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Research Article

Effects of Feeding Frequency and Dietary Protein/Carbohydrate Ratios on Gilthead Seabream (*Sparus aurata*) Intestinal Functionality and Health

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The present study evaluated the effects of feeding frequency (FF) and dietary protein/carbohydrate (P/CH) ratios on intestinal histomorphology, microbiota profile, and digestive and oxidative stress-related enzyme activities of gilthead seabream (*Sparus aurata*). To this purpose, two practical diets were formulated: one with 50% P and 10% CH (P50/CH10) and other with 40% P and 20% CH (P40/CH20). Triplicate groups of fish with 9.1 ± 0.01 g were fed these diets for 60 days until visual satiation at a FF of 1, 2, or 3 meals per day. Distal intestine histomorphology was not affected by diet composition or FF. However, the pyloric caeca (PC) of fish fed 1 meal per day presented more gut fold height alterations than the other groups, except in fish fed diet P50/CH10 3 meals per day, where no changes was observed. Fish fed diet P40/CH20 3 meals per day also presented higher PC submucosa cellularity than the other groups. Fish fed diet P40/CH20 presented a higher number of operational taxonomic units, microbial richness, and diversity indices than fish fed diet P50/CH10. Amylase was the only measured digestive enzyme affected by the experimental conditions, presenting higher activity in fish fed diet P50/CH10 once per day. Glucose-6-phosphate dehydrogenase activity was lower in fish fed 2 meals per day than only 1. While catalase activity was lower in fish fed 2 than 3 meals per day. Glutathione reductase activity was the only measured parameter affected both by dietary P/CH ratio and FF, being inferior in fish fed once per day the P50/CH10 diet than the P40/CH20 diet and, also in the P50/CH10 diet, to fish fed 1 than those fed 3 meals per day. Overall, no major interactions was observed between dietary P/CH ratio and FF; however, a P40/CH20 diet fed 2 meals per day might be recommended for gilthead seabream juveniles.

1. Introduction

The intestine, as the complex multifunctional organ, that is, assumes great importance in the overall performance of fish [1]. It was already established that one of the most important factors to maintain intestinal health is the use of balanced diets which fulfil the basic nutritional species requirements [2]. Carnivorous fish, such as gilthead seabream (*Sparus aurata*), evolved to digest highly digestible and nutritionally dense diets, rich in proteins (P) and low in carbohydrates (CH) [1]. Accordingly, dietary protein requirements of gilthead seabream are between 45 and 55%, depending on the

life stage, while only up to 20% CH can be used in the diets without causing major negative effects [3–6]. Dietary macronutrients can have an impact on intestinal health and functionality depending on levels and ratios between nutrients [7]. Therefore, it is important to understand how dietary nutrient ratios affect intestinal functionality and health. For instance, in gilthead seabream, although differences in intestinal histomorphology and microbiota diversity were not observed in fish fed different dietary P/CH ratios, differences were reported in digestive enzymes activity and oxidative-related parameters [8–12].

Feeding frequency (FF) optimization also helps to improve fish growth, health, and welfare [13]. The FF may modulate intestinal feed transit, digestion rate, and nutrient utilization efficiency, thus impacting growth, gut functionality, and health. In juvenile gilthead seabream, it was observed that although daily FF did not change the feed transit speed and the time that feed was in the intestine, it affected pepsin and trypsin activity [14, 15]. Furthermore, also in gilthead seabream, an increase in daily α-amylase and lipase activities was observed when FF increased from 1 to 2-3 meals per day, although these differences tended to disappear when activities were reported per meal [16].

The effects of FF on intestinal function and health have not yet been well-explored in gilthead seabream, and only scarce and diverse results are available for other fish species. For instance, in Nile tilapia (*Oreochromis niloticus*) and arapaima juveniles (*Arapaima gigas*), changes in FF did not affect the activities of digestive enzymes [17, 18], while in Lebranche mullet (*Mugil liza*) and white seabream (*Diplodus sargus*) juveniles, FF affected some digestive enzyme activities [19, 20]. Dolly Varden char (*Salvelinus malma*) juveniles fed increasing FF (up to 6 meals per day) presented higher serum malondialdehyde (MDA, usually used as a marker of lipid peroxidation) content [21], while blunt snout bream (*Megalobrama amblycephala*) juveniles fed 3 or 4 meals per day presented lower liver MDA content in comparison with those fed with lower (1 or 2) or higher (5 or 6) meals per day [22]. Regarding the effects of FF on intestinal histomorphology, in lumpfish (*Cyclopterus lumpus*), the severity of the inflammation increased in fish fed daily compared to fish fed only 3 or 4 days a week [23]. Nile tilapia fed on alternate days presented higher intestinal microbial biodiversity than fish fed every third day or kept unfed [24].

While results regarding FF effects on intestine functionality and health are disperse and seem contradictory and dependent on fish species, our recent observation that 2 or 3 meals a day improved growth of gilthead seabream juveniles fed P50/CH10 or P40/CH20 diets, when compared with only 1 meal a day [25], led us to inquire if FF manipulation might improve intestine functionality and health. In fact, it is known that FF affects CH utilization improving feed utilization and growth [26–28]. However, studies on intestine functionality and health, which might explain those improvements are lacking. Actually, there are only two studies in fish assessing simultaneously the effects of P/CH ratios and FF on parameters related with intestinal functionality, namely, in digestive enzymes, and none is in gilthead seabream [29, 30]. Thus, the current study aimed to evaluate the effects of FF (1, 2, or 3 meals per day) and dietary P/CH ratio (P50/CH10 or P40/CH20) on gilthead seabream intestinal histomorphology, microbiota diversity, and digestive and oxidative stress status.

