




## Article

# Impact of Plant-Based Diets on Hepatosomatic Index, Circulating Globulins and Growth in Rainbow Trout (*Oncorhynchus mykiss*)

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**Abstract:** Serum proteins are essential for maintaining osmotic pressure, regulating pH, transporting metabolites, and supporting immune responses. Studying the effects of plant-based diets on these proteins is crucial to understanding their impact on fish health and immune function. **Methods:** This study was conducted in a recirculation system, with rainbow trout distributed across nine tanks and fed three diets: fishmeal (control), medium plant meal (MPM; 40% of fishmeal replacement), and high plant meal (HPM; 80% of fishmeal replacement). Plasma protein and plasma glucose levels were measured at the initial and final stages, under both fasting (24 h after fast) and postprandial (6 h after fed) conditions, to assess dietary impacts. Additionally, the hepatosomatic index (HSI) was calculated at the end of the experiment to evaluate potential liver adaptations to the diets. **Results:** The initial protein and glucose levels were similar across all groups. However, by the end of the treatment, the fishmeal-fed group showed significantly higher total protein and globulin levels, while the albumin levels remained consistent across diet types. A significant interaction between sampling time and dietary treatment in fish reduced the clarity of dietary effects on postprandial plasma glucose levels. Furthermore, the HSI was significantly lower in fish fed the high plant meal (HPM) diet compared to the fishmeal and medium plant meal (MPM) groups, suggesting potential metabolic adaptation in response to plant-based diets. **Conclusions:** Replacing fishmeal with plant-based proteins in rainbow trout diets reduces total globulin and protein concentrations but leaves albumin unaffected, alongside reductions in fasting and postprandial glucose levels. The low growth and different HSI in fish fed plant-based diets highlights potential liver stress, emphasizing the need for further research to optimize sustainable aquaculture feeds while maintaining fish health and performance.



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**Keywords:** aquaculture; plant meal; plant-based diets; hepatosomatic index (HSI); rainbow trout; globulins; glucose

**Key Contribution:** This manuscript highlights significant findings regarding the impact of plant-based diets on growth; HSI; plasma proteins in salmonids.

## 1. Introduction

Optimizing fish growth while decreasing reliance on fish meal and fish oil in the commercial fish feed industry is essential for enhancing aquaculture sustainability. Feed represents over 55% of variable costs in intensive fish farming, making the development of cost-effective feed formulations a priority. Significant progress has been made in decreasing reliance on wild fisheries for fishmeal and fish oil, which helps alleviate pressure on marine resources [1]. In this context, plant-based proteins have increasingly replaced a substantial portion of fishmeal in carnivorous fish diets, providing a more sustainable and environmentally friendly alternative [2]. However, while these plant-based diets offer clear sustainability advantages, they also pose challenges, including nutritional imbalances stemming from lower levels of essential amino acids, reduced digestibility, and the presence of anti-nutritional factors (ANFs) that can hinder nutrient absorption and adversely affect fish health [3].

The liver plays a central role in nutrient metabolism, detoxification, and immune regulation in fish, making it a vital organ for maintaining overall health and performance. The hepatosomatic index (HSI), which measures the relative size of the liver to body weight, serves as an important indicator of hepatic metabolic status and adaptive responses to dietary changes [4]. Altered HSI values may signal liver enlargement due to increased metabolic demands or stress, often associated with plant-based diets that require the liver to metabolize higher levels of carbohydrates [5,6]. Understanding how changes in diet composition, including replacing fishmeal by using plant-derived feedstuff, impact liver function is crucial for designing feeds that support health and growth without compromising fish welfare.

Total protein (TP) concentration in blood, including globulins and albumins, has been regarded as a key parameter for monitoring overall fish health [7]. This is especially true since globulins transport molecules like hormones and vitamins, regulate osmotic pressure, support enzyme activity, and play a pivotal role in immune responses, particularly immunoglobulins [8,9]. Immunoglobulins (Ig), such as IgM, IgT, and IgD, function as antibodies that neutralize pathogens like bacteria and viruses in teleost fish [10]. In addition, albumins help maintain colloidal osmotic pressure for fluid balance and act as carriers for hormones, fatty acids, and drugs, playing a key role in nutrient transport and metabolism [11]. Thus, evaluating globulin and albumin concentration provides complementary insights regarding the nutritional and immunity status, and overall physiology, making them relevant indicators of health in fish farming.

Significant efforts have been dedicated to elucidating the cellular, metabolic, and endocrine mechanisms that regulate glucose homeostasis in carnivorous fish. Research indicates that factors such as limited adaptability in digestive and glucose transport capacities, complex hormonal regulation, hepatic metabolism, and suboptimal glucose utilization in peripheral tissues contribute to inadequate glucose regulation in these species. Consequently, this suggests a constrained ability to utilize carbohydrates for protein sparing in farmed carnivorous fish. This limitation becomes particularly relevant when considering the incorporation of plant-based diets as alternatives to traditional fishmeal formulations [3].

With the increasing inclusion of plant-based proteins in the diets of carnivorous fish like rainbow trout, it is essential to understand how these dietary changes influence the composition and concentration of circulating globulins. Research indicates that shifts in protein sources can affect immunoglobulin levels, which are critical for the fish's immune function, as well as glucose metabolism [7]. Investigating these effects will provide valuable insights into the overall health and growth performance of rainbow trout in plant-based feeding regimes, helping to inform future dietary formulations that balance sustainability with nutritional adequacy. Additionally, monitoring changes in the hepatosomatic index

(HSI) alongside these parameters offers a more comprehensive understanding of how plant-based diets impact hepatic and systemic physiology [4–6].

