MEDDOCS Open Access Publisher

ISSN: 2640-1223

Journal of Veterinary Medicine and Animal Sciences

Open Access | Research Article

Partial Replacement of Fish Meal with Three Different Protein Sources in Rainbow Trout Oncorhynchus mykiss: Response of Intestinal Digestive Enzymes

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Received: Jan 17, 2022

Accepted: Feb 16, 2022

Published Online: Feb 21, 2022

Journal: Journal of Veterinary Medicine and Animal Sciences

Publisher: MedDocs Publishers LLC

Online edition: http://meddocsonline.org/

Keywords: Digestive enzymes; Bacterial protein sources; Pea; Plant protein sources; Rainbow trout.

Abstract

Objective: In fish feeding formulation, high interest has recently been addressed to protein sources alternative to Fish Meal (FM); nevertheless, the response of digestive enzymes to dietary changes is not fully known. To fill this gap, a study was undertaken in rainbow trout (Oncorhynchus mykiss), to explore the effects on intestinal enzymes (trypsin, chymotrypsin, carboxypeptidases A and B, amylase) of three experimental diets, containing a plant protein source (pea protein concentrate-PPC), a microbial protein source (bacterial protein meal-BPM) or a mix of PPC and BPM (MIX), in partial (50%) replacement of FM.

Methods: Two feeding regimes (apparent satiation or rationed feeding at 1.4% of the fish wet biomass) and three times (1, 3 and 24 hours) after ingestion were tested. Enzyme activities were measured using conventional biochemical methods for digestive enzymes determinations.

Results: PPC administered to satiation lead to not significantly higher protein utilization than FM, as suggested by proteolytic enzymes trypsin, chymotrypsin, carboxypeptidase A. In fish fed rationed regime, PPC induced carboxypeptidase B peak and moderate trypsin increase. BPM stimulated trypsin in fish fed both feeding regimes and carboxypeptidase A in those fed to satiation. MIX diet varied significantly trypsin, carboxypeptidase B and amylase, increasing carboxypeptidase A and decreasing chymotrypsin in fish fed to satiation.

Cite this article: Gai F, Caruso G, Dabbou S, Muglia U, Gasco L. Partial Replacement of Fish Meal with Three Different Protein Sources in Rainbow Trout Oncorhynchus mykiss: Response of Intestinal Digestive Enzymes. J Vet Med Animal Sci. 2022; 5(1): 1104.

Conclusion: Digestive enzyme patterns were generally unaffected by the tested protein sources, suggesting that they could partially replace FM without detrimental effects on rainbow trout digestive processes.

Introduction

In intensive aquaculture the shortage of fish meal sources as the main ingredient of animal feed, mainly related to the overfishing and the increase in human demand, has resulted in the search for alternative protein sources [1-3]. Microbes (including bacteria, yeast and algae) have become potential candidates to be included in fish feeds as ingredients in partial replacement of fish meal [4-6]. In addition, the introduction into the dietary composition of some protein sources derived from plants has attracted increasing attention [1,3,7-15]. Plant ingredients are generally considered as good potential substitutes of fish meal thanks to their wide availability and low price [16,17]; nevertheless, their suitability as dietary FM substitutes in aqua feeds is still a matter of scientific debate, since their use can have some constraints. A first constraint is related to their lower protein and amino acid content compared to FM [17] that can result in a lower growth performance when plant proteins are included at high percentages into the diet; for this reason, the inclusion rates of plant protein feeds are generally quite limited.

Being highly carnivorous fish, salmonids need a high-quality protein source for their optimum health and growth performance. Among potential plant raw materials, lupin, peas and canola meal have been described as feasible ingredients for the partial replacement of FM in salmonid feeds [18]. Pea (*Pisum sativum*) is a species belonging to Leguminosae fairly marketed within the European Community; it is characterized by a high starch content (> 50% of dry matter), by a low crude protein content (<25% of dry matter) and high energetic value (14.3 kJ/g) [14,19].