2. Materials and Methods

2.1. Experimental Conditions and Sampling. Two plant-feedstuff- (PF-, 77%) based, isolipidic (17% crude lipids), and isoenergetic (20 kJ g^{-1}) diets with different P/CH ratios were formulated. One diet included 50% P and 10% CH, while the other diet included 40% P and 20% CH. The main source of CH used was wheat meal, while fish oil was the main lipid source. The composition of the experimental diets and proximate analysis is presented in Table 1.

The experimental trial was performed at the Marine Zoology Station of the University of Porto (Portugal) in a recirculating water system equipped with 18 fiberglass tanks (100 L water capacity), thermo-regulated to 24 ± 1°C, with a salinity of 36.0 ± 1.0 g L^{-1}, dissolved oxygen of 6.0 ± 0.5 mg L^{-1}, and where each tank was supplied with a continuous flow of filtered seawater (6.0 L min^{-1}). Gilthead seabream (*Sparus aurata*) juveniles were acquired from Sonríonansa Pesués (Cantabria, Spain). After a quarantine period of 19 days, 360 fish with a mean individual initial body weight of 9.10 ± 0.01 g were randomly distributed by 18 tanks (20 fish per tank). The diets and different FF were randomly assigned to triplicate groups. Fish were fed by hand for 60 days, 6 days a week, until visual satiation, at a FF of 1 meal (09 : 00), 2 meals (09 : 00 and 17 : 00), or 3 meals (09 : 00, 13 : 00, and 17 : 00) per day.

At the end of the trial, 8 fish from each tank were sampled 5 h after the morning meal, euthanized with a sharp blow to the head, and dissected on ice-cold trays. Three fish were sampled for collection of the distal intestine (DI, distinguished from the mid intestine by an enlarged diameter and darker mucosa) and pyloric caeca (PC) for histology evaluation. Samples were rinsed in phosphate-buffered saline, blotted dry with a paper towel, fixed in Bouin (code 57211, Thermo Scientific-Richard-Allan Scientific, Kalamazoo, USA) for 24 h, and then transferred to ethanol (70%) until further processing. The whole intestine with PC and intestinal content from 3 other fish was collected, immediately frozen in liquid nitrogen, and stored at -80°C until the analysis of digestive enzyme activity and lipid peroxidation (LPO). The remaining 2 fish were sampled under aseptic conditions to collect mucosa for microbiota characterization. Autochthonous microbiota samples were obtained by scraping the internal intestinal mucosa surface, immediately frozen in liquid nitrogen, and stored at -80°C until microbiota characterization.

2.2. Histological Processing and Morphological Evaluation. The DI and PC samples were processed and sectioned using standard histological techniques and stained with hematoxylin and eosin. Samples were evaluated as indicated by Krogdahl et al. [31], through a blinded semiquantitative analysis

Table 1: Composition of the experimental diets and proximate analysis.

	Diets	
	P50/CH10	P40/CH20
Ingredients (% DM)		
Fishmeal[1]	15.6	12.5
Fish oil[2]	14.0	14.7
Soybean meal[3]	25.0	20.0
Corn gluten[4]	20.0	15.0
Wheat gluten[5]	11.4	6.4
Wheat meal[6]	9.4	26.2
Monocalcium phosphate[7]	0.7	1.0
Lysine[8]	0.1	0.5
Taurine[9]	0.2	0.2
Vitamin mix[10]	1.0	1.0
Mineral mix[11]	1.0	1.0
Binder[12]	1.0	1.0
Choline chloride (50%)	0.5	0.5
Proximate analysis (% DM)		
Dry matter	93.6	93.0
Crude protein	51.9	42.2
Crude fat	17.5	17.4
Ash	6.0	5.4
Starch	9.8	17.4
Gross energy (kJ g^{-1})[a]	20.8	19.8

CH: carbohydrates; CP: crude protein; D: diet; DM: dry matter; GL: gross lipid; P: protein. [1]Sorgal. S.A. Ovar. Portugal (CP: 73.5% DM; GL: 17.0% DM). [2]Sorgal. S.A. Ovar. Portugal. [3]Sorgal. S.A. Ovar. Portugal (CP: 54.3% DM; GL: 1.8% DM). [4]Sorgal. S.A. Ovar. Portugal (CP: 70.0% DM; GL: 3.3% DM). [5]Sorgal. S.A. Ovar. Portugal (CP: 84.2% DM; GL: 1.0% DM). [6]Sorgal. S.A. Ovar. Portugal (CP: 13.8% DM; GL: 1.1% DM). [7]Sorgal. S.A. Ovar. Portugal. [8]Feed-grade lysine. Sorgal. S.A. Ovar. Portugal. [9]Feed-grade taurine. Sorgal. S.A. Ovar. Portugal. [10]Vitamins (mg kg^{-1} diet): retinol acetate. 18000 (IU kg^{-1} diet); cholecalciferol. 2000 (IU kg^{-1} diet); alpha tocopherol acetate. 35; sodium menadione bisulphate. 10; thiamin-HCl. 15; riboflavin. 25; calcium pantothenate. 50; nicotinic acid. 200; pyridoxine HCl. 5; folic acid 10; cyanocobalamin. 0.02; biotin. 1.5; ascorbic acid. 50; inositol. 400. Premix. Lda.. Viana do Castelo. Portugal. [11]Minerals (mg kg^{-1} diet): copper (II) sulphate. 5; ferrous carbonate. 40; fluorine. 1; potassium iodide. 0.6; magnesium oxide. 500; manganese oxide. 20; sodium selenite. 0.3; zinc oxide. 30; Minerals content (%): Calcium. 17; Phosphorus. 13; Potassium. 6; Cloride. 7; Sodium chloride. 4. Premix. Lda. Viana do Castelo. Portugal. [12]Liptosa. Madrid. Spain. [a]Gross energy calculated based on theoretical values (CP : 23.6 kJ g − 1 ; GL : 39.5 kJ g − 1 ; carbohydrates : 17.2 kJ g − 1, 23.6 × %dietary CP) + (39.5 × % dietary GL) + (17.2 × %dietary CH).

