Article

Hemp Meal (*Cannabis sativa*) as an Alternative Dietary Protein Source for European Perch (*Perca fluviatilis*)

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Simple Summary

The production of European perch is becoming increasingly advanced. Unfortunately, it is still associated with high costs due to the high proportion of fish meal required in the feed. We investigated whether hemp meal could replace it without harming the health of the fish. For ten weeks, the perch were fed a diet containing 0, 10, 20 or 30% hemp meal. The study revealed that there were no negative effects on growth, physical condition, muscle composition, blood parameters, or digestive organ structure. The diet containing 20% hemp meal provided the best overall growth and feed utilization. Fish receiving hemp meal also showed mainly beneficial changes in the intestinal lining, which aids digestion and nutrient absorption. Overall, hemp meal can safely replace some of the animal protein in the diet of perch, with around 20% inclusion appearing to be the most promising. The use of hemp meal can reduce feed costs, improve sustainability and reduce dependence on fish meal in fish farming.

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Abstract

The production of European perch (*Perca fluviatilis*) has become notably refined, with numerous physiological and nutritional studies conducted in recent years. However, it is still an expensive undertaking due to the high amount of animal protein required in the feed. Therefore, we investigated the possibility of using hemp meal (HM) as an alternative source of protein in extruded feed for perch reared in recirculating aquaculture systems

(RASs). Perch fry (initial body weight of 68.1 g) was divided into four groups (HM0, HM10, HM20, HM30; 100 fish in each) and fed with diets containing different levels of HM (0, 10, 20 or 30%, respectively) for 10 weeks. Overall, dietary inclusion of HM did not affect body parameters, muscle composition, or blood parameters, nor did it cause any serious histopathological lesions. Nonetheless, basic production indices (SGR, FCR, PER) all peaked in the HM20 group, and predominantly positive changes in intestinal mucosa were found in all three HM-inclusion groups. Furthermore, the expression patterns of several genes in the intestine and liver were different in groups HM20-30 than in HM0-10. Lastly, hepatic activities of alkaline phosphatase (ALP) and glutathione peroxidase (GPX) diminished with increasing dietary HM inclusion levels. In summary, there were no negative effects of HM on the homeostasis of studied fish or, more specifically, the physiology of their digestive organs. When accounting for minor tendencies in the results, the dietary inclusion of hemp meal at 20% turned out to be the most promising fish meal alternative for the European perch.

Keywords: blood biochemistry; digestive enzymes; fish meal replacement; gene expression; histology; plant protein

1. Introduction

The aquaculture industry remains the leading global consumer of fish meal (FM), even despite its decreasing use in fish and shellfish feed in recent decades [1]. Traditionally, FM has accounted for about 68% of the global supply of protein for aquafeed formulation, mostly due to its favorable amino acid profile, but also the contents of essential fatty acids, nucleotides, phospholipids, minerals, as well as water- and fat-soluble vitamins. All of this contributes to increased palatability and digestibility of feed [2]. However, FM is becoming decreasingly available because it originates from catch fisheries, where the overexploitation of wild stocks along with ongoing climate change (e.g., increasing frequency of the El Niño phenomenon) not only limit the final yield, but also drive the cost of FM. Therefore, it is essential for the future development of aquaculture to search for long-term sustainable, alternative sources of protein, and incorporate them in produced feed [3]. They can come from both plants and animals (e.g., insects [4]), and there are also possibilities to use bacteria or fungi [5]. The relevance of such dietary ingredients, however, depends on their chemical composition and how it matches the nutritional demands of each species. Nevertheless, there are other risks that must be accounted for to enable the production of safe, sustainable and functional aquafeed [5].

At the turn of the millennium, the search concentrated mainly on ingredients derived from terrestrial plants [6], and significant progress in terms of incorporating them in fish feed had already been made before 2006, especially for omnivores [7]. Back then, they were the preferred replacement for FM due to their much lower prices. Unfortunately, dietary plant protein can impair the health and overall performance of fish, mainly due to anti-nutritional factors contained therein. It also upsets the balance of macronutrients and trace elements in which FM is rich [7]. Naturally, in order to achieve sustainable and efficient fish production, it is crucial to formulate diets that result in high growth rates and good health but also have low environmental impacts. However, this is especially difficult when substituting FM with unconventional and novel ingredients [8].

Industrial hemp (*Cannabis sativa*) is becoming a significant part of agriculture worldwide, with an expected compound annual growth rate of 16.1% through 2034 [9]. Its cultivated area in the European Union alone increased by 75% between 2015 and 19. In

terms of environmental impact, hemp is valued for its prominent CO_2 binding capacity, but also for preventing soil erosion, enhancing local biodiversity, and having low pesticide requirements. Hemp fiber is used in textiles, construction, biofuel production, and many new, innovative applications, while its seeds are used for human and animal consumption. In the context of aquaculture, the inclusion of hemp seeds in striped bass (*Morone saxatilis*) feed yielded promising results [10], and there were also benefits from the use of hemp protein and oil for other commercial species, such as cobia (*Rachycentron canadum*), common carp (*Cyprinus carpio*), and Nile tilapia (*Oreochromis niloticus*) [11–13].

Hence, it should be acknowledged that industrial hemp is currently among the plants which are of greatest interest to aquafeed producers. Following that line of reasoning, the purpose of this study was to test the feasibility of using hemp meal (HM) as an alternative source of protein in an extruded feed for the European perch (*Perca fluviatilis*), which is an omni-carnivorous freshwater species [14] that shows high promise in the context of European aquaculture diversification [15–18].

2. Materials and Methods

2.1. Experimental Design, Feed and Fish

Hemp protein powder (defatted, milled, and sifted hemp seed cake) was the key dietary component used for experimental feed formulation (Table 1). Four isoenergetic (isoproteinous and isolipidous) extruded feeds with different HM inclusion levels (0%, 10%, 20%, 30%; Table 2) were developed and prepared at the Department of Ichthyology and Aquaculture (University of Warmia and Mazury in Olsztyn, Poland), and their proximate composition was determined with the use of standardized methodology [19]. Dry matter was assessed by drying in an oven at 105 °C for 24 h. Crude protein and fat were measured using Kjeldahl's and Soxhlet's methods, respectively. Crude ash was determined gravimetrically by measuring the loss of mass after combustion in a muffle furnace at 550 °C for 12 h. Crude fiber was estimated using the AOAC 973.18 method. Carbohydrate content (i.e., nitrogen free extract, NFE) was calculated by subtracting the five already determined components from 100%. Gross Energy (GE) in dry matter was then calculated accordingly: $GE = 0.01 \times (2.385 \times \text{protein} + 3.891 \times \text{fat} + 1.715 \times \text{NFE})$.

