishmeal (Fishmeal 70LT; Exalmar, Lima, Peru). Experimental diets were isonitrogenous, isolipidic, and isoenergetic (Table 1).

Diets were manufactured by Sparos Lda. (Olhão, Portugal). Main ingredients were ground (below 250 μm) in a micropulverizer hammer mill (SH1; Hosokawa Micron, B.V., Doetinchem, The Netherlands). Powder ingredients and oils were then mixed according to the target formulation in a paddle mixer (RM90; Mainca, S.L., Granollers, Spain). All diets were manufactured by temperature-controlled extrusion (pellet sizes: 0.8 and 1.5 mm) by means of a low-shear extruder (P55; Italplast, S.r.l., Parma,, Italy). Upon extrusion, all feed batches were dried in a convection oven (OP 750-UF; LTE Scientifics, Oldham, UK) for 4 h at 45°C. Samples of each diet were taken for proximate composition analysis (Table 1).

Table 1. Ingredient list and proximate chemical composition of experimental diets

	Diet		
Ingredient	0% SDPP[1]	3% SDPP	6% SDPP
Fishmeal 70LT[2]	36.9	33.7	30.5
Fishmeal 60[3]	12.5	12.5	12.5
SDPP (AP820P)[4]	0.0	3.0	6.0
CPSP 90[5]	8.3	8.3	8.3
Squid meal[6]	6.0	6.0	6.0
Wheat gluten[7]	5.0	5.0	5.0
Micronized soybean meal[8]	5.3	5.3	5.3
Wheat meal	7.5	7.5	7.5
Pea starch	8.5	8.5	8.5
Fish oil[9]	7.4	7.4	7.4
Vitamin and mineral premix[10]	1.0	1.0	1.0
Choline chloride	0.1	0.1	0.1
Binder (guar gum)	1.0	1.0	1.0
Soy lecithin	0.5	0.5	0.5
Composition,[11] %			
CP	51.2	50.4	52.1
Fat	12.4	12.6	12.3
Ash	10.9	10.2	10.9
Fiber	1.3	1.3	1.3
Moisture	7.6	9.6	9.1
GE,[12] kJ/kg	2,000.9	1,977.9	1,978.5

[1]SDPP = spray-dried plasma from porcine blood.

[2]Peruvian fishmeal LT: 71% CP and 11% fat (Exalmar, Lima, Peru).

[3]Fair average quality fishmeal: 62% CP and 12% fat (Cofaco, Ponta Delgada, Portugal).

[4]Spray-dried plasma from porcine blood (AP820P): 76% CP and 2.4% fat (APC Europe, SA, Granollers, Spain).

[5]Soluble fish-protein concentrate (CPSP 90): 84% CP and 12% fat (Sopropêche, Boulogne-Sur-Mer, France).

[6]Super prime squid meal: 80% CP and 3.5% fat (Sopropêche).

[7]VITEN: 85.7% CP and 1.3% CF (Roquette, Lestrem, France).

[8]Micronized soybean meal: 51.7% CP and 2.1% fat (Sorgal SA, Ovan, Portugal).

[9]Marine oil omega 3 (Henry Lamotte Oils GmbH, , Germany).

[10]PVO40.01 premix for marine fish (Premix Lda., Viana do Castelo, Portugal). Vitamins (per kg diet): 100 mg DL-alpha tocopherol acetate, 25 mg sodium menadione bisulfate, 20,000 IU retinyl acetate, 2,000 IU DL-cholecalciferol, 30 mg thiamin, 30 mg riboflavin, 20 mg pyridoxine, 0.1 mg B_{12}, 200 mg nicotinic acid, 15 mg folic acid, 1,000 mg ascorbic acid, 500 mg inositol, 3 mg biotin, 100 mg calcium panthotenate, 1,000 mg choline chloride, and mg betaine, 500. Minerals (per kg diet): 2.5 mg cobalt sulfate, 1.1 mg copper sulfate, 0.2 g ferric citrate, 5 mg potassium iodide, 15 mg manganese sulfate, 0.2 mg sodium selenite, 40 mg zinc sulfate, 0.6 g magnesium hydroxide, 1.1 g potassium chloride, 0.5 g sodium chloride, and 4 g calcium carbonate.

[11]Based on analysis of 3 samples per diet.

[12]Gross energy content was estimated by using the following equation: total carbohydrate × 17.2 J/kg, fat × 39.5 J/kg, and protein × 23.5 J/kg.