focusing on changes in (1) widening and shortening of the mucosal fold heights, (2) increased cellularity of the connective tissue and widening of lamina propria and submucosa, (3) infiltration of mixed leucocyte population (namely, intraepithelial lymphocytes and eosinophilic granular cells) in both the above-mentioned layers, (4) nucleus position and hypervacuolization within the enterocytes, and (5) increased number of goblet cells per analyzed area. The number of goblet cells was counted in each selected area/section, previously measured, as in the following equation:

$$\begin{aligned}\textbf{\textit{Goblet cells (GC) frequency}} = [&(\textbf{\textit{n}}^{\circ}\text{ of }\textbf{\textit{GC on section 1}} \div \textbf{\textit{area from section 1}}) \\ &+ (\cdots) + (\textbf{\textit{n}}^{\circ}\text{ of }\textbf{\textit{GC on section 4}} \\ &\div \textbf{\textit{area from section 4}})] \div 4.\end{aligned} \quad (1)$$

The 4 most intact villus sections were evaluated on each cut. The score 1 was given to the tissue with the least changes, and subsequent scores (up to 5) accounted for increasing histomorphology alterations, as described by Penn et al. [32]. The presence of goblet cells equal to the average was assigned with score 1. Scores 2, 3, 4, and 5 were assigned to sections where the presence of goblet cells was, respectively, 25%, 50%, 75%, or 100% above average. Digital image obtention and measurement of the selected areas were done with the Zen software (Blue edition; Zeiss, Jena, Germany). Three individual histological cuts were evaluated from each of nine fish ($n = 9$) within each experimental condition.

2.3. Microbial Diversity Analysis. Intestinal mucosa samples of the two fish per tank were pooled to reduce individual variation. DNA extraction, PCR amplification, polymorphism analyses of 16S rRNA genes by denaturing gradient gel electrophoresis (DGGE), bands excision, and reamplification were performed as described by Castro et al. [11] with slight modifications. Namely, samples were homogenized in a Precellys Evolution tissue homogenizer (Bertin Technologies SAS, Montigny-le-Bretonneux, France). Each PCR product was loaded on an 8% polyacrylamide gel with a denaturing gradient of 30 to 60% of 7 M urea/40% formamide. Amplicons were sequenced to identify microbiota operational taxonomic units (OTUs). Phylogenetic analysis to identify the closest known species was done as described in Castrol et al. [11]. Only sequences higher than 100 bp reads and a query coverage of 85-100% were considered for a valid identification.

2.4. Enzymatic Activities and Lipid Peroxidation (LPO). Fish intestines were homogenized (Ystral homogenize -Laboratory Series X10, Ballrechten-Dottingen, Germany) in 4 parts of ice-cold 50 mM Tris-HCl buffer (pH 7.8) containing 0.1 mM EDTA (ref. E5134, Sigma-Aldrich, Sintra, Portugal) and 0.1% (*v*/*v*) Triton X-100 (ref. T8787, Sigma-Aldrich, Sintra, Portugal). After centrifugation of homogenates (30 000*g*, 30 min, 4°C), the supernatants were recovered and stored at -80°C until use. All enzyme activities were measured at 37°C in a Multiskan GO microplate reader (model 51119200; Thermo Scientific, Nanjing, China) according to the specific assay conditions.

α-amylase (EC 3.2.1.1), lipase (EC 3.1.1.3), and total alkaline proteases activities were measured as described by Couto et al. [33]. Superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), glucose-6-phosphate dehydrogenase (G6PD; EC 1.1.1.49), glutathione peroxidase (GPX; EC 1.11.1.9), and glutathione reductase (GR, EC 1.6.4.2) activities were evaluated as described by Guerreiro et al. [34]. The optimal substrate and protein concentrations

Table 2: Details of the score-based evaluation of distal intestine histomorphology of gilthead seabream fed the experimental diets at different feeding frequencies.

P/CH ratio	P50/CH10			P40/CH20			SEM	p value
FF	1	2	3	1	2	3		
Intestine fold height	2.44	2.00	2.22	2.78	2.78	3.22	0.15	0.16
LP-width	1.89	2.33	1.89	1.89	2.00	1.89	0.06	0.14
LP-cellularity	1.89	2.11	1.67	1.44	1.67	1.44	0.09	0.25
SM-width	2.00	2.00	1.78	1.78	1.78	2.00	0.06	0.59
SM-cellularity	1.11	1.56	1.44	1.33	1.11	1.11	0.07	0.25
GCs	1.44	1.00	2.00	1.22	2.11	1.89	0.17	0.14
IELs	1.00	1.00	1.00	1.00	1.00	1.00	0.00	1.00
EGCs	1.00	1.00	1.00	1.00	1.00	1.00	0.00	1.00
Ent.- nucleus alignment	1.89	2.33	2.33	2.22	2.00	2.22	0.08	0.44
Ent.-vacuolization	1.00	1.00	1.00	1.00	1.00	1.00	0.00	1.00
Mean score	1.57	1.63	1.63	1.57	1.64	1.68	0.03	0.72

Values presented as means ($n = 9$) and pooled SEM. The results were analyzed using Kruskal-Wallis followed by all pairwise comparisons, and the significance values were adjusted using Bonferroni correction for multiple tests. No significant differences was found. CH: carbohydrate; EGC: eosinophilic granulocytes presence; Ent.: enterocytes; FF: feeding frequency; GC: goblet cell presence; IEL: intraepithelial leucocyte infiltration; LP: Lamina propria; P: protein; SEM: standard error of the mean; SM: submucosa.