Table 1. Proximate composition (% of wet matter) and producers/suppliers of feed ingredients.

	Protein	Fat	Fiber	Ash	Carboh.	Product	Producer/Supplier
Hemp meal	50.0	11.2	-	-	26.6	hemp protein powder	Allive Europe, Lithuania
Fish meal	70–72	<12.0	-	10–18	28.2	FF Classic	FF Skagen, Denmark
Soybean PC	56.0	2.0	3.5	7.0	23.5	HP 300 protein concentrate	Hamlet Protein, Denmark
Wheat flour	12.0	1.5	-	-	71.0	Szymanowska (type 480)	Polskie Młyny, Poland
Fish oil	-	-	-	-	-	marine fish byproducts	ROL-PASZ, Poland
Gluten	79.4	6.0	0.0	-	6.3	made from wheat	Barentz Polska, Poland
Proglobulin	78.0	2.0	-	9.0	-	dried blood plasma protein	Darling Ingredients, USA
Blood meal	>90.0	<2.0	-	<4.0	<5.0	made from poultry	Darling Ingredients, USA
Premix	-	-	-	-	-	Dolmix Pstrąg 1.5%	DOLFOS, Poland

For the experiment, 400 domesticated European perches with an average initial body weight (IBW) of 68.1 g were obtained from the pond farm of the Fishery Experimental Plant in Żabieniec (Poland). They were evenly distributed into eight tanks (0.3 m^3) of a recirculating aquaculture system (RAS) and divided into four feeding groups, two tanks each (i.e., 2 × 50 fish; mean IBW for tanks ranged 65.4–74.6 g). Water parameters were controlled

daily at the outflow from the storage tank (T = 20.6 ± 0.9 °C, O_2 = 9.22 ± 0.98 mg L^{-1}, pH = 7.0 ± 0.12, total ammonia < 0.05 mg L^{-1}, NO_2 < 0.001 mg L^{-1}). The groups were named according to the dietary inclusion level of HM in the administered feed: HM0, HM10, HM20, and HM30. The daily feeding rate equaled 1% of the estimated biomass. The experiment lasted for 10 weeks.

Table 2. Formulations and proximate composition of the experimental diets.

	HM0	HM10	HM20	HM30
Ingredients (% of dry matter)				
Hemp meal	0	10.0	20.0	30.0
Fish meal	39.5	32.3	28.2	17.7
Soybean PC	9.5	10.0	4.0	3.5
Wheat flour	20.0	18.0	17.0	15.8
Fish oil	13.3	13.0	12.5	12.5
Gluten	4.0	4.0	4.6	5.5
Proglobulin	4.0	4.0	5.0	6.0
Blood meal	4.0	4.0	4.0	5.4
Premix	5.7	4.7	4.7	3.7
Proximate composition (% of wet matter)				
Dry matter	92.6	92.7	92.6	92.6
Protein	46.0	46.0	46.0	46.0
Fat	18.0	18.0	18.0	18.0
Ash	9.1	7.7	7.2	5.4
Fiber	0.7	2.7	4.4	6.3
NFE [1]	18.8	18.3	17.0	16.9
GE [2] (MJ kg^{-1})	21.2	21.1	20.9	20.9

[1] nitrogen free extract; [2] gross energy.

2.2. Sampling, Growth Indices and Proximate Composition

After the experimental period, the fish were placed in propofol solution and subjected to morphometric analysis (final body weight, FBW, and final caudal length, FCL).

Growth performance indices were determined as follows:

- Fulton's condition factor (K) = 100 × (FBW × FCL^{-3});
- Viscerosomatic index (VSI) = 100 × (weight of viscera × FBW^{-1});
- Hepatosomatic index (HSI) = 100 × (weight of liver × FBW^{-1});
- Specific growth rate (SGR) = 100 × [(ln mean FBW − ln mean IBW) × $days^{-1}$];
- Feed conversion ratio (FCR) = feed intake × weight $gain^{-1}$;
- Protein efficiency ratio (PER) = weight gain × crude protein fed^{-1}.

Then, tissue samples were obtained for different analyses and fixed accordingly.

For the proximate analysis of muscles, 15 fish from each tank were fileted and skinned. The muscles of five fish were then pooled to constitute one sample (three per tank, n = 6). The dry matter, crude protein, fat, and ash contents of muscles were all determined using standard methods, as described above for feed composition analysis.

For blood biochemistry, blood samples from five fish per tank (n = 10) were collected from the caudal vein using a syringe, then immediately centrifuged for 30 s at 15,800 rpm and the supernatant was frozen in −80 °C. Meanwhile, different pieces of liver, anterior

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and posterior intestine were collected from five fish per tank (*n* = 10) for histological, gene expression and enzymatic analyses. Tissues for histology were fixed in Bouin's fluid for 24 h, then kept 70% ethanol in 4 °C. Lastly, samples for both genetic and enzymatic analyses were immediately frozen in liquid nitrogen, and were stored in −80 °C.

2.3. Blood Biochemistry

Blood plasma was analyzed using a Catalyst Dx chemical analyzer (Idexx Laboratories, Westbrook, ME, USA) on dedicated test slides (custom panels). The following parameters were assessed: total protein (TP), albumin (ALB), globulins (GLOB), glucose (GLU), total cholesterol (TC), triglycerides (TG), alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP). Each plasma sample was thawed only once at room temperature, and all measurements were performed simultaneously to eliminate multiple freeze/thaw cycles.

2.4. Histological Analysis of Digestive Organs

Intestinal and hepatic samples were subjected to standard histological processing, and the prepared paraffin blocks were cut to a thickness of 6 μm. The slides were then stained using AB-PAS (Alcian blue combined with periodic acid and Schiff's reagent) for identification of hepatic lipid deposition, as well as identification of acidic and neutral mucous cells in the intestine [20], as well as with PAS only, for assessment of accumulated hepatic glycogen. The stained slides were used for histomorphometric evaluation using a Nikon Eclipse Ni-E microscope with NIS Elements v. 5.30.00 image analysis software (Nikon Corporation, Tokyo, Japan).