Animals, Experimental Conditions, and General Procedures

Gilthead sea bream fry (average 1.3 g) were purchased from a commercial hatchery (Piscimar, Andromeda Group, Burriana, Spain) and transported by road to IRTA-Sant Carles de la Rapita research facilities (Sant Carles de la Rapita, Spain), where they were acclimated for 10 d. Before the onset of the trial, all fish were anesthetized (tricaine methanesulfonate [**MS-222**], 150 mg/L) and individually weighed for initial body weigth; ($\mathbf{BW_i}$) and measured for standard length (**SL**) to the nearest 0.1 g and 1 mm, respectively. Water temperature and pH (pH meter 507; Crison Instruments, Barcelona, Spain), salinity (MASTER-20T; ATAGO Co., Ltd., Tokyo, Japan), and dissolved oxygen (OXI330; Crison Instruments) were 22.4 ± 0.4°C, 7.1 ± 0.01, 36 mg/L, and 7.1 ± 0.3 mg/L (mean ± SD), respectively. Water flow rate in experimental tanks was maintained at approximately 9.0 L/min via a recirculation system (IRTAmar; IRTA, Barcelona, Spain) that maintained adequate water quality (total ammonia and nitrite were ≤0.10 and 0.4 mg/L, respectively) through UV, biological, and mechanical filtration. Photoperiod followed natural changes according to the season of the year (June to August; 40°37′41″ N). Each diet was tested with 4 replicates in 400-L cylindroconical tanks (150 fish per tank and initial density 0.5 kg/m^3) for a period of 60 d. Diets were distributed 8 times/d by automatic feeders (ARVO-TEC T Drum 2000; Arvotec, Huutokosk, Finland) at the rate of 2.5% of the stocked biomass, which approached apparent satiation.

Sampling to monitor fish growth took place monthly from the onset of the feeding period. For that purpose, all fish in each tank were netted and anesthetized with 150 mg MS-222/L, and their wet BW (g) and SL (cm) were determined. At the end of the trial (60 d), all fish from each tank were measured for their final BW ($\mathbf{BW_{f,\ g}}$) and final SL ($\mathbf{SL_f}$; cm); in addition, 80 specimens (fasted overnight) per experimental condition (20 per replicate) were sacrificed with an overdose of anesthetic for assessing the histological organization and integrity of the intestinal mucosa (n = 5), proximate carcass composition (n = 5), activity of antioxidative stress enzymes in the intestine (n = 5), and levels of nonspecific hematological immune parameters (n = 5).

Fish growth and feed utilization from different experimental groups was evaluated by means of the G:F and the following indices:

specific growth rate in BW ($\mathbf{SGR_{BW}}$; **%**) = $\mathbf{[(\ln BW_f - \ln BW_i) \times 100]}$/time (d) and Fulton's condition factor (**K**) = $(BW_f/SL^3) \times 100$.

The K is a morphometric index that estimates fish's body condition, which is determined by measuring the weight and length of individual fish. This approach assumes that heavier fish of a given length are in better condition (Sutton et al., 2000). For determining the body proximate composition of fish, sacrificed fish were homogenized and small aliquots were dried (120°C for 24 h) to estimate water content. Fat content from feed and fish was quantified gravimetrically after extraction in chloroform/methanol (2:1) and evaporation of the solvent under a stream of N followed by vacuum desiccation overnight (Folch et al., 1957). Protein content was determined according to Lowry et al. (1951). Ash contents were determined by keeping the sample at 500 to 600°C for 24 h in a muffle according to the AOAC (1990). All chemical analyses were performed in triplicate per fish and feed samples.