Table 3: Details of the score-based evaluation of pyloric caeca histomorphology of gilthead seabream fed the experimental diets at different feeding frequencies.

P/CH ratio	P50/CH10			P40/CH20			SEM	p value
FF	1	2	3	1	2	3		
Intestine fold height	1.78^{b}	1.11^{a}	1.44^{ab}	1.89^{b}	1.13^{a}	1.11^{a}	0.09	0.01
LP-width	2.00	2.00	2.00	2.11	2.25	2.00	0.03	0.15
LP-cellularity	1.89	1.89	2.00	2.11	1.88	1.89	0.07	0.91
SM-width	1.78	2.00	1.78	1.67	2.13	2.22	0.09	0.39
SM-cellularity	1.00^{a}	1.11^{a}	1.22^{a}	1.11^{a}	1.25^{a}	1.67^{b}	0.06	0.01
GCs	1.11	1.44	1.67	1.33	1.63	1.33	0.12	0.77
IELs	1.00	1.00	1.00	1.00	1.00	1.00	0.00	1.00
EGCs	1.00	1.00	1.11	1.00	1.00	1.00	0.02	0.43
Ent.- nucleus alignment	2.44	2.67	2.67	2.33	2.50	2.78	0.07	0.42
Ent.-vacuolization	1.67	1.22	1.67	1.67	1.63	1.33	0.08	0.35
Mean score	1.57	1.54	1.66	1.62	1.64	1.63	0.03	0.74

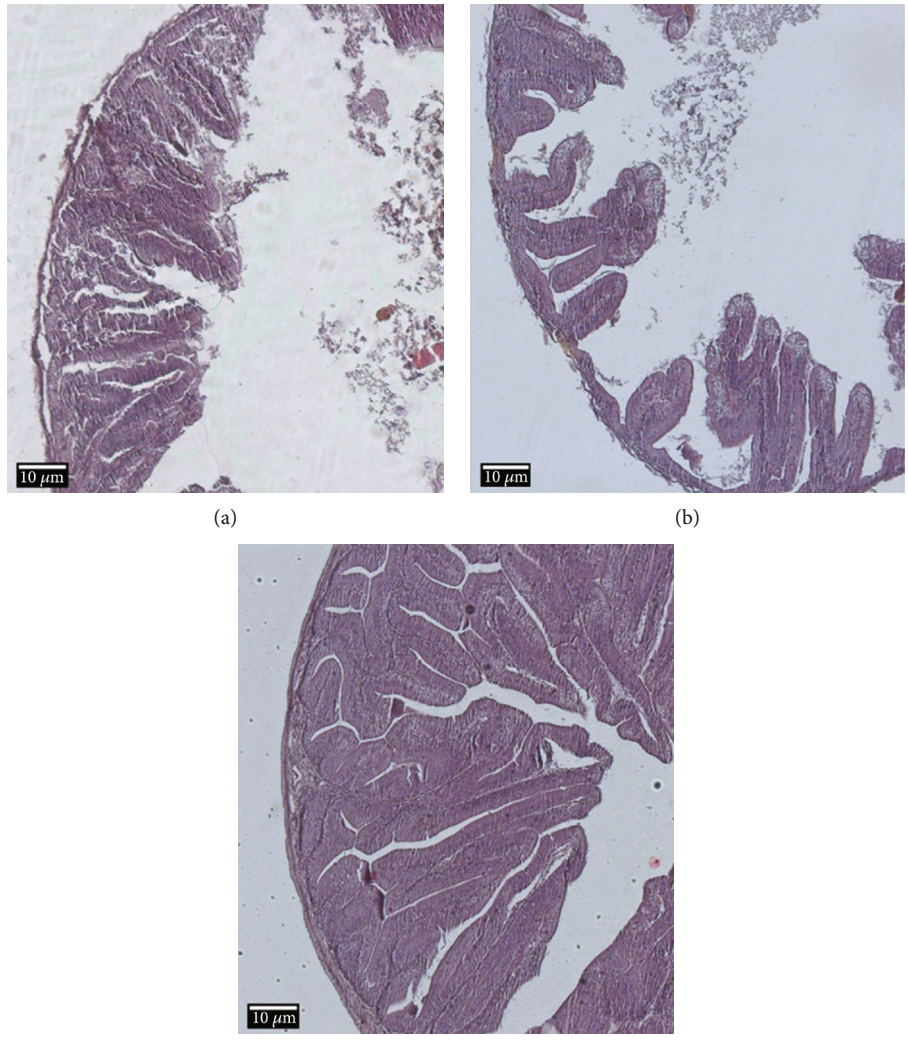
Values presented as means ($n = 9$) and pooled SEM. Different lowercase letters indicate statistical differences between experimental conditions groups as analysed by the Kruskal-Wallis followed by all pairwise comparisons. The significance values were adjusted by Bonferroni correction for multiple tests. CH: carbohydrate; EGC: eosinophilic granulocytes presence; Ent.: enterocytes; FF: feeding frequency; GC: goblet cell presence; IEL: intraepithelial leucocyte infiltration; LP: Lamina propria; P: protein; SEM: standard error of the mean; SM: submucosa.

for the measurement of the maximal activity for each oxidative stress enzyme were established by preliminary tests. The molar extinction coefficients used for H_2O_2 and NADPH were 0.039 and 6.22 mM^{-1} cm^{-1}, respectively.