The following parameters were measured manually in the intestine: the height of mucosal folds of the anterior (AFH) and posterior (PFH) sections, the width of middle lamina in the anterior (AWLP) and posterior (PWLP) sections, the height of enterocytes in the anterior (AEH) and posterior (PEH) sections, and the height of their supranuclear part in the anterior (ASH) and posterior (PSH) sections [21]. The parameters were measured 20 times in each of the 10 sampled individuals per group (*n* = 200).

In the liver, hepatocyte area (HA) and nuclear area (HNA) were measured by taking 100 measurements from each of the 10 individuals per group (*n* = 1000). The hepato-nuclear index (HNI) was also calculated for each measured cell [22]. Meanwhile, the quantification of lipid and glycogen deposits was performed automatically using ImageJ v. 1.53k [23]. Ten images of each hepatic parenchyma were taken uniformly using the same optical settings and magnification (field of view size of 0.084 mm^2); this principle was applied to both the AB-PAS and PAS-stained slides (*n* = 100 for each). A common threshold for white areas was established using AB-PAS images (lipid deposition, LD), and the same was done separately for the magenta color in the PAS-stained images (glycogen deposition, GD). The results of both analyses were expressed as % values of the entire field of view.

2.5. Gene Expression Analysis of Digestive Organs

Total mRNA was extracted from enteric and hepatic samples using a NucleoSpin RNA kit (Macherey-Nagel, Düren, Germany, cat. 740955.50). The yield of isolated RNA was estimated spectrophotometrically (NanoDrop Technologies, Wilmington, DE, USA), and its integrity was evaluated electrophoretically (separation on a 1% agarose gel with ethidium bromide). Afterwards, 1 μg of total RNA was used as starting material for cDNA synthesis (Maxima First Strand cDNA Synthesis Kit for RT-qPCR, with dsDNase; Thermo Fisher Scientific, Waltham, MA, USA). The expression levels of five housekeeping genes (*actb*, *gapdh*, *gusb*, *hprt1* and *b2m*), six genes of interest in the intestine (*il1b*, *il6*, *slc15a1b*, *slc27a4*, *pparaa* and *fads2*) and five in the liver (*slc15a1b*, *slc27a4*, *pparaa*, *fads2* and *leptin-like*) were assessed using primers specifically designed in the Primer-Blast online

software, https://www.ncbi.nlm.nih.gov/tools/primer-blast/, accessed on 14 February 2026; (National Library of Medicine, Bethesda, MD, USA), which were synthesized by Nexbio (Lublin, Poland). All necessary information about the studied genes and primers was compiled in Table 3. Real-time qPCR was carried out using components from a kit (HOT FIREPol EvaGreen qPCR Mix Plus, no ROX; Solis BioDyne, Tartu, Estonia, cat. 08-25-00020) and HMLC-grade oligonucleotide primers. The total reaction volume of 15 µL contained: 3 µL Master Mix, 2 × 0.225 µL primers (0.225 mM), 10.05 µL RNase free water and 1.5 µL of cDNA template. A Rotor Gene 6000 thermocycler (Corbett Research, Mortlake, Australia) was used and the amplification was carried out using the following protocol: (1) one cycle at 95 °C for 15 min (enzyme activation); (2) 35 cycles of 95 °C for 10 s (denaturation), 59 °C for 20 s (annealing) and 72 °C for 10 s (elongation); (3) one cycle at 72 °C for 7 min (product stabilization). The melting curve was performed over 70–95 °C at 0.5 °C intervals. Negative controls (no cDNA template) were included for each reaction, and the identities of PCR products were confirmed through direct sequencing (Nexbio). The mean expression of all five housekeeping genes was used to normalize the expression of the analyzed genes of interest.

Table 3. Oligonucleotide primer sequences for RT-qPCR.

Gene	Full name of Gene	GenBank No.	Sequence 5′→3′	Amplicon bp
il1b	interleukin 1, beta	XM_039779729.1	TGACTTCGACCTGTCTCAAGC ATCCTGAACGTCCGTTGTGT	94
il6	interleukin 6,	XM_039821186.1	GAGTACCCCGGCAACTCAAT GCCACGGTTTCTCATCTTTCG	90
slc15a1b	solute carrier family 15 member 1b	XM_039793389.1	GAGCACTGTAGGTCAAGCGA AGGTAATCCTCATTGGCCCTG	111
slc27a4	solute carrier family 27 member 4	XM_039780588.1	TATCTTCGAGGGGACTGGGG CCACCACGTCACCATCCTTA	110
pparaa	peroxisome proliferator-activated receptor alpha a	XM_039791719.1	GAGAGTTTCAGCCCCCTCAA CTTCTGAAGAAACCCTTGCAGC	107
fads2	fatty acid desaturase 2	XM_039808830.1	CCATGATTTCCCGCCGTCAG CGTGACTCTCCAAAATCCTGATGA	141
leptin-like	leptin-like	XM_039809530.1	TGCTGCACGTTTTGAGTGTG GACCAGTAGGGACCTGGAAA	133
Housekeeping genes				
actb2	actin, beta 2	XM_039824642.1	CTGCGGAATCCATGAGACCA CTGGTGGGGCAATAATCTTGA	188
gapdh	glyceraldehyde-3-phosphate dehydrogenase	XM_039805259.1	GGGACCCCGCTAACATCAAA CCTTCAAGTGAGCAGAAGCC	102
gusb	glucuronidase, beta	XM_039777627.1	GGCATCGCAGATAGTCGCAG AGAGGCATGGTTCTTGTCCC	96
hprt1	hypoxanthine phosphoribosyltransferase 1	XM_039813961.1	TTGACGGGCAAGAATGTCCT CTGGTCGGTAGCCAACACTT	157
b2m	beta-2-microglobulin-like	XM_039791199.1	CTTCAACAGCCAAAGAATCGCC CACGTGACAGATGAGGGTGT	92

2.6. Analysis of Hepatic Enzymes

Frozen liver samples were homogenized in distilled water and centrifuged at 14,000 *g* for 10 min at 4 °C. The activity of alkaline phosphatase (ALP) was tested with a SPINREACT (Girona, Spain, cat. 41242) kit, while glutathione peroxidase (GPX) and superoxide

dismutase (SOD) were tested with RANDOX (Crumlin, United Kingdom, cat. S504 and D125) kits. To standardize enzymatic activity, the total protein concentration of the samples was measured according to the common Lowry method. Enzymatic activity measurements were carried out according to the instructions provided. Each sample was analyzed in triplicate at 37 °C, and enzyme activity was expressed as the number of micromoles of reaction product per minute in one gram of protein (U g^{-1} of protein). The analyses were performed in 96-well plates using an Infinite 200 Pro spectrophotometer (Tecan, Grödig, Austria).