It has a good potential as a dietary protein source for temperate and warm water fish such as European sea bass [20,21,22] milkfish [23], and silver perch [24] and also for salmonid fish [25,26]. In gilthead seabream, the nutritional properties of pea - namely the low protein content and imbalanced amino acid composition - allow its dietary inclusion as a partial replacement of fishmeal, reaching percentages up to 20% [27,28]. In addition, like soybean, pea seeds contain a source of Antinutritional Factors (ANF) such as tannins, lectins and protease inhibitors that constrain its use as a dietary ingredient [14,15,29-31]. Pea digestibility can be improved by extrusion and de-hulling, which can also inactivate the ANF [24,32]. In rainbow trout *Oncorhynchus mykiss*, very little is still known on the effects on the digestive enzyme patterns caused by this alternative plant source. In the light of this consideration, in this species the use of pea seeds as a source of protein in partial replacement of fish meal deserves to be further investigated. Moreover, in feed formulation, increased attention has recently been given to explore the suitability of bacterial-derived proteins as dietary ingredients. When used at low inclusion rates, these did not produce any significant effects on fish growth either on intestinal microbiota [33,34]. In particular, Protorsan, which is a byproduct of L-glutamic acid produced by fermentation, consists of dehydrated bacterial bodies of *Corynebacterium glutamicum melassecola*, a bacterium able to degrade sugar-containing substrates such as molasses or starch hydrolysates into glutamic acid. Protorsan, which contains high levels of peptidoglycan as components of the bacterial cell wall, has been tested as a feed stimulant in diets for sea bream [33] due to its content of glutamic acid (12.32%) as a palatability enhancer; in rainbow trout its experimentation as a dietary ingredient has started [35]. This study focuses on the response of digestive enzyme patterns of *O. mykiss* to the use of pea, bacterial protein meal and a mixture of both these feed ingredients as protein sources in alternative to FM.

Materials and methods

All the experiments were carried out in accordance with the Directive 2010/63/EU. All manipulations were performed on fish specimens completely anaesthetized.

Experimental design

Three experimental diets were prepared by including pea protein concentrate (PPC, provided by AgriMarin Nutrition, Stavanger, Norway), Protorsan (provided by Mazzoleni Prodotti Zootecnici, Cologno al Serio, Italy) further indicated as Bacterial Protein Meal (BPM), or a Mixture of both protein concentrates (MIX), respectively, in partial replacement of Fish Meal (FM) A control diet, based on FM, was included in the trial. The feed formulation and proximate composition of the diets are shown in Table 1.

Table 1: Ingredients and proximate composition of the three experimental diets (pea protein concentrate-PPC; bacterial protein meal-BPM and mixed diet-MIX) used for rainbow trout Oncorhynchus mykiss in comparison to control diet containing fish meal (FM).

Diets	FM	PPC	BPM	MIX
Ingredients (%)				
Herring fish meal[a]	50.00	25.00	25.00	25.00
Protorsan[b]	0.00	0.00	25.00	12.50
Pea protein concentrate[c]	0.00	30.00	0.00	15.00
Corn meal	23.00	12.00	24.00	17.50
Fish oil	10.00	12.50	11.00	12.00
Corn gluten	8.00	12.00	6.00	9.00
Lignum sulphate	6.00	6	6	6
Mineral mixture[d]	1.50	1.25	1.50	1.50
Vitamin mixture[e]	1.5	1.25	1.50	1.50
Proximate composition (%DM)				
Dry matter (% fresh matter)	96.6	95.90	92.50	96.10
Crude protein	45.4	45.20	45.90	45.20
Ether extract	17.3	16.50	14.60	17.00
Ash	12.00	9.20	8.90	9.20
Crude fiber	1.90	1.90	2.00	1.90
Nitrogen free extracts[f]	23.40	27.20	28.60	26.70
Gross energy (MJ/kg DM)[g]	21.50	22.20	21.80	22.10

[b]Mazzoleni Prodotti Zootecnici, Cologno al Serio, Italy: DM 92%, CP 67%, EE 6%, ash 3.8%, CF 1%.