CAT and SOD were expressed as units (U) per mg of soluble protein, and all other enzymes were expressed as mU/mg protein. Except for SOD, whose activity unit was defined as the amount of enzyme needed to produce 50% inhibition of the ferricytochrome C reduction rate, and one unit (U) of enzyme activity was defined as the amount of enzyme needed to catalyse the hydrolysis of 1 μmol/min of substrate at assay temperature (37°C). Protein concentration was measured according to Bradford [35] using Bio-Rad Protein Assay Dye Reagent (ref. 5.000.006, Amadora, Portugal), with albumin bovine serum (ref. A4503, Sigma-Aldrich, Sintra, Portugal) as standard.

Malondialdehyde (MDA) concentration was measured as described in Couto et al. [33]. Results were expressed as nmol MDA per g of tissue, calculated from a calibration curve.

2.5. Statistical Analysis. All data are presented as the mean and standard error of the mean (SEM). Statistical analysis was done using SPSS 25 software package for Windows (IBM® SPSS® Statistics, New York, USA). Data were tested for normality by the Shapiro-Wilk test and homogeneity of variances by the Levene test. When normality was not verified, data were transformed before ANOVA.



(a) (b)

(c)

Figure 1: Detail of the alterations observed on intestine fold height in the pyloric caeca of gilthead seabream fed P50/CH10 diet or P40/CH20 diet, 1 meal per day (a and b), comparing with those fed P50/CH10 diet, 2 meals per day (c). Images with haematoxylin-eosin staining captured at 10x magnification.

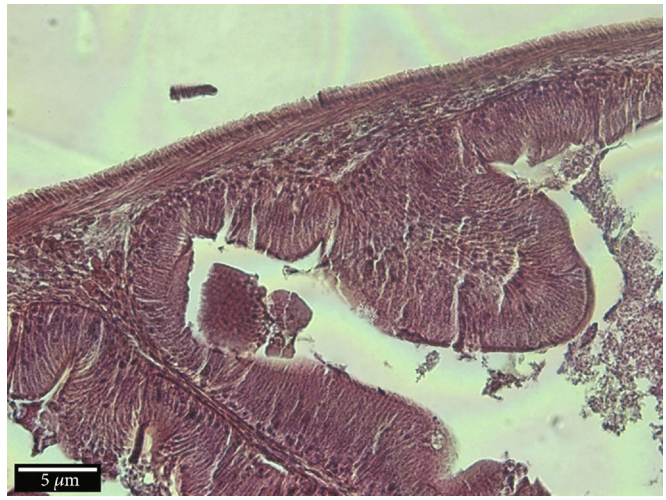
The enzyme activity data and LPO were analyzed by two-way ANOVA, with the dietary P/CH ratio and FF as factors. In the case of interaction between factors, one-way ANOVA was performed for the P/CH ratio within each FF, and FF within each P/CH ratio. Significant differences among groups were determined by the Tukey's multiple range test. Differences were considered statistically significant when $p < 0.05$. Since data for histomorphology evaluation were not normal nor homogenous, a nonparametric Kruskal-Wallis test followed by all pairwise comparisons was performed, and the significance values were adjusted by using the Bonferroni correction for multiple tests.

Statistical analysis related with the DGGE was performed as described in Castro et al. [11]. Intestine microbiota data were then subjected to two-way ANOVA with P/CH ratio and FF as factors, as described for the other parameters.

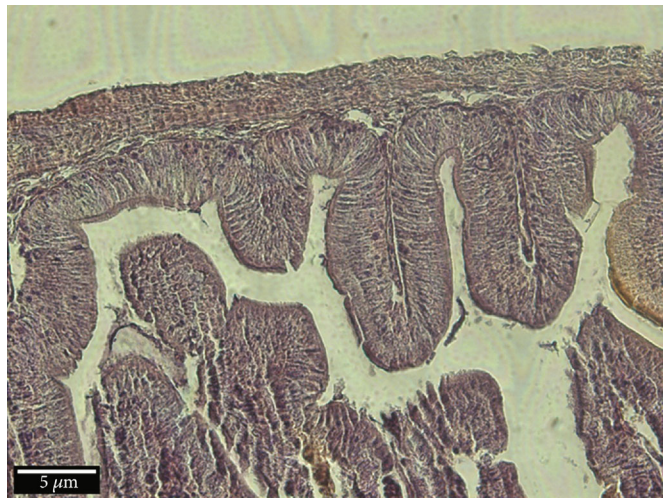
3. Results

The results of the growth trial were not the goal of the present study being presented in Basto-Silva et al. [25]. Shortly, feed intake was increased in fish fed the P40/CH20 diet and 2 or 3 meals per day, while fish fed 1 meal per day presented higher protein efficiency ratio (PER), feed efficiency (FE), and nitrogen retention (NR), but lower final fish weight than the other groups. Furthermore, the P40/CH20 diet led to an increase in PER and NR and a decrease in FE compared to fish fed the P50/CH10 diet.

3.1. Intestinal Histomorphology. Experimental diets and FF did not affect the histomorphology of the DI (Table 2). However, the PC of fish fed 1 meal per day presented a higher fold height compared to the remaining experimental conditions, except for fish fed the P50/CH10 diet 3 meals per day



(a)



(b)

Figure 2: Detail of the alterations observed on submucosa cellularity in the pyloric caeca of gilthead seabream fed P40/CH20 diet, 3 meals per day (a), comparing with those fed P50/CH10 diet, 1 meal per day (b). Images with haematoxylin-eosin staining captured at 40x magnification.