Organization and Integrity of the Intestinal Mucosa

For assessing the impact of the SDPP on the intestine and functionality, sacrificed fish were dissected on a glass plate maintained at 0 to 4°C. The intestine was sampled for measuring alkaline phosphatase (**AP**), an intestinal brush border enzyme used as marker of intestinal integrity and maintenance of intestinal homeostasis (Lallès, 2010). Purification and quantification of AP was conducted as previously described in Gisbert et al. (2009), and AP specific activity was expressed as international units per milligram of protein. Soluble protein of crude enzyme extracts was quantified by the Bradford method (Bradford, 1976) using BSA as a standard. For histological purposes, the mid intestine from 20 fish per dietary treatment was dissected and fixed in 4% buffered formaldehyde (pH = 7.4), dehydrated in a graded series of ethanol, cleared with xylene, embedded in paraffin, and cut in serial sections (3 to 5 µm thick). Transverse sections of the posterior intestine were connected to a light microscope (Leica DM LB; Leica Microsystems, Wetzlar, Germany) and photographed (Olympus DP70 Digital Camera; Olympus Imaging Europa GmbH, Hamburg, Germany). Digital images were processed and analyzed using an image analysis software package (ANALYSIS; Soft Imaging Systems GmbH, Münster, Germany). Measurements of total goblet cell number (full and empty) and villi height were based on the analysis of 8 to 10 randomly chosen fields from the intestinal mucosa of 20 fish per dietary group (Gisbert et al., 2013). Goblet cell counts in intestinal villi were expressed over a contour length of 100 µm, whereas villi height and width was calculated according to Escaffre et al. (2007).

Intestinal Lipid Peroxidation and Oxidative Stress Enzymes Levels

Quantification of lipid peroxidation in the intestine was conducted using the thiobarbituric acid reactive substances method described by Solé et al. (2004). In brief, lipid peroxidation was measured using 200 μL of the homogenate mixed with 650 μL of methanol and 1-methyl-2-phenylindole (solution stock of 10.3 m*M*) in acetonitrile:methanol (1:3; vol/vol) and 150 μL of 37% HCl. This mixture was incubated for 40 min at 45°C, cooled on ice for 10 min, and centrifuged at 21,000 × *g* for 10 min at 4ºC to remove protein precipitates. Absorbance was read at 586 nm, and the amount of peroxidized lipids (in nmol malondialdehyde/100 g tissue; wt/wt) was evaluated by means of a calibration curve made of a standard solution of 1,1,3,3-tetramethoxypropane (10 m*M*). Homogenized samples, prepared for the determination of the levels of lipid peroxidation, were used to measure antioxidant enzyme activities. Catalase (**CAT**; Enzyme Commission [**EC**] number 1.11.1.6) activity was measured in sampled tissues by the decrease in absorbance at 240 nm (extinction coefficient [**e**] = 40 M^{-1} cm^{-1}) using 50 m*M* H_2O_2 as substrate (Aebi, 1974). Glutathione S-transferase (**GST**; EC 2.5.1.18) activity was assayed by the formation of glutathione chlorodinitrobenzene adduct at 340 nm (e = 9.6 mM^{-1} cm^{-1}), using 1 m*M* 1-chloro-2,4-dinitrobenzene and 1 m*M* glutathione as substrates (Habig et al., 1974). Glutathione reductase (**GR**; EC 1.8.1.7) activity was determined by measuring the oxidation of nicotinamide adenine dinucleotide phosphate reduced (**NADPH**) at 340 nm (e = 6.22 m*M*–1 cm–1), using 20 m*M* glutathione disulphide and 2 m*M* NADPH as substrates (Carlberg and Mannervik, 1975). Total glutathione peroxidase (**GPX**; EC 1.11.1.9) was determined according to Günzler and Flohé (1985) by measuring the consumption of NADPH at 340 nm (e = 6.22 m*M*–1 cm–1), using 75 m*M* glutathione and 8.75 m*M* NADPH as substrates. Oxidative stress enzyme activities were expressed as specific enzyme activities (nmol min –1 mg protein–1), whereas soluble protein was determined by the Bradford method (Bradford, 1976). All assays were conducted in triplicate at 25°C, and absorbance was read using a spectrophotometer (Tecan Infinite M200; Tecan Group Ltd., Männedorf, Switzerland).