[c]AgriMarin Nutrition, Stavanger, Norway: DM 90%, CP 55%, starch 9%, EE 2%, ash 6%.

[d]Mineral mixture (g or mg/kg diet): Bicalcium phosphate 500 g, calcium carbonate 215 g, sodium salt 40 g, potassium chloride 90 g, magnesium chloride 124 g, magnesium carbonate 124 g, iron sulphate 20 g, zinc sulphate 4 g, copper sulphate

3 g, potassium iodide 4 mg, cobalt sulphate 20 mg, manganese sulphate 3 g, sodium fluoride 1g, (Granda Zootecnica, Cuneo, Italy).

[e]Vitamin mixture (IU or mg/kg diet): DL-a tocopherol acetate, 60 IU; sodium menadione bisulphate, 5 mg; retinyl acetate, 15000 IU; DL-cholecalciferol, 3000 IU; thiamin, 15 mg; riboflavin, 30 mg; pyridoxine, 15 mg; B12, 0.05 mg; nicotinic acid, 175 mg; folic acid, 500 mg; inositol, 1000 mg; biotin, 2.5 mg; calcium panthotenate, 50 mg; choline chloride, 2000 mg (Granda Zootecnica, Cuneo, Italy).

[f]Calculated as 100-(%Crude protein +%Ether extract +%Ash +%Crude fiber).

[g]Determined by calorimetric bomb.

The Dry Matter (DM) (#930.15) and the ash (#924.05) were assessed according to the Association of Official Analytical Chemists procedures [36]. The total Nitrogen (N) content was determined using a nitrogen analyzer (Rapid N III; Elementar Analysen system GmbH, Hanau, Germany) according to the Dumas method and the gross energy was measured using an adiabatic calorimetric bomb (C7000; IKA, Staufen, Germany). All the diets were isonitrogenous (CP 45 %) and isoenergetic (22 MJ/kg DM). They were manufactured in the laboratory at the Experimental Station of the Department of Agriculture, Forestry, and Food Sciences of the University of Torino. The grounded ingredients and fish oil were thoroughly mixed; water was then added to the mixture to attain an appropriate consistency for pelleting. Pellets were obtained using a 3.5 mm die stainless meat grinder (Platone, Torino, Italy), then dried in a stove overnight at 50°C, and then refrigerated at +5 °C until their use.

The experiment was conducted on a stock of juvenile *O. mykiss* obtained from a private hatchery (Bassignana, Cuneo, Italy), which was kept in the facility of the Department of Agricultural, Forest, and Food Sciences of the University of Torino. During the feeding trial, the ethical principles indicated by the European Commission Directive (2010/63/EU) on the care and use of animals for scientific purpose were applied. All work was conducted with the formal approval of the Institutional animal care committee. A stock of 1200 *O. mykiss* juveniles (initial mean body weight 114.6 ± 0.2 g) were individually weighed to obtain a homogeneous stock of fish and randomly distributed into 24 fibre-glass tanks (0.5 m^3) supplied by an open-water circuit; a water flow rate of 25 l/ min was applied. Temperature and dissolved oxygen values were 13 ± 1 °C and 7.0 ± 0.5 mg/l, respectively.

The adopted experimental design was that reported in a previous study [35], bi-factorial with four diets x three replicates x two feeding regimes (4x3x2). After a period of acclimatisation to the tanks and diets of 2 weeks, the feeding trial lasted 77 days. Feeds were distributed manually, 6 days per week, twice a day, according to two different administration modalities: to apparent satiation ("ad libitum" feeding regime) or to rationed feeding regime (at 1.4% of the wet biomass). Care was made to check that feed was ingested and not rejected at each feeding time.