## 2. Materials and Methods

### 2.1. Fish and Rearing Conditions

This study was conducted in a freshwater recirculation system at Unidad Experimental de Acuicultura, Universidad Católica de Temuco, Region de la Araucanía, Chile. The fish (*O. mykiss*;  $182 \pm 1.5$  g) were obtained from a commercial freshwater fish farm (Piscícola Huililco, Pucón, Región de la Araucanía, Chile). The fish were randomly distributed in nine 800 L fiber-glass tanks (55 fish per tank) supplied with freshwater at a constant temperature ( $12 \pm 1.5$  °C) and aeration was continuously provided via air stones to maintain the dissolved oxygen levels near  $90 \pm 5\%$  oxygen saturation. The fish were acclimated to experimental conditions under a natural photoperiod and fed ad libitum, twice a day at 8:00 h and 14:00 h, with an acclimation diet. Water quality physiochemical parameters (i.e., oxygen, temperature, pH and nitrate concentration) were checked on a daily basis.

### 2.2. Feeding Trial

Three experimental diets fulfilling the National Research Council nutritional requirements of *Oncorhynchus mykiss* [3] were produced by extrusion cooking with a laboratory twin-screw extruder at the CETA Pilot Plant Santiago, RM, Chile. The experimental diets were formulated to be isonitrogenous, isolipidic, and isocaloric. A fishmeal diet (FM) was used as the control diet. In the two experimental diets—medium plant meal (MPM) and high plant meal (HPM)—fishmeal was replaced at levels of 40% and 80%, respectively, using a blend of plant proteins, mainly soy protein concentrate, wheat gluten, and micronized extruded soybean meal (Table 1). Rapeseed oil was used to balance lipid content among the experimental diets. The diets were stored at 4 °C, while daily portions were weighted and kept in plastic bags at  $-20$  °C until use. Briefly, each experimental diet was fed to triplicate tanks, and the feeding ratio was fixed at 1.5% (BW day<sup>-1</sup>) of the tank biomass to ensure similar feed intake between the experimental groups (Figure 1). In order to reduce unnecessary stress in fish, every two weeks, after 24 h of fast, 30% of the fish in each tank were lightly anesthetized with 30 mg/L of benzocaine (20%; BZ<sup>®</sup>-20; Veterquímica, Santiago, RM, Chile) and individually weighed to adjust the feeding rate during the trial; however, every four weeks, all fish were individually weighed to track growth performance during the feeding trial.

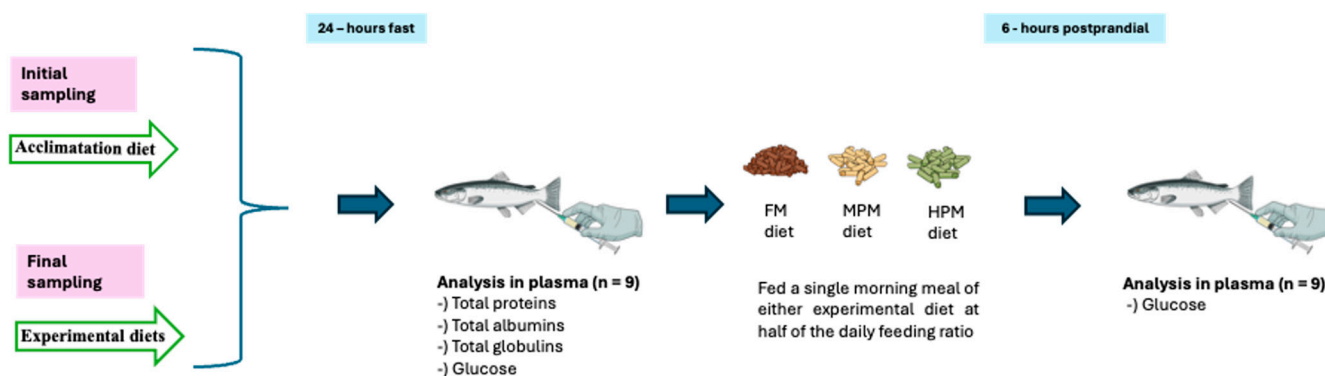
**Table 1.** Ingredients and nutrient composition of the experimental diets fed to rainbow trout for 12 weeks.

Ingredients (%)	Diets		
	FM Diet	MPM Diet	HPM Diet
Super Prime fish meal <sup>1</sup>	60.0	36.0	12.0
Soy protein concentrate <sup>2</sup>	0.0	9.0	18.0
Wheat gluten <sup>2</sup>	0.0	9.0	18.0
Extruded micronized soybean meal <sup>3</sup>	0.0	9.0	18.0
Wheat meal <sup>4</sup>	15.0	12.0	7.5
Alpha cellulose <sup>2</sup>	4.0	2.0	0.0
Rapeseed oil <sup>5</sup>	18.0	19.0	20.0
Vitamin Premix (including vitamin C) <sup>3†</sup>	1.6	1.6	1.6
Mineral Premix <sup>3‡</sup>	0.15	0.15	0.15
Choline chloride (70%) <sup>3</sup>	0.57	0.57	0.57
Dicalcium phosphate <sup>3</sup>	0.0	0.6	2.5

Table 1. Cont.

Ingredients (%)	Diets		
	FM Diet	MPM Diet	HPM Diet
L-Methionine <sup>3</sup>	0.0	0.1	0.4
Lysine <sup>3</sup>	0.0	0.3	0.6
Astaxanthin <sup>6</sup>	0.08	0.08	0.08
Chromium oxide (Cr <sub>2</sub> O <sub>3</sub> ) <sup>7</sup>	0.6	0.6	0.6
<i>Feed nutrient composition (as-is, %)</i>			
Dry matter	94.6	95.2	94.0
Crude protein	46.3	47.7	47.7
Fat	22.6	22.4	22.3
Ash	10.0	8.2	7.4
Fiber	1.4	1.1	1.0
NFE	16.3	15.8	15.6
Gross energy (MJkg <sup>-1</sup> )	22.5	22.9	22.7