Nonspecific Hematological Immune Parameters

Blood (500 μL) was taken from anesthetized fish by caudal puncture with lithium-heparinized syringes and immediately centrifuged (2,000 × *g* for 20 min at 4°C) to separate serum. The lysozyme activity in serum was measured according to the method of Ellis (1990). Briefly, a sample of 0.05 mL serum was added to 1.4 mL of a suspension of *Micrococcus lysodeikticus* (0.2 mg/mL) in a 0.1 *M* sodium phosphate buffer (pH 6.8). The reaction was conducted at 25°C and absorbance was measured at 530 nm after 0.5 and 4.5 min in a spectrophotometer, using egg-white lysozyme as standard. Each unit (kilounits/mL) is defined as the amount of sample causing a decrease in absorbance of 0.001 per min. The hemolytic assay for alternative complement pathway (**ACP**) was determined following the technique described by Sunyer et al. (1995) with minor modifications for ELISA plates. The results are expressed in alternative complement units per ml, which are defined as the titer at which 50% hemolysis is produced. For the bacteriolytic test, bacteria (*Escherichia coli*) were grown for 20 h in 20 mL of lysogeny broth at 37°C in an orbital incubator at 200 rpm. A 1:100 bacterial suspension was chosen to give an optical reading of 0.5 to 0.6 at a wavelength of 540 nm when added to the serum dilution (1:1 bacterial suspension:serum dilution) and blank with sterile Luria-Bertani medium. The mixture was placed for 1 h at 37°C on an orbital incubator (200 rpm). To study the bactericidal kinetics of fish serum, a 0.5-mL aliquot was withdrawn at intervals of 30 min and read at 540 nm with a microplate reader (Tecan Infinite M200; Tecan Group Ltd., Barcelona, Spain). Results are given as fold increase of the absorbance. All chemicals and reagents used for evaluating different hematological immune parameters were purchased from Sigma-Aldrich (Madrid, Spain).

Statistical Analyses

The mean values of BW_f, SL_f, and K were expressed as mean ± SD. The calculation was based on individual BW_f, SL_f and K values of all the fish belonging to the same treatment, and consequently, the SD describes the dispersion of the individual values. The mean values of survival, specific growth rate, G:F, specific enzyme activities, hematological parameters, intestinal goblet cell number and villi height, and proximate biochemical composition were expressed as mean ± SEM. These parameters were calculated using the values of the replicates (n = 4 for each treatment), and the SEM quantifies the error in calculating the mean of the population from the tank values. Data expressed as percentage were arcsin ($x^{1/2}$) transformed. Results were compared by means of 1-way ANOVA (data normally distributed) and, when significant differences were detected ($P < 0.05$), the Tukey multiple-comparison test was used to detect differences among experimental groups. All statistical analyses were performed using SigmaPlot version 12.0 (Systat Software Inc., Chicago, IL).

Table 2. Survival, growth performance (final BW [BW_f], final standard length [SL_f], specific growth rate [SGR], Fulton's condition factor [K], and G:F) and carcass chemical composition of gilthead sea bream (*Sparus aurata*) fingerlings fed experimental diets containing different levels of spray-dried plasma from porcine blood (SDPP)

	Diet		
Item	0% SDPP	3% SDPP	6% SDPP
Survival rate,[1] %	97.8 ± 0.6	98.0 ± 0.8	98.6 ± 0.4
Growth performance			
BW_f[2]	30.2 ± 0.7[b]	33.8 ± 1.7[a]	32.2 ± 1.7[ab]
SL_f[2]	10.4 ± 0.1[b]	10.7 ± 0.1[a]	10.7 ± 0.1[a]
K[2]	2.66 ± 0.04[b]	2.75 ± 0.03[a]	2.65 ± 0.04[b]
$SGR_{0\ to\ 60\ d}$,[1] %/d	5.21 ± 0.02[b]	5.43 ± 0.05[a]	5.35 ± 0.05[ab]
G:F[1]	1.09 ± 0.04[b]	1.24 ± 0.03[a]	1.23 ± 0.06[a]
Composition,[3] %			
CP	68.4 ± 0.3	69.3 ± 0.2	68.6 ± 0.2
Fat	23.4 ± 0.3	23.8 ± 0.3	24.8 ± 0.2
Ash	2.5 ± 0.2	2.5 ± 0.2	2.5 ± 0.2

[a,b]Values with different superscripts differ ($P < 0.05$).

[1]Based on 4 replicate tanks per diet with 145 to 150 fish per tank.

[2]Mean BW_f, SL_f, and K values were calculated using the individual values from all fish within the same dietary treatment (4 replicate tanks per diet with 145 to 150 fish per tank).

[3]Based on 20 fish per diet (4 replicate tanks per diet with 5 randomly selected fish per tank).