Sampling of the digestive organs and treatment for enzymatic analyses

At the end of the feeding trial, the fish were collected; five specimens per tank were sampled at different post-prandial times (1, 3 and 24 h from last meal), anaesthetized, weighed and sacrificed with a lethal dose (100 mg/l) of MS-222 (Sigma-Aldrich). The intestinal tract of each specimen was removed as a whole and without washing kept at -20 °C until analysis. After removal of their contents, the thawed samples of the whole intestine (anterior, middle and posterior) were subjected to homogenization in a Potter-Ultraturrax in physiological solution (in a 1:50 w/v ratio) and centrifuged at 3000 rpm x 20 min at + 5 °C. The obtained supernatant - considered as enzyme extract - was divided into 1.5 ml aliquots in Eppendorf vials at -20 °C.

Enzymatic assays

For the determination of enzymatic activities, the methods previously described by Caruso et al. [37] were used Specific details are reported below.

Trypsin activity was determined using the method of Hummel [38] modified by Rick [39], with p-Toluenesulfonyl-L-Arginine Methyl Ester (TAME) as the substrate. The reaction started with the addition of enzyme extract (0.05 ml) to a mixture of 0.15 ml of 10.0 mM TAME solution and 1.3 ml of 46.0 mM Tris buffer at pH 8.10 with 11.5 mM CaCl2 buffer. The change in absorbance was measured at 247 nm during 3 min at 25.0 °C. Trypsin activity was reported as 1Unit = 0.001 Absorbance increase per minute.

Chymotrypsin activity was determined using the method of Hummel [38] modified by Rick [40], with N-Benzoyl-L-Tyrosine Ethyl Ester (BTEE) as the substrate. The reaction started with the addition of enzyme extract (0.05 ml) to a mixture of 0.70 ml of BTEE (80.0 mM in 50 % methanol) and 0.75 ml of 80.0 mM Tris buffer at pH 7.80 with 0.1 M $CaCl_2$ buffer. The change in absorbance was measured at 256 nm during 3 min at 25.0 °C. Chymotrypsin activity was reported as 1Unit = 0.001 Absorbance increase per minute.

Carboxypeptidases A and B were measured using Hyppuryl-L-arginine and Hyppuril-L-phenylalanine as the substrates, respectively [41]. For carboxypeptidase A, 0.15 ml of enzyme extract were mixed with 1.35 ml of 1.1 mM hyppuryl-L-phenylalanine in 27.5 mM Tris buffer 0.11 M NaCl at pH 7.60. For carboxypeptidase B, 0.15 ml of enzyme extract were mixed with 1.35 ml of 1.1 mM hyppuryl-L-arginine in 27.5 mM Tris buffer 0.11 M NaCl at pH 7.60. The change in absorbance was measured at 254 nm during 3 min at 25.0 °C. Carboxypeptidases A and B were reported as 1Unit = 0.001 Absorbance increase per minute.

Amylase activity was determined using the method of Bernfeld [42] modified by Rick [43]. The reaction mixture, containing 0.05 ml of enzyme extract, 1.0 ml solution of soluble starch (1 % in phosphate buffer pH 6.9) as the substrate and 1.0 ml of 20 mM phosphate buffer at pH 6.9 with 10 mM NaCl, was first incubated for 10 minutes at 25.0 °C. After addition of Dinitrosalicylate (DNS) reagent (2.0 ml of 1 % DNS, 30 % sodium potassium tartrate), the mixture was placed in boiling water for 5 minutes and cooled at room temperature for 30 minutes. Absorbance of the mixture was determined at 540 nm, using maltose for calibration. Alpha-amylase activity was reported as 1Unit = 1 microgram of maltose released per minute.

The measured enzymatic values were normalized to the protein content, determined according to the method by Lowry et al. [44], and reported as specific activities (Units of enzyme/mg protein).