2.7. Statistical Analysis

The obtained datasets were subjected to normality (Shapiro–Wilk) and homogeneity (Levene) tests. Consequently, the Kruskal–Wallis test with Dunn's test for multiple comparisons (muscle composition, blood plasma indices, gene expression, hepatic enzymes) or One-Way ANOVA with Fisher's post hoc test (FBW, FCL, K, VSI, HSI and all histological parameters) were used to identify statistically significant differences between groups (at $p < 0.05$). The STATISTICA v. 13 software (TIBCO Software, USA) was used to perform all analyses.

3. Results

3.1. Basic Parameters and Muscle Composition

The individual fish body parameters were compiled in Table 4. Final fish size was similar in all experimental groups, as there were no significant differences in FBW and FCL. However, fish from groups HM20 and Hm30 had a significantly higher K factor than those in HM10 ($p = 0.0372$ and 0.0496, respectively) and a significantly higher VSI than fish from HM0 ($p = 0.0385$ and 0.0306, respectively). In contrast, fish from HM0 and HM10 had a significantly higher HSI than their counterparts in HM20 ($p = 0.0002$ and 0.0150, respectively) and –HM30 ($p < 0.0001$ and $p = 0.0005$, respectively).

Table 4. Body parameters of European perch fed diets with different hemp meal contents.

	HM0	HM10	HM20	HM30
IBW [1] (g)	67.3 ± 2.1	67.0 ± 0.6	66.4 ± 1.4	71.9 ± 3.8
FBW [2] (g)	94.9 ± 0.1	92.4 ± 1.2	94.8 ± 0.6	94.9 ± 4.0
FCL [3] (cm)	16.7 ± 0.1	16.8 ± 0.0	16.7 ± 0.0	16.9 ± 0.4
K factor [4]	1.99 ± 0.05 ^{ab}	1.98 ± 0.04 ^{b}	2.06 ± 0.08 ^{a}	2.06 ± 0.05 ^{a}
VSI [5] (%)	8.33 ± 0.54 ^{b}	8.95 ± 0.2 ^{ab}	9.42 ± 1.16 ^{a}	9.47 ± 0.06 ^{a}
HSI [6] (%)	2.44 ± 0.16 ^{a}	2.19 ± 0.53 ^{a}	1.76 ± 0.14 ^{b}	1.56 ± 0.26 ^{b}

[1] initial body weight; [2] final body weight; [3] final caudal length; [4] Fulton's condition factor; [5] viscerosomatic index; [6] hepatosomatic index; Results are presented as means ± SEM. ^{a,b} means with differing superscript letters are significantly different at $p < 0.05$.

The final survival rate was relatively high in all groups (93–97%) (Table 5). When accounting for the survival rate and IBW, it became apparent that the inclusion of HM in the diet affected the SGR, FCR and PER of perch in a peak-and-drop manner, as the highest values of the three indices occurred in group HM20, while the lowest occurred in HM30 (Table 5).

Lastly, there were no statistically significant differences in terms of proximate composition of muscles (Table 6).

Table 5. Growth indices of European perch fed diets with different hemp meal contents.

	HM0	HM10	HM20	HM30
Survival (%)	93.0 ± 1.4	97.0 ± 1.4	96.0 ± 0.0	95.0 ± 7.1
SGR [1] (% day^{-1})	0.49 ± 0.04	0.46 ± 0.01	0.51 ± 0.04	0.40 ± 0.02
FI [2] (g)	2632 ± 88	2757 ± 9	2687 ± 126	2870 ± 195
FCR [3]	2.55 ± 0.50	2.45 ± 0.21	2.20 ± 0.28	3.30 ± 0.99
PER [4]	0.86 ± 0.17	0.90 ± 0.09	0.99 ± 0.11	0.70 ± 0.22

[1] specific growth rate; [2] feed intake; [3] feed conversion ratio; [4] protein efficiency ratio. Results are presented as means ± SEM.

Table 6. Proximate composition [% of wet weight] of muscles of European perch fed diets with different hemp meal contents.

	HM0	HM10	HM20	HM30
Dry matter	23.2 ± 0.0	23.9 ± 0.0	24.1 ± 0.4	24.0 ± 0.6
Protein	20.9 ± 0.6	21.3 ± 0.5	20.9 ± 0.3	21.3 ± 0.1
Fat	1.1 ± 0.0	1.1 ± 0.0	1.1 ± 0.1	1.2 ± 0.1
Ash	1.5 ± 0.0	1.6 ± 0.0	1.6 ± 0.0	1.6 ± 0.1

3.2. Results of Blood Biochemistry

There were no statistically significant differences between groups of the studied blood parameters, and there were no emerging trends within the data (Table 7).

Table 7. Blood plasma indices of European perch fed diets with different hemp meal contents.

	HM0	HM10	HM20	HM30
TP [1] (g L^{-1})	53.0 ± 8.6	52.4 ± 5.9	53.9 ± 7.0	53.2 ± 6.4
ALB [2] (g L^{-1})	16.2 ± 3.0	16.4 ± 3.0	17.0 ± 2.8	16.6 ± 3.2
GLOB [3] (g L^{-1})	36.8 ± 7.0	36.0 ± 5.7	36.9 ± 6.6	36.5 ± 5.5
ALB/GLOB	0.5 ± 0.1	0.5 ± 0.2	0.5 ± 0.2	0.5 ± 0.2
GLU [4] (mmol L^{-1})	6.4 ± 2.8	5.4 ± 1.9	6.8 ± 2.9	5.8 ± 2.0
TC [5] (mmol L^{-1})	6.2 ± 1.0	5.8 ± 0.8	6.2 ± 0.9	5.9 ± 0.8
TG [6] (mmol L^{-1})	9.0 ± 2.9	8.8 ± 2.8	9.0 ± 2.8	7.9 ± 2.6
ALT [7] (U L^{-1})	33.5 ± 27.8	25.8 ± 26.9	39.3 ± 34.6	31.8 ± 32.5
AST [8] (U L^{-1})	155 ± 85	144 ± 115	170 ± 122	152 ± 111
ALP [9] (U L^{-1})	16.6 ± 10.6	13.7 ± 7.5	15.7 ± 8.8	15.4 ± 8.4

[1] total protein; [2] albumin; [3] globulins; [4] glucose; [5] total cholesterol; [6] triglycerides; [7] alanine aminotransferase; [8] aspartate aminotransferase; [9] alkaline phosphatase. Results are means ± SD.