RESULTS

At the end of the trial, no statistically significant differences in survival were found among gilthead sea bream fingerlings fed different experimental diets (Table 2). The dietary inclusion of SDPP affected growth performance; fish fed 3% SDPP were 10.5% heavier in BW_f and 2.8% longer in SL_f ($P < 0.05$) than those fed the control diet (0% SDPP), whereas fish fed 6% SDPP showed BWf values intermediate between the former groups (Table 2). Similarly, SGRBW values were greater in fish fed 3% SDPP ($P < 0.05$) than in fish fed 0% SDPP, whereas those animals fed 6% SDPP showed intermediate values (Table 2). The K was also affected by the level of inclusion of SDPP in diets, being greater in fish fed 3% SDPP ($P < 0.05$) in comparison to those fed the 0 and 6% SDPP diets (Table 2). The inclusion of SDPP in diets for gilthead sea bream fry improved G:F values (Table 2); thus, fish fed 3 and 6% SDPP showed greater G:F values ($P < 0.05$) than those fed the control diet (0% SDPP). The inclusion of SDPP in diets for gilthead sea bream did not affect the chemical composition (i.e., CP, fat, or ash content) of fish carcass (Table 2).

The histological examination of the intestinal mucosa in fish fed different diets demonstrated that inclusion of the SDPP in feeds for gilthead sea bream fry did not affect the length and width of intestinal villi (Fig. 1). Fish fed 6% SDPP had the greatest number of goblet cells counted over a contour length of 100 μm of intestinal epithelium (0.85 ± 0.03) followed by those fed 3% SDPP (0.73 ± 0.03, whereas fish fed the control diet (0% SDPP) had the lowest number of goblet cells in their intestinal mucosa (0.59 ± 0.02; $P < 0.001$; Fig. 1). No differences in the specific activity of AP from enterocytes were found among fish fed diets containing different levels of SDPP (Fig. 1).

The levels of lipid peroxidation and activity of selected anitoxidative stress enzymes from the intestine are shown in Table 3. Thiobarbituric acid reactive substance levels in the intestine were similar regardless of the diet tested. However, the inclusion of the SDPP in feeds for gilthead sea bream fry affected the specific activity of CAT, GR, and GST in the intestine ($P < 0.001$). Fish fed 3% SDPP had the lowest specific activity values for CAT, GR, and GST ($P < 0.001$) in comparison to fish fed the control diet (0% SDPP), whereas no statistically significant differences were detected among experimental groups with regards to GPX activity.

Nonspecific immune responses were evaluated in gilthead sea bream fingerlings in relation to dietary supplementation of SDPP by evaluation of selected blood parameters (Table 4). Fish fed diets containing 3 and 6% SDPP showed greater levels of lysozyme ($P < 0.05$), ACP, and bactericidal activity in serum than fish fed the control diet (0% SDPP).

DISCUSSION

A major challenge for aquaculture production is to identify and validate stable, predictable, and high-quality sources of alternative proteins for aquafeeds. In addition, there is a trend toward developing functional feeds to optimize animal performance as well as operational efficiency. In this sense, gathered information from terrestrial farmed species (van Dijk et al., 2001; Campbell et al., 2003; Ferreira et al., 2009; Frugé et al., 2009; Cho and Kim, 2011) indicate that spray-dried blood and also SDP might be high-quality protein sources for aquafeeds (Lee and Bai, 1997; Johnson and Summerfelt, 2000; Tacon, 2005). However, despite the fact that blood meal and blood products have been shown to be cost-effective nutrient sources for farmed fish and shrimp, it is estimated that less than 5% of total global manufactured aquafeeds (21 million t in 2005) use blood meal within their feeds (2 to 5% average dietary inclusion level), which is mainly used as a cost-effective source of highly digestible animal protein, as a fishmeal replacer, and pellet coloring agent. Aquaculture feed manufacturers that incorporate blood meal and blood products in their feeds are mainly concentrated in Asia and North and South America. In