Statistical analyses

The experimental design involved as sources of variability the "diet" factor (three experimental diets + 1 control) and the "feeding regime" factor (administration to apparent satiation *versus* rationed feeding regime), with three repeated measurements, corresponding to the three digestion times (1, 3 or 24 hours after feeding). A Two-ways ANOVA test with replicated measurements was applied to the dataset, to assess the significance of differences related to the diet, to the feeding modality or to diet X feeding interaction. A probability level of 0.05 was considered as the threshold value to consider as significant the obtained F values.

Results

Growth parameters

The growth performance in terms of Weight Gain (WG) and Specific Growth Rates (SGR) of the same diets assayed in this research was determined by Gai et al. [35] in a previous study performed on rainbow trout fed experimental diets in alternative to FM. Significant differences (P<0.01) in WG depending on the diet were found; WG ranged from 39.5 to 113.5 in fish fed ad libitum with BPM and FM dietary groups respectively, while it ranged from 40.6 to 100.4 in fish fed at rationed regimen with the same diets. Similarly, significantly different (P<0.01) SGR were observed, with values ranging from 0.40 to 0.90 in fish fed ad libitum with BPM and FM, respectively; and from 0.40 to 0.85 in those fed at ratio in the same dietary groups. These results were explained by the lowest crude protein digestibility recorded in the BPM group that could be due to a negative effect of bacterial membrane and cell wall components on protein digestibility, as observed in previous studies in rainbow trout fed single-cell proteins from brewer's yeast [45].

Enzymatic data

The specific enzymatic activities measured in our study (mean value from 3 replicated assays ± standard deviation) are shown in Figure 1, comparing "apparent satiation" to "rationed" feeding regimes.