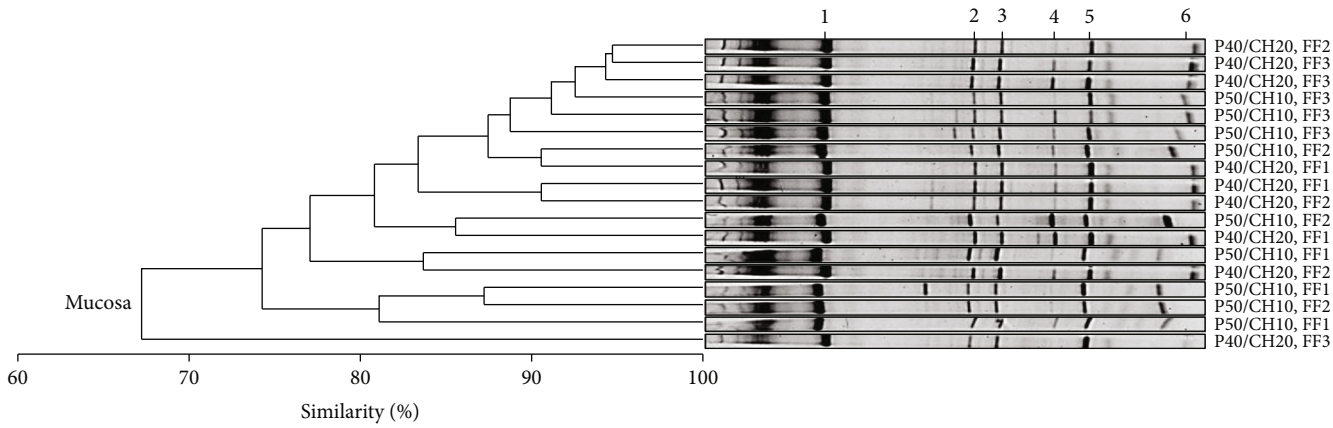


Figure 3: Dendrogram and PCR-DGGE fingerprint of the intestines' autochthonous microbiota of gilthead seabream fed the experimental diets at different feeding frequencies. The bacterial species identified and described in Table 4 correspond to the sequenced gel bands represented in this figure by numbers (1-6).

Table 4: Ecological parameters obtained from PCR-DGGE fingerprints of the intestines' autochthonous microbiota of gilthead seabream fed the experimental diets at different feeding frequencies.

P/CH ratio	P50/CH10			SEM	P40/CH20			SEM
FF	1	2	3		1	2	3	
OTUs	9.67	11.33	13.33	0.63	13.00	13.67	12.67	0.45
Richness[1]	0.56	0.65	0.77	0.04	0.75	0.80	0.73	0.03
Diversity[2]	2.10	2.28	2.43	0.06	2.43	2.45	2.37	0.04
SIMPER similarity (%)[3]	78.83	80.89	90.02	1.99	81.02	79.05	77.71	2.74

Two-way ANOVA	P/CH ratio	FF	I	P/CH ratio		FF		
				P50/CH10	P40/CH20	1	2	3
OTUs	0.02	0.13	0.07	A	B	—	—	—
Richness[1]	0.02	0.13	0.06	A	B	—	—	—
Diversity[2]	0.02	0.20	0.06	A	B	—	—	—
SIMPER similarity (%)[3]	0.30	0.39	0.29	—	—	—	—	—

Values presented as means and pooled SEM ($n = 3$ per treatment pooled from 6 fish). The results were analyzed by using two-way ANOVA, followed by the Tukey's test. Different uppercase letters indicate significantly different P/CH ratios. CH: carbohydrates; FF: feeding frequency; I: interaction; OTUs: average number of operational taxonomic units; P: protein; SEM: standard error of the mean. [1]Margalef species richness: d = (S-1)/log(N). [2]Shannon's diversity index: $H^{'} = -\sum(\text{pi}(\ln\text{pi}))$. [3]SIMPER: similarity percentage within group replicates.

Table 5: Closest known species identified from the DNA sequencing of the autochthonous intestinal bacteria communities of gilthead seabream fed the experimental diets at different feeding frequencies.

Band	Closest known species (BLAST)	Phylum	Similarity (%)	Accession number of nearest neighbor
1	*Lactobacillus aviarius* subsp. *aviarius*	Firmicutes	100	LC071825.1
2	*Lactobacillus acidophilus*	Firmicutes	100	MT645504.1
3	Uncultured bacterium from environmental samples	n/a	98	EU009390.1
4	Uncultured bacterium from environmental samples	n/a	86	LC031369.1
5	*Pseudomonas* sp.	Proteobacteria	100	MK033128.1
6	Uncultured bacterium from environmental samples	n/a	100	KY857639.1

where no changes were observed (Table 3, Figure 1). Furthermore, fish fed the P40/CH20 diet 3 meals per day showed higher cellularity of the submucosa (SM) compared to the remaining experimental conditions (Figure 2).

3.2. Microbiota Diversity. The Bray-Curtis dendrogram and PCR-DGGE fingerprint analysis showed that the intestine bacterial communities maintained a similarity of up to 60% (Figure 3). However, no clustering was detected between samples from different experimental diets or FF. The average number of OTUs, microbial richness, and diversity indices were higher in fish fed the P40/CH20 diet, while the similarity index was not affected by the dietary composition or FF (Table 4). Sequence analysis of DGGE selected bands showed that the dominant autochthonous bacteria detected corresponded to uncultured bacteria not yet assigned to a specific taxon or were most closely related to genera belonging to the phylum Firmicutes and Proteobacteria, namely, *Lactobacillus* sp. and *Pseudomonas* sp., respectively (Table 5).