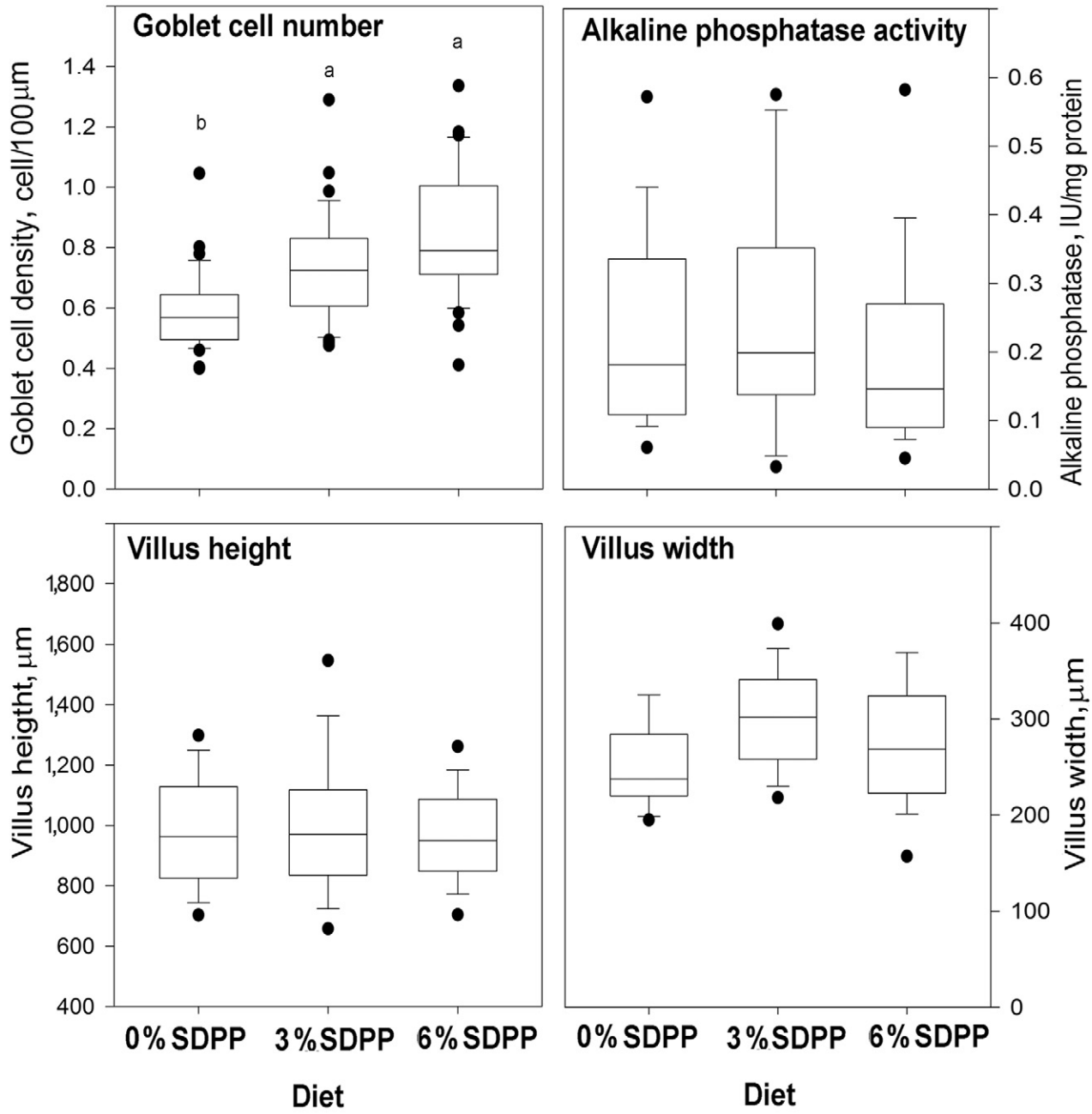

Figure 1. Box and whisker plots of the villi width and height, intestinal goblet cell number, and specific activity of alkaline phosphatase from the brush border of enterocytes of gilthead sea bream (*Sparus aurata*) fingerlings fed experimental diets containing different levels of spray-dried plasma from porcine blood (SDPP). Measurements of total goblet cell number (full and empty) and villi height and width were based on the analysis of 8 to 10 randomly chosen fields from the intestinal mucosa of 20 fish per dietary group (5 fish per replicate tank). Alkaline phosphatase specific activity was calculated from 4 replicate tanks per diet with 5 fish tank. [a,b]Different letters denote that means for the experimental groups differ ($P < 0.001$). The solid line represents the mean value for each set of samples, whereas dots represent the outliers.

Europe, the use of porcine blood products and blood meal has been legally accepted by European Union regulation since 2005 (Anonymous, 2013; Tacon, 2005) and its use has been increased since that time.