<sup>1</sup> Lota Protein, Concepción, VIII Región, Chile. <sup>2</sup> Oregon Chem Group, Santiago, RM, Chile. <sup>3</sup> QualityPro, Santiago, RM, Chile. <sup>4</sup> Molino La Estampa, Santiago, RM, Chile. <sup>5</sup> Kirkland Signature, Costco Wholesale Corporation, Issaquah, Washington, USA. <sup>6</sup> Atacama Bio Natural Products S.A.C., Atacama, Chile. <sup>7</sup> Merck KGaA, Darmstadt, Germany. <sup>†</sup> per kg dry diet: vitamin A 24,000 IU; vitamin D 11,200 IU; vitamin E 240 IU; vitamin C 4.8 g; thiamine 15 mg; riboflavin 30 mg; nicotinic acid 25 mg; pyridoxine 15 mg; cyanocobalamin 0.05 mg; pantothenic acid 50 mg; biotin 0.5 mg; folic acid 1.58 mg. <sup>‡</sup> Contributed in mg kg<sup>-1</sup> of diet: iron 52.5 mg; potassium 3 mg; manganese 75 mg; zinc 154.5 mg; copper 3 mg; iodine 0.6 mg; cobalt 0.3 mg; selenium 0.2 mg.



**Figure 1.** Schematic experimental design. Initial sampling: At the beginning of the feeding trial, fish were fasted for 24 h before sampling. Blood was collected from three fish per tank ( $n = 9$  per treatment) to determine total protein, albumin, globulin, and fasting plasma glucose concentrations. Following this, the fish were fed a single morning meal of their respective experimental diet at approximately 0.75% of the daily feeding ratio, and another set of three fish per tank ( $n = 9$  per treatment) was sampled 6 h postprandially to assess initial postprandial glucose levels. Final sampling: The same procedure was repeated at the end of the feeding trial (week 12). Additionally, the fish were individually weighed at the beginning of the trial, as well as at weeks 4, 8, and 12.

### 2.3. Chemical Composition of Fish and Diets

Chemical analyses of diets and fish were conducted following the AOAC methods [12] at Instituto de Nutrición y Tecnología de los Alimentos (INTA), University of Chile. Briefly, dry matter was determined by drying samples overnight (12 h) in an oven at 105 °C to a constant weight. Crude protein content was determined according to the Kjeldhal method (the nitrogen-to-protein conversion factor used was 6.25) using a nitrogen analyzer. Crude fat in diets was determined following the Mojonnier method (acid hydrolysis) (AOAC Official Method 935.38, 925.32, 922.06.). Ash content was determined by incineration at 600 °C for 4 h. Crude fiber was measured gravimetrically following the Weende method (AOAC Official Method 978.10). Nitrogen-free extract (NFE) was estimated as follows: % NFE = % DM – (% EE + % CP + % ash + % CF). Diet gross energy was determined using

a bomb calorimeter at Universidad de La Frontera, Temuco, Chile (IKA, Model C2000 Basic, Brazil). Hepatosomatic index (HIS) was estimated as  $(100 \times (\text{liver weight}/\text{body weight}))$ . Lipid retention was calculated to evaluate the efficiency with which dietary lipids were incorporated into the fish's body. The formula used was as follows:

$$\text{Lipid Retention (\%)} = 100 \times [(\text{Final Body Weight} \times \text{Final Body Lipid Content}) - (\text{Initial Body Weight} \times \text{Initial Body Lipid Content})] / \text{Lipid Intake}.$$

#### 2.4. Sampling Procedure

During the 12-week feeding trial, samples were collected at two time points: the beginning and the end (Figure 1). After a 24 h fast from the acclimation diet, a total of 27 fish from the initial population were sampled for blood collection to obtain initial reference values of total proteins, albumins, globulins and glucose. This fasting sampling time was set-up since previous work has reported an adequate pre-sampling period for measuring the serum levels of several metabolites, including glucose, in rainbow trout [13].

Next, fish were fed a single morning meal of either experimental diet at half ( $\sim 0.75\%$ ) of the daily feeding ratio ( $\text{BW day}^{-1}$ ) with a 15 min gap between tanks to account for blood sampling timing, and thus ensuring that sampling occurred at the same postprandial time in each tank. Three fish per tank were sampled 6 h postprandially, coinciding with the peak in blood glucose in trout [14], for initial postprandial glucose determination. At the end of the trial, three fish per tank ( $n = 9$  per treatment, totaling 27 fish) were sampled after a 24 h fast from their respective experimental diets to determine final values of total proteins, albumins, globulins, and basal glucose. Then, another three fish per tank ( $n = 9$  per treatment, totaling 27 fish) were sampled 6 h postprandially after being fed their respective experimental diets for the determination of final postprandial plasma glucose concentrations. The sampled fish were euthanized with an overdose ( $200 \text{ mg L}^{-1}$ ) of tricaine methanesulfonate (MS 222; Argent Chemical Laboratories, Redmond, WA, USA). Blood samples were collected in EDTA-pretreated tubes and centrifuged at  $4000 \times g$  for 5 min at  $4^\circ\text{C}$ . The plasma layer was removed and stored on ice. Plasma aliquots for testing were then stored at  $-80^\circ\text{C}$ .