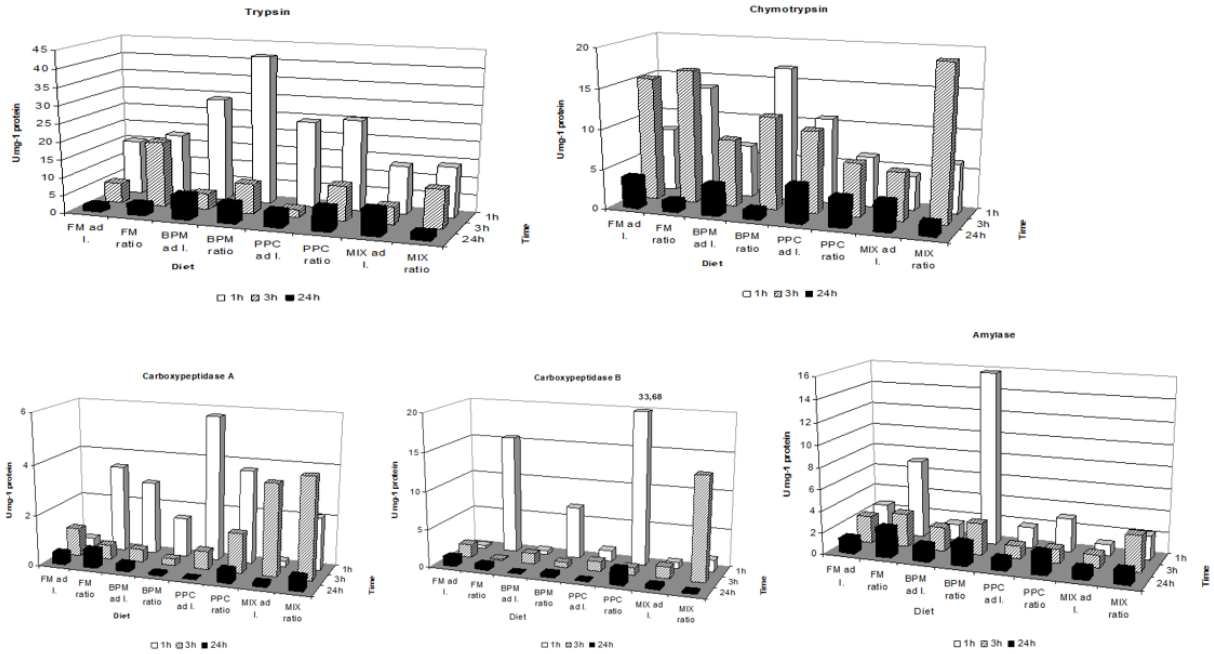

Figure 1: Specific activity values of digestive enzymes (mean from 3 replicates) measured in the intestine of rainbow trout *Oncorhynchus mykiss* specimens fed diets [administered to apparent satiation or "ad libitum", indicated as "ad l.") or at rationed feeding regime (indicated as "ratio")] supplemented with Pea Protein Concentrate (PPC), Bacterial Protein Meal (BPM) and a Mix of both dietary ingredients (MIX), in comparison with a control group fed a control diet containing fish meal (FM).

The levels of trypsin varied from 2.09 ± 0.10 to 29.05 ± 0.50 U/mg protein for fish fed diets administered to apparent satiation, while from 2.07 ± 0.50 to 41.64 ± 6.35 U/mg protein in fish fed at the rationed Feeding regime. Compared to the control diet (FM), all the experimental diets resulted in increased tryptic activity, which was particularly stimulated by the BPM diet administered to both apparent satiation or rationed regimes. Peak values in this enzymatic activity were recorded already 1 hour after feeding; at 3 or 24 hours after feeding, tryptic activity levels progressively decreased in fish fed the FM diet in the rationed group as well as in the group fed to apparent satiation, showing conversely a slight increase in fish fed all the other alternative diets administered to satiation.

Chymotrypsin values were comprised between 3.75 ± 0.10 and 15.42 ± 1.80 U/mg protein in fish fed to apparent satiation, and between 1.25 ± 0.40 and 19.40 ± 3.80 U/mg protein in fish fed at rationed regime. Compared to FM diet, lower enzyme activity values were measured in fish fed the experimental diets, especially by the MIX and BPM diets when administered to apparent satiation, and by the PPC diet at rationed regime. Peaks in enzymatic activity occurred generally 3 hours after ingestion.

Carboxypeptidase A showed values ranging from 0.03 ± 0.01 to 5.73 ± 1.85 U/mg protein in fish fed to apparent satiation regime, while they ranged from 0.08 ± 0.01 to 3.98 ± 1.90 U/mg protein in those fed at rationed regime. On average, higher enzyme values than in, fish fed FM diet were found in fish fed all the experimental diets, particularly the PPC diet administered both to apparent satiation and rationed regimes. Conversely, the BPM diet rationed administered resulted in a decrease in carboxypeptidase A values. Enzyme values peaked 1 or 3 hours after feeding.

Carboxypeptidase B concentrations varied from 0.08 ± 0.01 to 1.73 ± 0.60 U/mg protein and from 0.19 ± 0.83 to 33.68 ± 4.70 U/mg protein in fish fed to apparent satiation and rationed regime, respectively. Peaks in digestive activity were found 3 hours or 1 hour after apparent satiation or rationed administration, respectively. Following administration of the BPM diet at both the feeding regimes, carboxypeptidase B levels generally decreased compared to those recorded in fish fed the FM diet. The effects produced by the PPC administration on the levels of this enzyme were sometimes contrasting, i.e. causing decreased activity values when fish were fed to apparent satiation and increased enzyme levels when it was rationed administered.