3.3. Results of Histological Analysis of Digestive Organs

Histological analysis revealed significant changes in the structure of the anterior intestine. No extensive histopathological changes were found, but in the HM20 and HM30 groups local degenerative changes occurred at the apexes of intestinal folds, and epithelial mucous cells were less abundant (Figure 1A–D). Compared to HM0, the AFH of the HM10-30 groups was significantly higher (all $p < 0.0001$), while their AEH was concurrently smaller (all $p < 0.0001$), and the ASH was also smaller in groups HM20 and HM30 than in HM0 and HM10 (all $p < 0.0001$). The significantly widest AWLP was found

in individuals from the HM0 group (all $p < 0.0001$), while the narrowest was found in the HM10 group (all $p < 0.0001$), with HM20 and HM30 showing intermediate values (Table 8).

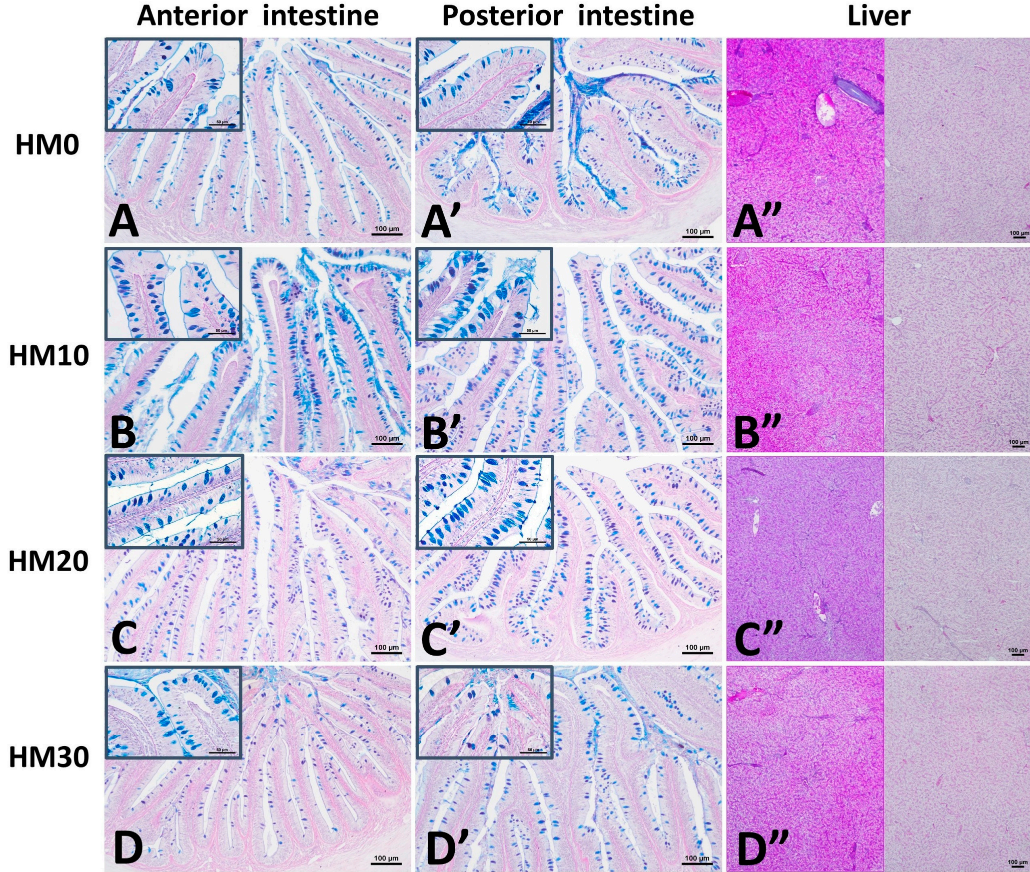

Figure 1. Histological structure of the anterior intestine (**A**–**D**), posterior intestine (**A′**–**D′**) and liver (**A″**–**D″**) of European perch fed diets with different hemp meal contents. The magnified inset images reveal the structure of the intestinal epithelium with pronounced mucous cells (from light blue to dark blue). The images of the liver visualize glycogen deposition (**left**, magenta) and lipid deposition (**right**, non-stained) in the hepatic parenchyma. PAS staining (left halves of **A″**–**D″**), AB-PAS staining (all remaining images). Scale bars = 100 µm (50 µm in inlets).

The intestinal fold structure in the posterior section was like that in the anterior section (Figure 1A′–D′). The lowest PFH was found in the HM0 and HM30 groups (all $p < 0.0001$), while the highest was found in group HM10 (all $p < 0.0001$), the fish group which was also characterized by the largest PEH and PSH, as compared to HM0 ($p = 0.0016$ and $p < 0.0001$, respectively), HM20 (both $p < 0.0001$) and HM30 ($p < 0.0001$ and $p < 0.0029$). The widest PWLP occurred in HM10 and HM20, when compared to HM0 ($p = 0.0057$ and $p < 0.0001$, respectively) and HM30 (both $p < 0.0001$) (Table 8).

The livers of studied fish were characterized by highly variable deposition of both lipids and glycogen (Figure 1A″–D″). The HA found in HM20 was larger than in the other groups (all $p < 0.0001$) (Table 9). In contrary, group HM10 had a larger HN than HM20 ($p = 0.0006$). The HNI of group HM20 was lower than that of HM0 ($p < 0.0001$), HM10 ($p < 0.0001$) and HM30 ($p = 0.0052$). Meanwhile, the LD of group HM30 was lower than in HM0 ($p = 0.0002$), HM10 ($p < 0.0001$) and HM20 ($p < 0.0001$). Lastly, the GD was the highest in HM0 (all $p< 0.0001$), while in HM10 it was also lower than in HM20 and HM30 (both $p < 0.0001$) (Table 9).

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Table 8. Histological parameters of intestines of European perch fed diets with different hemp meal contents.