#### 2.5. Quantification of Plasma Metabolites

The metabolite analyses were performed at Núcleo Científico Tecnológico en Biorecursos, Universidad de La Frontera (BIOREN-UFRO). The plasma samples were thawed at room temperature. The following parameters were quantified: total proteins ( $20 \mu\text{L}$  with  $1000 \mu\text{L}$  mix reaction,  $580 \text{ nm}$ ) and albumin ( $10 \mu\text{L}$  with  $1000 \mu\text{L}$  mix reaction,  $578 \text{ nm}$ ), according to the protocol of the manufacturer (HUMAN Diagnostics, Wiesbaden, Germany) in a multimodal Synergy HT reader (BIOTEK, Winooski, VT, USA). Total globulins were obtained by the difference between total protein and the albumin content [15]. Glucose was determined by enzymatic oxidation through the action of glucose oxidase. The formation of  $\text{H}_2\text{O}_2$ , a product of the oxidation, reacts with 4-aminoantipyrine and phenol, generating quinoneimine, which is visualized by a violet color change. Then,  $10 \mu\text{L}$  of the sample/standard was incubated with  $1000 \mu\text{L}$  of the enzymatic reagent (phosphate buffer  $100 \text{ mmol/L}$ ; 4-aminoantipyrine  $0.25 \text{ mmol/L}$ ; phenol  $0.75 \text{ mmol/L}$ ; glucose oxidase  $15 \text{ KU/L}$ ; peroxidase  $1.5 \text{ KU/L}$ ; mutarotase  $2.0 \text{ KU/L}$ ; and sodium azide  $0.095\%$ ) for 10 min at  $25^\circ\text{C}$ . Read OD at  $500 \text{ nm}$ . Glucose standard:  $100 \text{ mg/dL}$  ( $5.55 \text{ mmol/L}$ ).

#### 2.6. Ethical Notes

This study was conducted in accordance with the guidelines of the Bioethics Committee of the Instituto de Nutrición and Tecnología de los Alimentos (INTA) at Universidad de Chile. The ethical approval code is 24808-INTA-UCH.

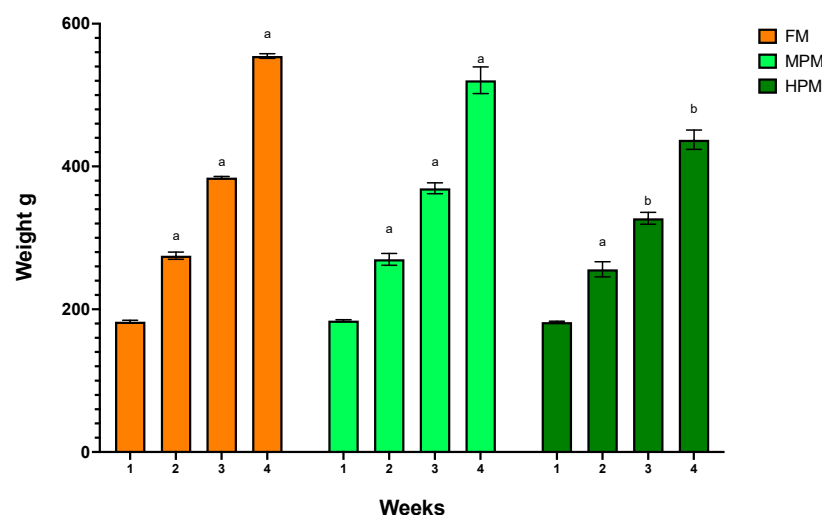
## 2.7. Statistical Analysis

Statistical analyses and graphs were performed using GraphPad Prism version 10.3.1 for Mac (GraphPad Software, Boston, MA, USA). Data were analyzed for normality (Kolmogorov–Smirnov test) and homoscedasticity (Levene’s test). One-way analysis of variance (ANOVA) was performed to detect a significant effect of the dietary treatments. Tukey’s HSD tests were performed as post hoc tests to identify experimental groups that differed significantly. The initial values for plasma concentrations of total proteins, albumins, globulins, and glucose under fasting conditions were included for reference only and were excluded from statistical analyses because the fish had been fed an acclimation diet prior to the initial sampling. A two-way ANOVA, considering sampling time and dietary treatments as two main factors, was conducted to detect a significant effect of these factors on postprandial plasma glucose concentration in fish. Statistical significance between treatments was assessed at a 5% level ( $\alpha = 0.05$ ).

## 3. Results

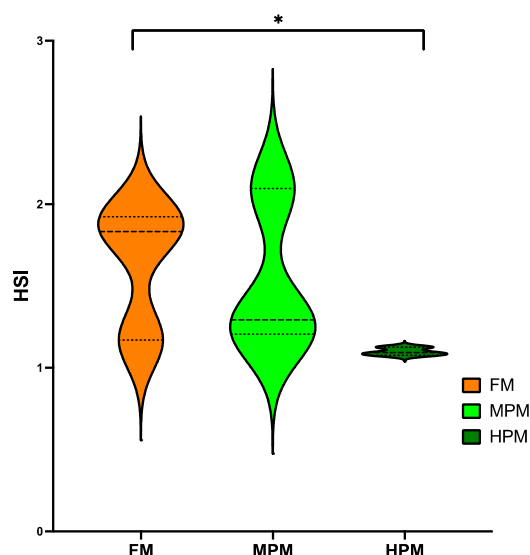
### 3.1. Fish Growth Performance

During the 12-week feeding trial with experimental diets for rainbow trout, growth parameters were monitored, and the results from the different experimental groups are presented in Figure 2. The figure illustrates the effect of plant-based diets on fish growth over this period, comparing three dietary treatments: fishmeal-based (FM), medium plant-meal-based (MPM), and high-plant meal-based (HPM). Fish fed the FM diet demonstrated the highest weight gain, followed by those on the MPM diet, while the HPM group exhibited the lowest growth. A statistically significant difference was observed between the FM and HPM groups, indicating that higher inclusion levels of plant-based ingredients negatively affect growth.