Blood byproducts have been successfully used in compound feeds since the early 1970s with no reported deleterious or negative effects on fish growth. In this sense, spray-dried blood cells have been successfully used as a fish meal replacer in feeds for rainbow trout (*Oncorhynchus mykiss*) with no loss in growth or feed efficiency (Luzier et al., 1995; Johnson and Summerfelt, 2000). Similarly, Lee and Bai (1997) showed that fish meal could be replaced by hemoglobin powder in juvenile Japanese eel (*Anguilla japonica*) diets, although the level of fish meal substitution was dependant on the dietary supplementation of several essential AA. In contrast, Martínez-Llorens et al. (2008) found that blood meal could substitute fish meal up to 15% (5%

of dietary inclusion) with no effect on performance of gilthead sea bream juveniles, whereas hemoglobin inclusion resulted in a reduction in fish growth. Similarly, Jahan et al. (2000) observed a decrease in growth in carp (*Cyprinus carpio*) fed diets containing 8 and 12% blood meal compared to those fed 3% blood meal. Although in this study SDPP was not used in the gilthead sea bream diets as a major raw material for fish meal replacement due to its relatively high price as a protein source, its inclusion at lower levels (3% SDPP) improved fish growth performance in terms of BW_f, SL_f, K, and SGR_{BW}, whereas at greater levels, the advantages of the dietary inclusion of SDPP were not as evident. These results obtained from fish are similar to those reported in weaned piglets where the beneficial effects of SDPP inclusion in diets were attributed to the high digestibility of this protein source (Bureau et al., 1999; van Dijk et al., 2001; Booth et al., 2005) as well as to an increase in feed ingestion rates (Grinstead et al., 2000; van Dijk et al., 2001; Ferreira et al., 2009). Under the present experimental results, fish were fed to near satiation. The greater growth of gilthead sea bream fingerlings fed 3% SDPP might be attributed to the greater nutritional value (globulin proteins) and digestibility of this blood byproduct compared to the fish meal of the control diet. This hypothesis is in agreement with the greater G:F values found in gilthead sea bream fed 3 and 6% SDPP in comparison to those obtained in fish fed the control diet (0% SDPP).

In addition to the benefits of dietary supplementation of SDPP in terms of growth, SDPP also improved health condition of terrestrial animals by enhancing its immune competence (Torrallardona et al., 2003; Moretó and Pérez-Bosque, 2009; Che et al., 2012) and reducing the redox status in the intestine (Gao et al., 2011) as well as increasing the animal's ability to cope with stress (Peace et al., 2011). In this context, several studies have reported the beneficial effects of using SDP on the immune response and disease resistance of shrimp (*Penaeus japonicus* and *Litopenaeus vannamei*; Russell and Campbell, 2000), European eel (*Anguilla anguilla*; Jensen and Nielsen, 2003), rainbow trout (Campbell, 2003), and grouper (Jensen and Nielsen, 2003). In the present study, the inclusion of SDPP in diets for gilthead sea bream fingerlings resulted in an increase of goblet cell density as well as an enhancement of the intestinal antioxidative stress enzymes. The intestine plays an important role in the ingestion and absorption of nutrients and provides a physiologic and immunologic barrier to a wide range of microorganisms and foreign substances (Lallès, 2010). Therefore, the expansion of the goblet cell population would benefit fish by providing an effective immune barrier against potentially pathogenic gut bacteria (McGuckin et al., 2011). Mucus produced by intestinal goblet cells can contain lysozymes, immunoglobins, lectins, crinotoxins, and antibacterial peptides that counter pathogens and toxins (Shephard, 1994) as well as playing a key role in the establishment of the commensal intestinal microbiota (Kim and Ho, 2010; McGuckin et al., 2011). Consequently, the increase in goblet cell density in the intestinal mucosa can be interpreted as an enhancement of the intestinal innate immune function in fish fed SDPP. Antioxidant enzymes are an important part of the antioxidant system of the organism, protecting the cells against oxidative stress. The modulation of the intestinal antioxidant defense system found in gilthead sea bream fed SDPP, as indicated by the changes of antioxidant enzyme activities in the intestinal mucosa, might have promoted the development and health of the intestine, also leading to a better growth performance, as Gao et al. (2011) described in piglets fed SDPP.

Table 3. Lipid peroxidation levels and activity of antioxidative stress enzymes from the intestine of gilthead sea bream (*Sparus aurata*) fingerlings fed experimental diets containing different levels of spray-dried plasma from porcine blood (SDPP)

	Diet		
Item[1]	0% SDPP	3% SDPP	6% SDPP
TBARS, nmol MDA/100 g	0.097 ± 0.022	0.121 ± 0.037	0.105 ± 0.023
CAT, nmol min^{-1} mg $protein^{-1}$	1.88 ± 0.15[a]	1.30 ± 0.10[b]	1.68 ± 0.12[ab]
GR, nmol min^{-1} mg $protein^{-1}$	1.73 ± 0.15[a]	1.01 ± 0.11[b]	1.59 ± 0.11[a]
GST, nmol min^{-1} mg $protein^{-1}$	476.8 ± 33.4[a]	351.0 ± 28.4[b]	506.5 ± 0.15[a]
GPX, nmol min^{-1} mg $protein^{-1}$	3.71 ± 1.43	3.25 ± 1.33	4.21 ± 1.6

[a,b]Values with different superscripts differ ($P < 0.001$).