	HM0	HM10	HM20	HM30
AFH [1] (μm)	695.56 ± 81.43 ^{b}	816.51 ± 96.15 ^{a}	822.64 ± 97.17 ^{a}	805.93 ± 114.83 ^{a}
AEH [2] (μm)	37.28 ± 3.16 ^{a}	33.91 ± 2.65 ^{b}	33.63 ± 3.50 ^{bc}	32.66 ± 3.16 ^{c}
ASH [3] (μm)	18.30 ± 2.40 ^{a}	18.52 ± 2.24 ^{a}	15.97 ± 2.12 ^{b}	15.65 ± 2.26 ^{b}
AWLP [4] (μm)	15.54 ± 5.38 ^{a}	10.80 ± 3.10 ^{d}	12.74 ± 3.60 ^{c}	13.96 ± 2.62 ^{b}
PFH [5] (μm)	487.15 ± 90.12 ^{c}	693.46 ± 77.71 ^{a}	621.73 ± 96.36 ^{b}	515.77 ± 80.33 ^{c}
PEH [6] (μm)	38.96 ± 4.44 ^{b}	41.71 ± 3.84 ^{a}	37.54 ± 3.15 ^{c}	37.94 ± 3.34 ^{c}
PSH [7] (μm)	17.05 ± 2.64 ^{c}	19.51 ± 2.67 ^{a}	17.35 ± 1.77 ^{c}	18.10 ± 3.46 ^{b}
PWLP [8] (μm)	12.05 ± 2.01 ^{b}	13.13 ± 1.98 ^{a}	13.60 ± 2.36 ^{a}	11.16 ± 4.09 ^{c}

Parameters of anterior intestine: [1] fold height, [2] enterocyte height, [3] enterocyte supranuclear height, [4] width of the lamina propria; parameters of posterior intestine: [5] fold height, [6] enterocyte height, [7] enterocyte supranuclear height, [8] width of the lamina propria; Results are presented as means ± SD. ^{a,b,c} means with differing superscript letters are significantly different at $p < 0.05$.

Table 9. Histological parameters of the liver of perch fed diets with different hemp meal contents.

	HM0	HM10	HM20	HM30
HA [1] (μm^2)	230.47 ± 81.38 ^{b}	224.79 ± 61.96 ^{b}	258.21 ± 105.23 ^{a}	200.87 ± 106.02 ^{c}
HN [2] (μm^2)	25.29 ± 5.81 ^{ab}	25.81 ± 6.86 ^{a}	24.34 ± 5.45 ^{b}	24.93 ± 5.58 ^{ab}
HNI [3] (%)	12.37 ± 5.57 ^{a}	12.27 ± 4.52 ^{a}	10.80 ± 4.59 ^{b}	11.76 ± 4.78 ^{a}
LD [4] (%)	43.62 ± 9.38 ^{a}	46.76 ± 13.21 ^{a}	47.21 ± 11.10 ^{a}	36.65 ± 6.76 ^{b}
GD [5] (%)	34.66 ± 4.63 ^{a}	23.69 ± 3.15 ^{c}	30.17 ± 4.07 ^{b}	30.72 ± 3.40 ^{b}

[1] total area of hepatocytes, [2] nuclear area of hepatocytes, [3] hepato-nuclear index, [4] lipid deposition, [5] glycogen deposition. Results are presented as means ± SD. ^{a,b,c} means with differing superscript letters are significantly different at $p < 0.05$.

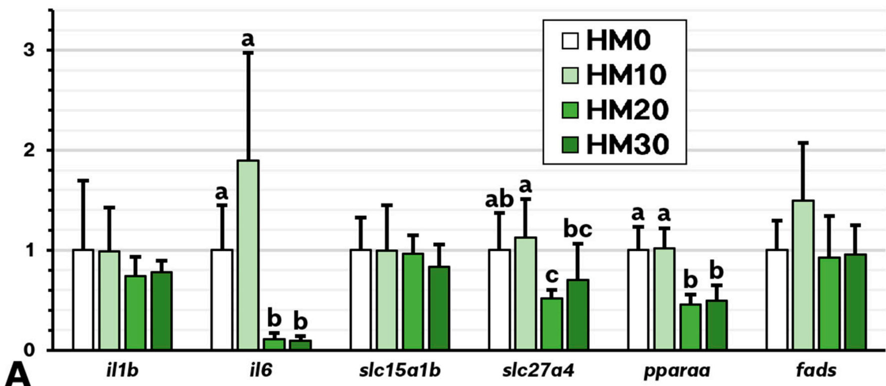
3.4. Results of Gene Expression Analysis of Digestive Organs

In the anterior intestine, the expressions of the *il6* and *pparaa* genes were significantly lower in groups HM20 and HM30 than in HM0 (p = 0.0089 and 0.0012 for HM20; p = 0.0015 and 0.0030 for HM30) and HM10 (p = 0.0007 and 0.0003 for HM20; p = 0.0001 and 0.0008 for HM30) (Figure 2A). The expression of *slc27a4* was also significantly higher in HM10 than in HM20 (p = 0.0002) and HM30 (p = 0.0247), and in HM0 it was also higher than in HM20 (p = 0.0030). In the posterior intestine, the expression of *slc15a1b* was several-fold higher in all four groups when compared to the transcript levels of the remaining studied genes, and it was significantly lower in group HM10 than in HM20 (p = 0.0032) and HM30 (p = 0.0466) (Figure 2B).

The hepatic expression of *pparaa* was significantly lower in HM0 and HM10 when compared to HM20 (p = 0.0009 and 0.0143, respectively) and HM30 (p = 0.0003 and 0.0067, respectively) (Figure 3A).

3.5. Results of Analysis of Hepatic Enzymes

The activity of ALP was significantly lower in groups HM20 (p = 0.0031) and HM30 (p = 0.0109) when compared to HM10 (Figure 3B). The activity of GPX was lower in HM30 than in HM0 (p = 0.0003) and HM10 (p = 0.0033). The activity of SOD remained similar.