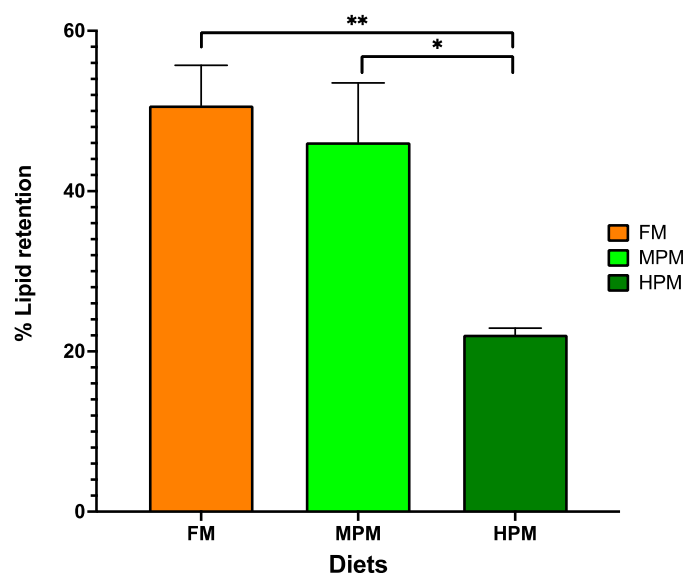
**Figure 2.** Weight monitoring of rainbow trout fed with three experimental diets for 12 weeks. Experimental diets: fish meal diet (FM), medium plant meal diet (MPM), and high plant meal diet (HPM). Letters (a,b) indicate significant differences.

The hepatosomatic index (HSI) was evaluated at the end of the 12-week feeding trial with different diets (Figure 3). The figure shows the final HSI for fish fed three diets: fishmeal (FM), medium plant meal (MPM), and high plant meal (HPM). The HSI values were highest in the FM group and decreased with higher inclusion of plant-based ingredients, with the HPM group displaying the lowest values.



**Figure 3.** Hepatosomatic index (HSI; %) in rainbow trout fed three experimental diets at the final stage of the 12-week feeding trial. Experimental diets: fish meal diet (FM), medium plant meal diet (MPM), and high plant meal diet (HPM). In the violin plot, the width of each curve represents the approximate frequency of data points in different value ranges. Violin plots combined with the overlaid box plot provide additional statistical information, such as medians, quartiles, and outliers, allowing for a more comprehensive understanding of both data distribution and central tendency within each group. The asterisk (\*) denotes a statistically significant difference between dietary treatments ( $p < 0.05$ ).

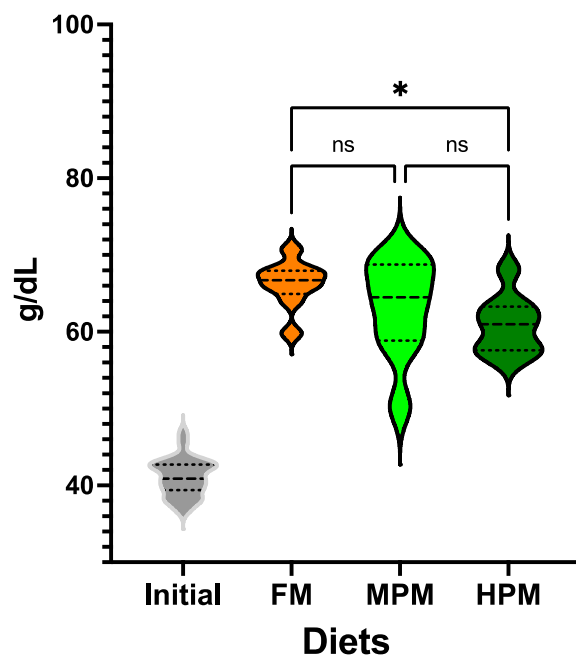
The percentage of lipid retention for fish fed the three diets was evaluated at the end of the experiment. Lipid retention was highest in the FM group and decreased with higher inclusion of plant-based ingredients, with the HPM group exhibiting the lowest retention values (Figure 4). A statistically significant difference was observed between the FM and HPM groups, indicating that plant-based diets negatively affect the retention of dietary lipids in rainbow trout whole body.



**Figure 4.** Lipid retention (%) in rainbow trout fed three experimental diets at the final stage of the 12-week feeding trial. Experimental diets: fish meal diet (FM), medium plant meal diet (MPM), and high plant meal diet (HPM). The asterisk (\*) and double asterisk (\*\*) denote a statistically significant difference between dietary treatments ( $p < 0.05$  and  $p < 0.01$ , respectively).

### 3.2. Protein Levels

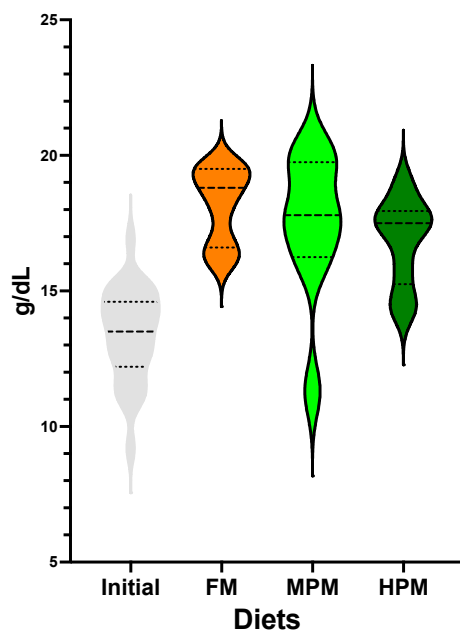
Protein levels were measured at two time points: first, immediately after the acclimation period and prior to the start of the experimental diets (initial), and second, at the end of the treatment period with the experimental diets (12 weeks—final). Total protein levels are shown in Figure 5. At the initial stage, all groups displayed similar serum protein levels; however, after the treatment with the experimental diets, this parameter was significantly altered. Notably, the fishmeal-fed group exhibited significantly higher protein levels compared to the group with the highest proportion of plant-based ingredients in the diet.