[1]TBARS = thiobarbituric acid reactive substances; MDA = malondialdehyde; CAT = catalase; GR = glutathione reductase; GST = glutathione S-transferase; GPX = glutathione peroxidase. Based on 20 fish per diet (4 replicate tanks per diet with 5 randomly selected fish per tank).

The results regarding the improvement of gut innate immune response in gilthead sea bream fingerlings fed SDPP were reinforced by those obtained from hematological parameters, which confirmed that the oral administration of SDPP enhanced the serum immune function in fish. Serum lysozyme is an important antibacterial defense molecule of the innate immune system produced by leukocytes. Certain nutrients and compounds can be supplemented in the feed to modulate the lysozyme activity of fish and it is generally associated with an increase in protection against a variety of bacterial infections (Saurabh and Sahoo, 2008). In the present study, lysozyme activity was increased in fish fed 3 and 6% SDPP, which might be attributed to the high content of immunoglobulins in this blood product (Torrallardona et al., 2003; Moretó and Pérez-Bosque, 2009; Che et al., 2012). Similarly, the serum bactericidal activity and the hemolytic assay for ACP were also en-

Table 4. Nonspecific immune responses in serum (lysozyme, bactericidal activity, and hemolytic assay for alternative complement pathway [ACP]) from gilthead sea bream (*Sparus aurata*) fingerlings fed experimental diets containing different levels of spray-dried plasma from porcine blood (SDPP)

	Diet		
Item[1]	0% SDPP	3% SDPP	6% SDPP
Lysozyme, kilounits/mL	45.5 ± 6.7[b]	65.5 ± 5.1[a]	68.3 ± 5.24[a]
Bactericidal activity, % Abs	58.5 ± 2.1[b]	64.5 ± 1.1[a]	67.7 ± 1.7[a]
ACP, ACH_{50} units/mL	140.5 ± 7.5[b]	175.1 ± 5.0[a]	179.4 ± 8.5[a]

[a,b]Values with different superscripts differ ($P < 0.05$).

[1]Abs = absorvance at 540 nm; ACH_{50} = alternative complement units per ml. Based on 20 fish per diet (4 replicate tanks per diet with 5 randomly selected fish per tank).

hanced in fish fed both diets containing SDPP in contrast to the control group, which showed values similar to those reported in the literature (Tort et al., 1996a,b, 1998). Both nonspecific immune responses are involved in killing and clearing of pathogenic organisms in fish (Ellis, 2001; Holland and Lambris, 2002). Therefore, complement activation is a multifaceted and complex process, involving a multitude of factors, which has a primary role in the innate immunity of fish, facilitating chemotaxis, opsonization, and pathogen destruction but is also linked to the acquired immune system because complement activation enhances B cell proliferation (Holland and Lambris, 2002; Kiron, 2012). Although the definitive evaluation of a dietary ingredient as an immunomodulator generally requires a challenge with an active pathogen, present data from gilthead sea bream seem to indicate enhancement of the blood nonspecific immune function, as demonstrated in shrimp and rainbow trout fed SDPP and challenged with the white spot syndrome virus and *Yersinia ruckeri*, respectively (Russell and Campbell, 2000; Campbell, 2003).

In conclusion, the results from this study indicated that the inclusion of SDPP in diets for gilthead sea bream fingerlings promoted growth performance (BW, SL, and K) and improved G:F values, although it did not change the chemical composition of fish carcass. In addition, SDPP affected the activity of the intestinal antioxidant defense system and increased the density of goblet cells in the intestinal mucosa, which may be interpreted as an enhancement of the intestinal health condition and innate immune function in fish fed SDPP. These results were supported by the enhancement of the serum nonspecific immune function observed in fish fed SDPP-containing diets. These results may be of practical significance for fish farmers, because the inclusion of SDPP in fish diets might not only reduce disease and stress in aquaculture systems but also improve their growth performance and efficiency during the rearing process.

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