Amylase values were comprised between 0.99 ± 0.10 and 2.67 ± 0.80 U/mg protein in fish fed to apparent satiation, while they ranged from 1.26 ± 0.40 to 15.83 ± 3.40 U/mg protein in fish under restricted feeding regime. Enzymatic values peaked after 3 or 1 hours after apparent satiation or rationed administration, respectively. Amylase levels decreased after apparent satiation administration of all the diets, especially of the MIX diet compared to the FM one. When the experimental diets were administered at rationed regime, the amylolytic activity decreased in fish fed the PPC and the MIX diets compared to those fed the FM diet, while the BPM diet resulted in increased enzyme secretion.

ANOVA results, reported in Table 2, showed that no significant changes occurred in relation to diet, feeding regime, or their interaction. Indeed, no significant differences were observed in the digestive patterns of fish fed the experimental diets compared to those fed the control diet.

Table 2: Outputs of two ways- ANOVA test performed on repeated measurements (1.3 and 24 hours after feeding) of digestive enzymes in rainbow trout *Oncorhynchus mykiss*. Reported are the F value and the probability level.

		Diet	Feeding	Diet x feeding interaction
Trypsin	F value	0.41	0.632	0.061
	Significance level (P)	0.727	0.438	0.98
Chymotrypsin	F value	0.33	0.477	0.498
	Significance level (P)	0.804	0.5	0.689
Carboxypeptidase A	F value	0.542	0.114	0.296
	Significance level (P)	0.66	0.74	0.828
Carboxypeptidase B	F value	0.43	2.929	0.401
	Significance level (P)	0.735	0.106	0.754
Amylase	F value	1.15	3.835	0.719
	Significance level (P)	0.359	0.068	0.555

Discussion

The present research aimed at evaluating the effects on digestive patterns of new protein sources for *O. mykiss* feeding through testing three different dietary formulations, that included a Plant Protein Source (PPC), a Bacterial one (BPM) and a mix of both in alternative to FM. The ultimate goal of this topic is to propose an eco-friendly alternative to the use of FM.

In *O. mykiss* inclusion up to 20% of peas in partial replacement of FM was reported to be unsuitable to improve growth performance and feed utilization [30].

According to the dataset obtained in the present study, *O. mykiss* fed the experimental diets having a percentage of FM replacement equal to 50% showed no significant changes in their enzymatic patterns, compared to fish belonging to the control group; however, the response of the digestive enzyme to the new dietary regime highlighted some specific trends. In fish fed a PPC-containing diet, administered to satiation as well as at rationed regime, the levels of proteolytic enzymes were slightly higher than those measured in control fish, with an increase measured 1 hour after feed ingestion for trypsin and 1 and 3 hours after feed ingestion for chymotrypsin. This result is in contrast with impairment of tryptic activity reported by Krogdhal et al. [46] in *O. mykiss* fed soybean proteinase inhibitors. The stimulating action of PPC appeared to be more evident on the secretion of the enzyme carboxypeptidase A, which showed peaks 1-3 hours after feed ingestion under the satiation regime. A similar effect was recorded for carboxypeptidase B in fish feed at rationed feeding regime. Conversely, in fish fed at this same regime, PPC induced a reduction in chymotrypsin levels.

Dietary supplementation with BPM resulted in a stimulating effect on the secretion of trypsin in fish fed both feeding regimes and of carboxypeptidase A in fish fed to apparent satiation. In fish fed at rationed regime, BPM induced a decrease in carboxypeptidase B, while it did not have effects on this enzyme when administered to apparent satiation. In a recent study [35] on *O. mykiss*, BPM was found to affect negatively growth performance and nutrient digestibility compared to a control (FM-fed) group, while PPC diet gave results similar to FM group. The low nutrient digestibility associated to BPM administration led us to hypothesize that *O. mykiss* specimens fed with BPM were more stimulated to secrete enzymes to compensate for the low digestibility.

No effects were produced on amylase levels by BPM and PPC in fish fed to satiation; this finding was in agreement with the dietary composition of these two diets. A similar result, namely the lack of significant changes in alpha-amylase levels, was observed by Santigosa et al. [47] in *O. mykiss* fed a mixture of plant protein sources in partial or total replacement of FM for 12 weeks.