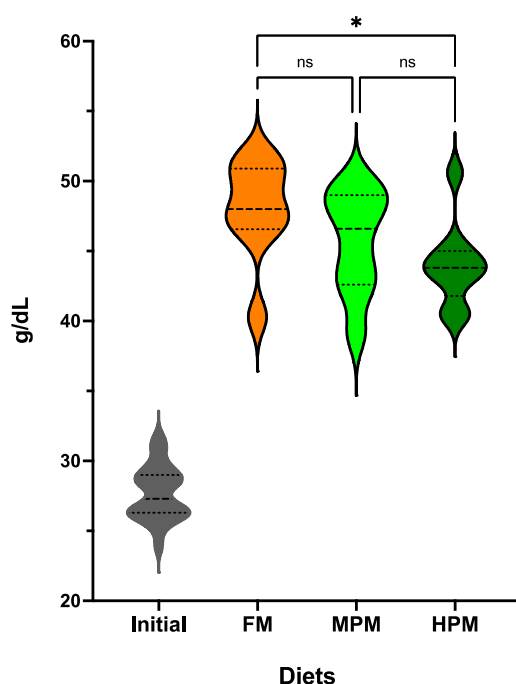
**Figure 5.** Total protein levels (g/dL) under fasting conditions in rainbow trout fed three experimental diets at the final stage of the 12-week feeding trial. Experimental diets: fish meal diet (FM), medium plant meal diet (MPM), and high plant meal diet (HPM). Violin plots combined with the overlaid box plot provide additional statistical information, such as medians, quartiles, and outliers, allowing for a more comprehensive understanding of both data distribution and central tendency within each group. The initial values were included for reference purposes only and were not used in statistical analysis as the fish were fed an acclimation diet prior to the initial sampling. The asterisk (\*) denotes a statistically significant difference between dietary treatments ( $p < 0.05$ ).

Albumin levels were also measured at the same time points as total protein levels. However, this parameter did not show any significant differences associated with any of the experimental diet groups (Figure 6). Furthermore, no changes in albumin levels were observed at the end of the treatment period.

Similarly, globulin levels were measured at the same time points as total protein levels, with the results presented in Figure 7. At the initial assessment, all groups demonstrated comparable globulin levels; however, following the treatment with the experimental diets, this parameter showed significant changes. Notably, the group fed with fishmeal (FM) exhibited substantially higher globulin levels compared to the group that received the highest proportion of plant-based ingredients in their diet (HPM).



**Figure 6.** Albumins levels (g/dL) under fasting conditions in rainbow trout fed three experimental diets at the final stage of the 12-week feeding trial. Experimental diets: fish meal diet (FM), medium plant meal diet (MPM), and high plant meal diet (HPM). Violin plots combined with the overlaid box plot provide additional statistical information, such as medians, quartiles, and outliers, allowing for a more comprehensive understanding of both data distribution and central tendency within each group. The initial values were included for reference purposes only and were not used in statistical analysis as the fish were fed an acclimation diet prior to the initial sampling.

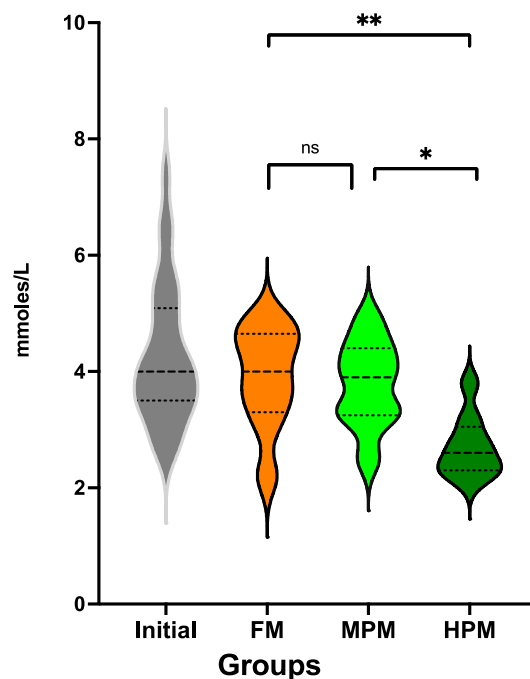


**Figure 7.** Globulins levels (g/dL) under fasting conditions in rainbow trout fed three experimental diets at the final stage of the 12-week feeding trial. Experimental diets: fish meal diet (FM), medium plant meal diet (MPM), and high plant meal diet (HPM). Violin plots combined with the overlaid box plot provide additional statistical information, such as medians, quartiles, and outliers, allowing for a more comprehensive understanding of both data distribution and central tendency within each group. The initial values were included for reference purposes only and were not used in statistical analysis as the fish were fed an acclimation diet prior to the initial sampling. The asterisk (\*) denotes a statistically significant difference between dietary treatments ( $p < 0.05$ ).

### 3.3. Glucose Levels

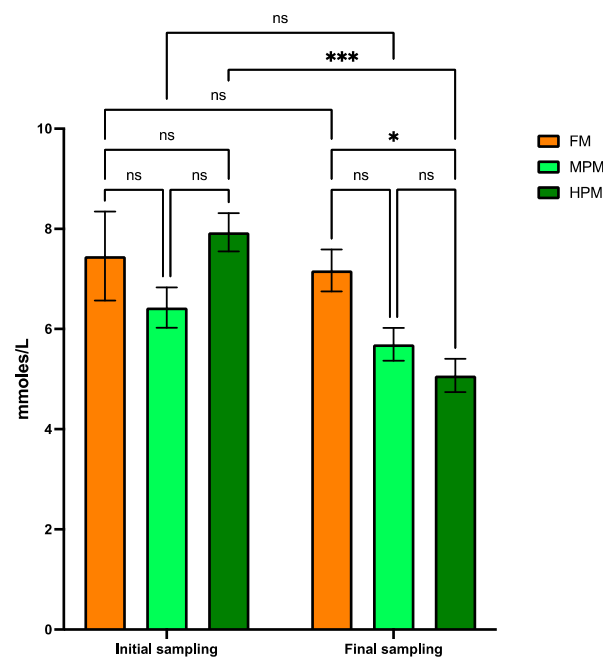
Glucose levels were measured at two time points: first, immediately after the acclimation period and prior to the start of the experimental diets (initial glucose), and second, at the end of the treatment period with the experimental diets (12 weeks—final glucose). Measurements were taken during both fasting and postprandial stages (6 h after feeding).