3.3. Digestive and Oxidative Stress-Related Enzymes and Lipid Peroxidation. The α-amylase activity was increased in fish fed diet P50/CH10 and also in fish fed 1 meal per day (Table 6). Total alkaline protease and lipase activities were not affected by diet or FF.

G6PD and CAT activities were affected by FF, but not by the dietary P/CH ratio (Table 7). Fish fed 2 meals per day presented lower G6PD and CAT activities than fish fed 1 or 3 meals per day, respectively. GR activity was higher in fish fed 3 meals than 1 meal per day, but only in fish fed P50/CH10 diet. Furthermore, in fish fed diet P50/CH10 1 meal per day GR activity was also lower than in fish fed the P40/CH20 diet in the same FF. GPX, SOD, and LPO were not affected by diets or FF.

4. Discussion

Potential interactions between the dietary P/CH ratio and FF on growth, feed utilization, and metabolism of CH were recently evaluated in gilthead seabream [25], as well as in gibel carp (*Carassius auratus gibelio*) and common carp (*Cyprinus carpio*) [29, 30]. While Cheng et al. [30] also presented data on α-amylase activity and Zhao et al. [29] on trypsin activity, this is the first study to determine the combined effects of the dietary P/CH ratio and FF on several

Table 6: Specific activity of digestive enzymes, α-amylase, lipase, and total alkaline protease activity (mU/mg protein) of gilthead seabream fed experimental diets at different feeding frequencies.

(a)

P/CH ratio	P50/CH10			SEM	P40/CH20			SEM
FF	1	2	3		1	2	3	
α-Amylase	663.8	407.0	391.8	39.9	441.1	370.5	373.1	26.3
Lipase	15.1	12.0	13.8	0.8	13.6	11.8	11.6	0.6
Total alkaline protease	624.3	669.8	582.5	19.7	674.8	611.5	662.4	20.7

(b)

Two-way ANOVA	P/CH ratio	FF	I	P/CH ratio		FF		
				P50/CH10	P40/CH20	1	2	3
α-Amylase	0.03	0.00	0.10	B	A	b	a	a
Lipase	0.18	0.12	0.71	—	—	—	—	—
Total alkaline protease	0.40	0.73	0.12	—	—	—	—	—

Values presented as means ($n = 9$) and pooled SEM. The results were analyzed by two-way ANOVA, followed by the Tukey's test. Different uppercase letters indicate significantly different P/CH ratios, and lowercase letters indicate significantly different feeding frequencies. CH: carbohydrates; FF: feeding frequency; I: interaction; P: protein; SEM: standard error of the mean.

parameters of intestinal morphology, functionality, and health of fish.

In the current study, neither the dietary P/CH ratio nor FF affected DI histomorphology, but some minor alterations were observed in the PC in fold-height and submucosa cellularity. This may suggest that PC was more sensitive than DI to the dietary treatments and FF imposed. However, the minor alterations observed in PC most probably did not have biological significance, since the PC mean score was similar between groups, and no correlation was observed between the remaining functionality and health intestine parameters. Previously, Couto et al. [36] also observed in gilthead seabream that dietary soy purified antinutrients affected the PC but not DI histomorphology. Likewise, the absence of DI histomorphology alterations in gilthead seabream fed with different P/CH ratio diets was previously observed by other authors [11, 37].

Gut microbiota composition is strongly influenced by dietary composition and FF either in mammals or fish [38–42]. Although changing the dietary P/CH ratio alters the available nutrients for bacteria fermentation, the associated changes in gut microbiota composition remain unclear [38, 40, 42]. In the current study, fish fed the diet P40/CH20 had an increased average number of OTUs, richness, and diversity indices. The present experiment does not allow us to conclude if these differences are due to the different amounts of protein or CH in the diet. In European seabass (*Dicentrarchus labrax*), a dietary increase of CH lead to increased gut microbiota diversity [43] and this may suggest that the results observed in the current study might also be related with the increased CH content of diet P40/CH20. However, in gilthead seabream, fish fed 0% or 20% of CH diets did not present differences in gut microbiota composition [11]. However, in that study, only the allochthonous microbiota was analyzed, whereas in the present study, we analyzed the autochthonous microbiota. Differences between the two studies might also be related to the CH source used: wheat meal in the current study and gelatinized starch in the study by Castro et al. [11], thus providing different substrates for bacteria proliferation [38, 40]. Besides these differences, the different outcomes might be connected to the different fish sizes used in both studies (9 g in the current study against 71 g in the study by Castro et al. [11]), as it is known that fish developmental stages influence microbiota composition [44].

In the current study, no differences was observed in the autochthonous gut microbiota with FF. Differently, Sherif et al. [41] observed in Nile tilapia that exchanging the feeding regime on a alternately weekly basis affected the intestine microbiota, changing the abundance and proportions of *Lactobacillus*, *Aeromonas*, *Pseudomonas*, and *Edwardsiella* spp. However, in the present study, gut microbiota composition was evaluated by DGGE, a technique that has relatively low resolution. Therefore, for a comprehensive assessment of dietary and FF effects on fish, further studies should be done using methods with a higher-resolution, as, for instance, next-generation sequencing.