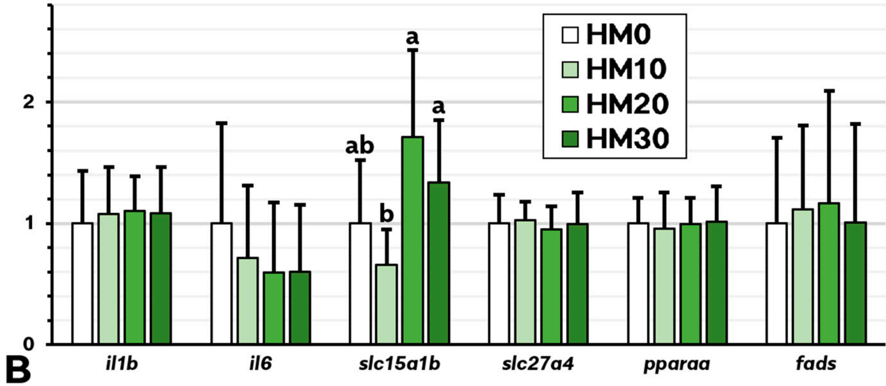


Figure 2. Normalized gene expression of European perch fed diets with different hemp meal contents: (**A**) in the anterior intestine, (**B**) in the posterior intestine, relatively to the mean of group HM0. [a,b] means with differing superscript letters are significantly different at $p < 0.05$.

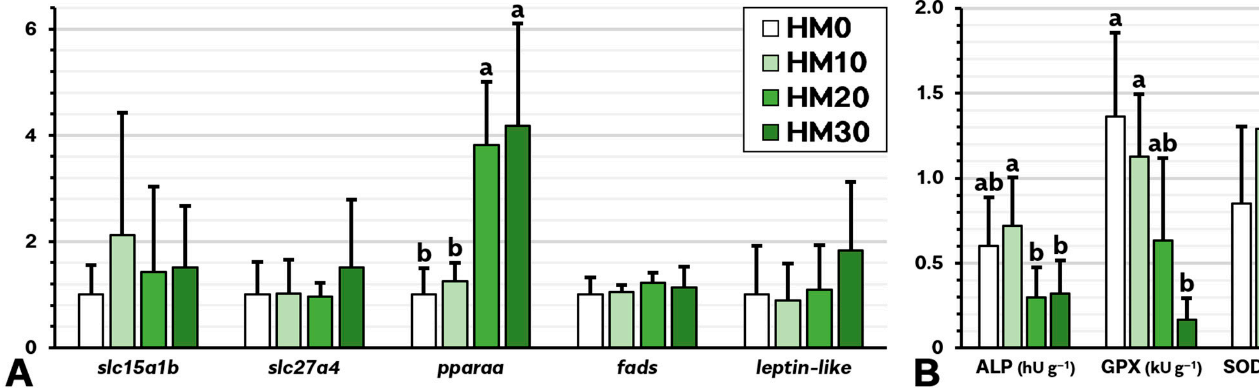


Figure 3. Normalized gene expression, relatively to the mean of group HM0 (**A**) and enzymatic activity (**B**) in the liver of European perch fed diets with different hemp meal contents. [a,b] means with differing superscript letters are significantly different at $p < 0.05$.

4. Discussion

Due to its alleged beneficial properties, industrial hemp has recently attracted the attention of feed producers, and the European Food Safety Authority's (EFSA) Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) opinionated that hemp seed (and cake) can be used in animal nutrition [24]. In fact, many relevant studies were already carried out on livestock, e.g., broiler chickens [25], but there is little

available data when it comes to the use of hemp products in fish diets. Hemp seeds are indeed a rich source of essential amino acids (especially arginine), and contain significant amounts of vitamin E, magnesium, phosphorus, potassium, iron, or zinc. By weight, hemp flour has high carbohydrate (50%) and protein (35%), and low fat (10%) contents, while further processed hemp meal (post oil extraction) can contain up to 70% protein [26]. *Cannabis sativa* seeds also contain cannabinoids, alkaloids, polyphenols and flavonoids, all of which contribute to their high antioxidant, anti-inflammatory, antimicrobial and immunomodulatory properties. Hemp seed-based diets improve animal memory and expression levels of anti-aging genes, while their polyunsaturated and omega-3 fatty acids contribute to lower cholesterol levels, preventing heart disease [27].

Dietary HM inclusion at up to 30% did not significantly affect the survival or growth rates of the European perch in the current study; however, the rearing indices (SGR, FER, PER) peaked for the 20% HM diet. In comparable experiments performed on juvenile fish, similar improvements were found for common carp fed a 10% hemp cake diet [12], striped bass given a 13% HM diet (20% FM replacement) [10], and cobia fed a 37.5% HM diet (40% FM replacement) [11]. In contrast, a 20% HM diet slightly restricted the growth of hybrid striped bass (*Morone chrysops* × *M. saxatilis*), but its proximate composition of muscles was unaffected [28], exactly as in the studied *P. fluviatilis*. Intriguingly, perch fed with the 20–30% FM feeds were characterized by higher VSI and K indices, and lower HSI. In the cobia study [11], the HSI and fat contents (muscle and whole-body) were raised due to the 37.5% HM diet, while protein and ash contents were reduced. In comparison, striped bass which were given a 13% HM diet showed slightly higher HSI and whole-body protein, and lower fat content [10]. All these contradictory results suggest that dietary HM affects basic body parameters of fish in a species-specific manner, rather than following a consistent pattern, implying that the underlying mechanisms are more complicated than initially presumed. However, HM processing, such as defatting, could also be the reason for such differing outcomes.

Biochemical analysis of blood serum is a preliminary diagnostic tool, which allows us to assess the physiological and nutritional state of fish [29]. Serum proteins have a wide range of functions, which is why total protein is a very revealing blood parameter [30], whereas triglyceride and cholesterol levels also reflect metabolic alterations [31]. Meanwhile, activity spikes of enzymes such as ALT, AST, or ALP point towards impaired liver function [32]. In this context, it appears that dietary inclusion of HM neither improved nor worsened the condition of European perch, as it did not have any significant effects on the studied plasma parameters [13,33,34].