The glucose levels for fish during the fasting stage, both at the initial and final points of the treatment, are presented in Figure 8. While all groups of fish exhibited similar glucose levels at the initial stage, significant differences were observed at the end of the experimental diets, with notable variations between the fishmeal (FM) diet and the plant-based diets (MPM; HPM).



**Figure 8.** Glucose levels (mmoles/L) under fasting conditions in rainbow trout fed three experimental diets at the final stage of the 12-week feeding trial. Experimental diets: fish meal diet (FM), medium plant meal diet (MPM), and high plant meal diet (HPM). Violin plots combined with the overlaid box plot provide additional statistical information, such as medians, quartiles, and outliers, allowing for a more comprehensive understanding of both data distribution and central tendency within each group. The initial values were included for reference purposes only and were not used in statistical analysis as the fish were fed an acclimation diet prior to the initial sampling. The asterisk (\*) and double asterisk (\*\*) denote a statistically significant difference between dietary treatments ( $p < 0.05$  and  $p < 0.01$ , respectively).

The two-way ANOVA analysis revealed postprandial glucose levels, measured 6 h after feeding, as shown in Figure 9. A significant interaction ( $p = 0.029$ ) between sampling time and dietary treatment explained 10.53% of the total variance in postprandial plasma glucose levels. Moreover, dietary treatment ( $p = 0.0474$ ) and sampling time ( $p = 0.0026$ ) accounted for 9% and 14.02% of the variance, respectively. Given the statistical significance of the interaction, assessing the independent effects of these factors is challenging.



**Figure 9.** Glucose levels (mmoles/L) under postprandial (6 h) conditions in rainbow trout fed three experimental diets at beginning and at the final stage of the 12-week feeding trial. Comparison was performed using two-way ANOVA. Experimental diets: fish meal diet (FM), medium plant meal diet (MPM), and high plant meal diet (HPM). The asterisk (\*) and triple asterisk (\*\*\*) denote a statistically significant difference between dietary treatments ( $p < 0.05$  and  $p < 0.001$ , respectively).

#### 4. Discussion

Carnivorous fish require a diet high in protein to ensure optimal growth and health. Fishmeal has traditionally been the primary protein source of choice in finfish diets. However, due to its skyrocketing prices in recent decades, significant efforts have been made to include more affordable alternatives, notably terrestrial plant proteins [16–20]. Replacing more than half of the fishmeal in salmonid species feed with terrestrial plant proteins may lead to undesirable effects in fish, including decreased growth rates [18], increased adiposity [18], distal enteritis [19,20], and reduce overall health [20]. Therefore, in salmonid nutrition research, there has been great interest in understanding the effects of terrestrial plant proteins-based diets on the growth performance, physiology, metabolism and immune system of fish.

The results of the 12-week feeding trial clearly indicate that the replacement of 80% of fishmeal with plant-based ingredients in rainbow trout diets significantly impacts growth performance (Figure 2). The trend observed in this study aligns with previous findings that show reduced growth performance in carnivorous fish when plant-based proteins partially or fully replace fishmeal in their diets [3,16]. However, the moderate growth observed in the MPM group suggests that partial replacement of fishmeal with plant-based ingredients may be feasible if diets are carefully formulated [20].

The observed changes in the hepatosomatic index (HSI) (Figure 3) suggest important physiological implications related to the inclusion of plant-based ingredients in aquafeeds. Lower HSI values in fish fed diets with a higher plant meal content may reflect shifts in hepatic metabolic activity or energy allocation. Similar results have been reported in other species, such as brown bullhead (*Ameiurus nebulosus*) [4] and red seabream (*Pagrus major*) [6]. In comparison to the FM diet (FMD), the plant-meal-based diets may induce various effects previously documented in fish, including slower growth, reduced feed efficiency, lower hepatosomatic index (HSI) and signs of tissue degeneration in the hepatic tissue [4,6,21,22]. Similar to our findings, Matulic (2020) reported that hepatosomatic

indices (HSI) were significantly lower in all experimental groups fed plant-based meal (PBM) diets compared to those fed fishmeal (FM), indicating liver hypotrophy in brown bullhead fed PBM diets [4]. The authors demonstrated that diets with a higher inclusion of PBM led to increased liver tissue reactions, including hepatocyte nuclear apoptosis and morphological disruptions. These findings highlight the need for careful formulation of plant-based diets to minimize adverse hepatic effects, ensuring they support fish health and metabolic efficiency in aquaculture systems.

The observed differences in lipid retention (Figure 4) highlight the challenges associated with replacing fishmeal (FM) with plant-based ingredients in aquafeeds. The negative effects of plant-based proteins on lipid digestibility in salmonids, resulting in slower growth and reduced feed conversion efficiency, have been well documented [23,24]. A primary mechanism underlying this issue is the disruption of bile acid homeostasis, which occurs either through diminished synthesis (caused by a lower cholesterol input) or heightened excretion via feces, thereby compromising fat digestion in these species [23,24]. The significant lower lipid retention in fish fed the high-plant-meal-based (HPM) diet compared to the FM group suggests a reduction in lipid digestibility, likely due to the nutritional limitations, including cholesterol, of plant-based ingredients. These results align with previous findings in other species, where diets with a high inclusion of plant-based ingredients have been shown to compromise lipid utilization [23,24]. It may be closely related to the lower hepatosomatic index (HSI) observed in these groups. A lower HSI suggests reduced hepatic activity or lipid storage, which aligns with the decreased lipid retention. The liver plays a central role in lipid metabolism, including lipid absorption, processing, and storage. Diets with reduced lipid digestibility, as seen in the HPM group, could lead to lower lipid availability for hepatic storage, reflected in a decreased HSI.