Similar to the present study, Cheng et al. [30] also assessed the combined effects of dietary P/CH ratios and FF on α-amylase activity in common carp. The authors tested diets with 3 P/CH ratios (P32/CH5, P30/CH10, and P28/CH20) fed 2 or 4 meals per day and, as in the present study, did not report any significant interaction between those two factors. However, as in the current study, fish fed the higher CH diet and the higher FF presented lower α-amylase activity. These results agree with previously reported results in gilthead seabream fed low P/CH ratio diets [12]. A possible explanation for these results is that in high CH diets, α-amylase molecules could be adsorbed by crude starch, thus inhibiting starch hydrolysis and, at the same time, accelerating intestinal transit speed, leading to a reduction in the time available for intestinal absorption

Table 7: Intestine specific activity of glucose-6-phosphate dehydrogenase (G6PD), glutathione peroxidase (GPX), glutathione reductase (GR) (mU/mg protein), catalase (CAT), superoxide dismutase (SOD) (U/mg protein), and lipid peroxidation (LPO) (nmol malondialdehyde g^{-1} tissue) of gilthead seabream fed the experimental diets at different feeding frequencies.

P/CH ratio	P50/CH10			SEM	P40/CH20			SEM
FF	1	2	3		1	2	3	
G6PD	14.1	8.6	12.1	1.0	16.7	10.8	12.0	0.9
GPX	7.9	8.5	19.3	1.9	12.8	17.4	11.6	2.0
GR	20.1^{Aa}	25.8^{ab}	31.6^{b}	1.5	33.1^{B}	23.1	31.2	2.1
CAT	28.0	18.6	44.4	6.4	18.2	15.3	55.3	9.9
SOD	663.2	782.4	776.3	45.2	807.6	709.8	784.1	53.9
LPO	51.5	61.5	42.4	4.2	50.8	46.8	45.3	2.9

Two-way ANOVA	P/CH ratio	FF	I	P/CH ratio		FF		
				P50/CH10	P40/CH20	1	2	3
G6PD	0.20	0.00	0.61	—	—	b	a	Ab
GPX	0.34	0.18	0.12	—	—	—	—	—
GR	0.21	0.02	0.02					
CAT	0.94	0.03	0.87	—	—	Ab	a	b
SOD	0.83	0.72	0.61	—	—	—	—	—
LPO	0.46	0.21	0.31	—	—	—	—	—

Values presented as means ($n = 9$) and pooled SEM. The results were analyzed by two-way ANOVA, followed by Tukey's test. Two-way ANOVA: if the interaction was significant, one-way ANOVA was performed for P/CH ratio within feed frequency and for feed frequency within P/CH ratio. In this case, significant differences were indicated in the upper part of the table. Different uppercase letters indicate significantly different P/CH ratios, and lowercase letters indicate significantly different feeding frequencies. CH: carbohydrates; FF: feeding frequency; I: interaction; P: protein; SEM: standard error of the mean.

[45]. Another explanation is that when fish are fed fewer meals per day, the higher feed load by meal promotes higher pancreatic secretion of α-amylase [19].

It could be expected that the change in the level of dietary protein affected proteolytic activity, as previously observed by García-Meilán et al. [9, 12] also in gilthead seabream. Nevertheless, no differences in total alkaline protease activity was observed in the current study. Similar to the present results, a lack of effects on proteolytic activity was also reported in gilthead seabream fed different dietary P/CH ratios [8]. The authors tested different levels of P, lipids, and CH in the diet and concluded that intestinal total proteolytic activity was only influenced by changes in dietary lipid, suggesting that proteolytic activity is more sensitive to changes in dietary fat than variations in dietary P or CH.

When the production of reactive oxygen species (ROS) is higher than the respective removal, LPO occurs. CAT reduces H_2O_2 to O_2 and H_2O, being more active when the production of H_2O_2 is high, while G6PD is involved in NADPH regeneration which is a coenzyme required for the normal functioning of CAT, GPX, and GR [46, 47]. In the current study, although LPO levels were not affected by the FF, lower G6PD and CAT activities were observed in fish fed 2 meals per day, which might indicate a reduction of total ROS production. The available data suggests that an intermediary FF contributes to improving the antioxidant capacity of fish. Accordingly, in juvenile Dolly Varden char, total antioxidant capacity increased with FF up to 5 meals per day, decreasing at higher FF [21]. Also, in blunt snout bream fed between 1 and 6 meals per day, the lowest hepatic CAT and GPX activities were detected in fish fed 3 or 4 meals per day, while the total antioxidant capacity was higher in these groups [22]. Similarly, in juvenile tiger puffer (*Takifugu rubripes*), fish fed 4 or 6 meals per day exhibited lower antioxidant enzyme activities, namely, SOD, CAT, and GPX activities, than those fed only 2 meals per day or continuous feeding [48].

In the current study, the dietary P/CH ratio did not have any major effect on LPO or antioxidant enzyme activities. This is similar to what was previously reported for gilthead seabream and European seabass [10, 49].

GR which catalyzes the NADPH-dependent regeneration of reduced glutathione from oxidized glutathione generated by GPX was the only oxidative stress-related enzyme presenting an interaction between dietary P/CH ratio and FF. Despite no differences was observed regarding GPX activity, GR results might suggest that fish fed diet P40/CH20 at 1 meal per day might be under a higher overall ROS production than fish fed diet P50/CH10 at the same FF. Within fish fed the P50/CH10 diets, the same seems true for fish fed 3 meals instead of 1 meal per day. However, since no other interactive effect was observed in the remaining stress oxidative-related enzymes, or any other measured parameter, it is not possible to draw any conclusion regarding the interactive effect of using different FF and P/CH ratios.

In conclusion, the present results indicate that there are no major interactions between the dietary P/CH ratios and FF with respect to the intestinal functionality and health of gilthead seabream. Present results further support the conclusion of Basto-Silva et al. [25] where a diet with a lower P/CH ratio (P40/CH20 vs. P50/CH10) fed 2 meals per day appears to be the most adequate strategy for gilthead seabream juveniles.

Data Availability

The data used to generate the results in this manuscript can be made available if requested to the corresponding author.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

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