Imbalanced nutrition and/or anti-nutritional factors in the diet change the morphology of intestinal mucosa of fish, impeding nutrient digestion and absorption, which can be assessed using histological methods. Common negative markers include: (a) height decreases in intestinal folds and supranuclear areas of enterocytes, (b) the widening of lamina propria, (c) an increase in the numbers of mucosal cells, and (d) an infiltration of immune cells into the epithelium [35]. In the studied perch juveniles, intestinal folds became longer in both the anterior and posterior sections in the two groups fed diets with 10% and 20% HM inclusion. In consequence, their mucosal surface likely became augmented, which usually improves feed utilization by enhancing the breakdown and pickup of nutrients [36,37]. Similar changes were observed in the posterior intestine of sharpsnout sea bream (*Diplodus puntazzo*) fed diets containing up to 48% pea protein concentrate [38]. This is truly important, because shortening of mucosal folds in the posterior intestine would have indicated an inflammatory state, as that part of the tract has additionally prominent immune functions [36]. In contrast, intestinal fold height was unaffected by high inclusion levels of soybean protein in the diets of rainbow trout (*Oncorhynchus mykiss*) [39],

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meagre (*Argyrosomus regius*) [40], and gilthead seabream (*Sparus aurata*) [41]. Meanwhile, even though the absorptive supranuclear area of enterocytes in the anterior intestine was much diminished in the HM20-30 groups, it was improved in the posterior intestine in groups HM10 and HM30. The latter observation opposes the results of the rainbow trout study [39], as well as those of a feeding experiment on Siberian sturgeon (*Acipenser baerii*) with lupin-containing diets [42]. Furthermore, shorter ASH was also found in our previous study on perch juveniles fed a FM diet containing 46% protein [43], which ultimately was deemed the most adequate of the tested compositions, with the current dietary formulations completed in accordance with those conclusions. Hence, it can be stated that the results of histological measurements in the intestine were generally favorable for the HM-containing feeding groups.

Histological measurements of hepatocytes are indirect markers of the liver's condition, with changes in cellular area being a result of lipid and/or glycogen accumulation levels [44], and with larger nuclei being an implication of improved total gene transcription rates [45]. In this context, the changes which occurred in the studied perch livers due to dietary HM inclusion were not severe; however, the changes in hepatocyte nuclear and cellular areas trended negatively in the HM20 group. Notably, the control group HM0 had the highest level of accumulated glycogen, while the lipid levels were significantly reduced only in the HM30 group. In comparison, hepatocyte glycogen storage increased in yellow perch (*Perca flavescens*) when a wheat gluten protein-based diet was supplemented with a lysine-glycine dipeptide, yet diminished (and lipid storage too) when both amino acids were added freely to the diet [46].

Increased expression of genes encoding pro-inflammatory cytokines, such as *il1b* and *il6*, relates to the activation of innate immunity in response to, e.g., infections, but also commonly occurs in fish fed low-FM diets [47]. However, in our study, there were no differences in the expression of *il1b*, while the *il6* expression was significantly reduced in the anterior (groups HM20-30) and also slightly in the posterior (HM10-30) parts of the intestines of fish fed diets with lower FM contents than the control group HM0. These observations are very consistent with *il1b* and *il6* levels reported in the anterior intestine of gilthead seabream offered a diet based on a mixture of plant meals [47]. On the other hand, diets based on raw or fermented soybeans brought an increase in the expression of pro-inflammatory cytokines in the posterior intestine of turbot (*Scophthalmus maximus*) [48]. Meanwhile, the expression of *slc15a1b*, encoding the membrane-bound peptide transporter PEPT1b, which plays an important role in intestinal absorption [49], was raised in the posterior intestine in perch groups fed with diets containing 20-30% HM. Similarly, this gene's expression increased in both the anterior and posterior intestine of rainbow trout four hours after feeding with a low FM diet [50], although a plant meal-based diet slightly reduced the expression of this gene in the anterior intestine of gilthead seabream [47]. Meanwhile, the expression of two genes important for the metabolism of fatty acids, *slc27a4* and *pparaa* [51,52], decreased in the anterior intestines of fish from groups HM20-30, suggesting that their absorption rate of lipids diminished due to the higher HM inclusion in their diets. In comparison, the expression of both these genes increased in the pyloric caeca of Atlantic salmon (*Salmo salar*) post-smolts offered a diet with plant meal and supplementary soy saponin [53]. Moreover, there were no differences between perch groups in both intestinal and hepatic expression of the fatty acid desaturase-encoding gene *fads*, contrasting with the results of studies on the gilthead seabream and Atlantic cod (*Gadus morhua*), both of which were given diets with 100% vegetable oils [54,55]. Furthermore, a diet composed entirely of plant protein and oil increased the intestinal and hepatic expression of *fads* in sea bass (*Dicentrarchus labrax*) [56]. In perch livers, the only significant difference among the studied genes occurred for the expression of *pparaa*, which was raised

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in groups HM20-30. In reference, although the hepatic *pparaa* levels of the Atlantic salmon fed with a plant-based diet were not altered, its *slc27a4* expression did increase [53], similar to the trend observed in our study in the HM30 group.

In the liver, antioxidative enzymes such as GPX and SOD, as well as the metabolic-oriented ALP, are negative markers of inflammation and stress [57,58]. For example, in common carp, increasing dietary supplementation levels with a crude leaf extract of *C. sativa* reversely lowered the hepatic activity of ALP [59], while the dietary addition of lactic acid reduced the activity of GPX [60]. Hence, as the hepatic ALP and GPX activities decreased in perch groups fed with the 20–30% HM-containing diets, and SOD remained relatively constant throughout, it can be concluded that the dietary addition of HM was beneficial in terms of this organ's homeostasis.

5. Conclusions

In summary, based jointly on the results of all performed analyses, it was concluded that the dietary addition of hemp meal did not impede the development of juvenile European perch and had no profoundly negative effects on the homeostasis of its digestive organs. Undeniably, further research is still required to assess the effects of such dietary plant protein on this generally carnivorous species, for instance when administered during its different life stages. Different feed components (e.g., lipid sources) should also be tested in combination with the hemp meal. Nevertheless, the trends observed within the data suggest that the 20% inclusion level of hemp meal in the feed (approx. 30% fish meal replacement rate) was the most adequate for the species among the tested dietary formulations. It is worth noting that this approach could also potentially reduce feed costs, as demonstrated for intensively cultured common carp [61]. It also aligns with a broader trend of using agricultural and food industry by-products as feed ingredients (i.e., following the principles of circular economy), and would also reduce the pressure exerted on wild fish stocks [62]. However, the use of such raw materials requires strict quality control (e.g., nutritional profile, assessment of antinutrients and chemical contaminants), as their composition and safety can vary significantly depending on their origin and processing technology [63].

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Data Availability Statement: The raw data supporting the conclusions of this article will be made available by the authors on request.

Conflicts of Interest: The authors declare no conflicts of interest.

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