Albumins and globulins are the two major types of proteins in the blood of animals [16]. Albumins are mainly involved in maintaining and regulating oncotic pressure in body fluids, and thus maintaining blood volume in animals. In addition, albumins also serve as transport proteins and participate in the regulation of pH in the blood [16]. In turn, all proteins not classified as albumins are collectively considered globulins, which primarily participate in the immune defense system of animals as immunoglobulins. However, globulins—including fibrinogen,  $\beta$ -lipoprotein, and vitellogenin—also play roles in other aspects of animal physiology, such as blood clotting, lipid transport, and embryo development, respectively [25].

Total protein levels are commonly used in fish health assessments, but specific protein fractions may offer more precise insights. In the study of Alfonso 2024 [11], they analyzed *Dicentrarchus labrax* and *Sparus aurata* to understand how different globulin fractions contribute to total blood protein and how these contributions vary over time. In *D. labrax*, beta1 globulins were the largest contributor (34.16%), followed by albumin (18.24%). In *S. aurata*, albumin was the primary contributor (23.39%), followed by beta1 globulins (19.71%). The contributions were relatively stable, with minor variations linked to environmental or individual factors, and larger fish had higher total protein levels. These findings underscore the need for further research into globulin dynamics to improve fish health monitoring. In the initial parameters in our study, albumins represented close to 30% of the total protein. Further, we observed that replacing fishmeal with increasing levels of a plant protein blend (i.e., soy protein concentrate, extruded soybean meal and wheat gluten) did not affect the concentration of total albumins in the plasma of fish (Figure 6) [26–28]. However, a significant decrease in both total globulin (Figure 7) and total protein (Figure 5) concentrations in the plasma of fish was observed, with the latter influenced by the drop in globulin levels, as the albumin levels remained unaffected. The effect of using plant meal in lieu of fish meal on blood parameters, including total globulins, immunoglobulins and total proteins, is not

consistent across the fish literature. Previous works have reported either a downregulation in the expression of genes encoding for immunoglobulins [26–28], and a decrease in the levels of immunoglobulins, and/or total globulins [28–31] and/or total proteins [29–36] in fish plasma. However, the opposite effect has also been observed in fish. Rumsey [37] reported that feeding rainbow trout a soybean-protein-based diet (i.e., soybean meal or soybean protein concentrate) caused an increase in the concentrations of total protein and immunoglobulins in the plasma of fish. Similarly, an increase in globulin and total plasma protein levels was observed in both rainbow trout [38] and common carp (*Cyprinus carpio*) [39] fed plant-derived proteins as a replacement for fishmeal. The reason for these contradictory effects between these reports remains unclear. However, it is possible that these opposite effects between these reports are associated with whether a single or several sources of plant proteins were used; thus, in those studies where an increase in globulin and total plasma protein levels was observed, the tested groups evaluated only one type of plant protein as a replacement for fishmeal. In contrast, in those studies where a decrease in globulin and total plasma protein levels was observed, the tested groups evaluated several sources of plant proteins. Interestingly, a recent study reported that plasma total protein concentrations were higher in fish with larger body size [11]; however, this was not observed when considering sampling time, suggesting a body mass effect rather than an age-related physiological influence on total plasma proteins. In line with these findings, we observed that the initial values for plasma concentrations of total proteins, albumins and globulins were lower than those detected in the control group at the end of the feeding trial. Since initial values were obtained from fish fed the acclimatation diet (similar formulation of the control diet), these differences suggest a fish size effect on these variables within experimental groups. However, due to these differences in fish size being caused by the diets, it is not possible to rule out the size effect on total proteins, albumins and globulins between the groups at the end of the trial.

From a metabolic perspective, studying rainbow trout is particularly interesting because they are considered “glucose intolerant” due to their poor utilization of glucose, which is phenotypically manifested by a prolonged postprandial hyperglycemia [40–42]. Thus, postprandial plasma glucose levels have been considered a variable of interest in this topic; however, less attention has been given to fasting plasma glucose levels. Most of the research on glucose homeostasis conducted in rainbow trout has used fishmeal-based or semi-purified diets as the primary protein source, with less attention given to the potential role of plant proteins in this regard. In this study we detected a significant interaction between sampling time and dietary treatment on the total variance observed in postprandial plasma glucose levels, and thus the real impact of plant-derived proteins, as an alternative for fishmeal replacement, in the postprandial plasma glucose level should be interpreted with caution. However, this is not true for plasma glucose concentration under fasting conditions, where replacing 80% of fishmeal (HPM diet, respectively) led to a significant decrease in its value compared with both the control group and fish fed the MPM diet at the end of the trial (Figure 8). These findings suggest that the type of protein used in diets with similar levels of carbohydrates (measured as NFE) and close to the optimal level range of carbohydrates for trout [42] matters when considering glucose homeostasis in fish under 24-h fasting conditions. Therefore, further research to improve our understanding on how alternative protein sources, mainly terrestrial plant proteins, influence glucose homeostasis in carnivorous fish is required, particularly in the context of a sustainable aquaculture industry.

## 5. Conclusions

The findings highlight the physiological and metabolic challenges associated with replacing fishmeal with plant-based ingredients in aquafeeds for rainbow trout. The observed reductions in growth performance, hepatosomatic index (HSI), and lipid retention emphasize the need to address these impacts to support fish health and metabolic efficiency. High replacement of fishmeal with plant-based proteins in rainbow trout diets reduces total globulin and protein concentrations while leaving albumin unaffected. Our results also suggest that the impact of a high-plant-protein diet on glucose homeostasis is more pronounced under fasting conditions than after feeding when fed diets with carbohydrates levels close to the optimal range for trout, highlighting the need for further research into plant protein effects on fish metabolism and immunity to support sustainable aquaculture.

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