The MIX diet, when administered to apparent satiation, was not associated to significant variations in trypsin, carboxypeptidase B and amylase, while resulted in increased carboxypeptidase A and decreased chymotrypsin. Digestive enzyme patterns in fish rationed fed were substantially similar to those recorded in fish fed to apparent satiation, except for increased chymotrypsin levels.

Considering the changes over time of digestive enzyme secretion, in the present investigation all the enzymatic patterns, and particularly trypsin, followed a general decreasing trend during the digestive process, except for chymotrypsin and carboxypeptidase B, whose enzymatic values increased 3 hours after feed ingestion. Only for fish fed to apparent satiation, a slight increase in tryptic values was measured 24 hours after ingestion; this could depend on the administration regime and not be a function of the administered diet, since in fish feed a restricted dietary regime enzyme secretion exhibited mostly a decreasing trend.

According to the overall observations, this study on digestive enzymes indicated a very good adaptive response of *O. mykiss* specimens to the experimental diets. The use of PPC, in partial replacement of FM, increased protein utilization, at least as es-

timated by the amount of proteolytic digestive enzymes. This observation is in contrast with the significant decrease of total protease activity after feeding with plant proteins reported by Santigosa et al. [47] in sea bream; the same authors [48,49] reported a delayed intestinal nutrient absorption in sea bream and rainbow trout fed with plant protein sources. Plant-based ingredients were found to cause reduced protein digestibility also in cod [50]. This effect likely related to ANF present in protein sources [29] which are reported to decrease enzymatic activity [51] and to induce intestinal inflammatory processes in Atlantic salmon fed soybean meal [52], or pea protein concentrate at high levels [53], whereas no significant histological alterations have been reported for lupin kernel meal-fed *O. mykiss* [9].

Conclusions

According to the digestive enzymatic patterns observed in this investigation, PPC did not cause apparent detrimental effects on the secretion of digestive enzymes. This finding confirmed what previously reported by Øverland et al. [31] in Atlantic salmon, where PPC was unable to affect protein digestibility compared to a FM reference diet. In the present study, in spite of high carbohydrate content of plant feed ingredients and particularly of field peas [14], PPC did not seem to affect amylase, whose secretion is rather stimulated by BPM in rationed fed *O. mykiss*. Therefore, PPC and BPM can be included in the formulation of practical diets for this fish species in partial replacement of FM without apparent detrimental effects on digestive processes. Further research using different dietary compositions and digestibility trials are, however, required to obtain deeper insights into the digestive physiology of *O. mykiss* and to suggest the examined ingredients as good, cheap protein dietary ingredients in alternative to FM.

Acknowledgments

The Authors thank Marco Ortoffi (ISPA, National Research Council), Luciano Sola, and Dario Sola for their careful management of the fish and Vanda Malfatto (DISAFA, University of Turin) for their technical assistance during the laboratory analysis.

This research was funded by Italian Ministry of Agriculture, Food, and Forestry Policy (MiPAAF) in the framework of the RENAI project (Tecnologie alimentari per la riduzione dell'impatto ambientale da azoto negli allevamenti intensivi italiani).

This study was carried out in strict accordance with the Directive 2010/63/EU on the protection of animals used for scientific purposes (Enforced in Italy on 29 March 2014). All experimental manipulations of fish were performed in a manner designed to minimize pain and discomfort.

Author contributions: Conceptualization, all the authors; methodology, F.G., G.C., S.D., and L.G.; formal analysis, F.G., G.C. and L.G.; investigation, F.G., G.C., S.D. and L.G.; data curation, F.G. and G.C.; writing-original draft preparation, all the authors; writing-review and editing, all the authors.; supervision, F.G. and G.C.; project administration, F.G. and L.G.; funding acquisition, F.G. and L.G. All authors have read and agreed to the published version of the manuscript.

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