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Biofunctional Characterization of Collagen from Orange-Spotted Grouper, *Epinephelus coioides*: Maintenance of Cartilage in Aged Zebrafish

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Abstract: In this investigation, collagen was successfully extracted from the skin and fins of the orange-spotted grouper, *Epinephelus coioides*, with recovery rates of 4.45% and 23.65% (dry weight basis), respectively. UV–Vis spectrophotometric analysis demonstrated distinct absorbance peaks at 222 nm and 217 nm for collagen isolated from the skin and fins, correspondingly. Both collagen sources were confirmed to be type I, characterized by the presence of two α-chains (α1 and α2), with glycine as the predominant amino acid, an absence of tryptophan, and a notable content of proline and hydroxyproline. The enzymatic hydrolysis of fin-derived collagen using pepsin yielded low-molecular-weight peptides (PHC), which were subsequently incorporated into the diet of 1.5-year-old zebrafish, either alone or in combination with glucosamine (GC), to assess their biological effects. After eight weeks of dietary supplementation, zebrafish fed PHC or the combined PHC + GC diets exhibited a significant upregulation of *sox9a* (*jef*) expression in spinal tissues, accompanied by a marked downregulation of *runx2*. Although the differences in swimming performance among the groups were not statistically significant, zebrafish that received PHC or the combined supplements demonstrated enhanced endurance compared to the control group. These results suggest that collagen sourced from grouper may have advantageous effects in supporting cartilage health in aged zebrafish. Furthermore, utilizing fish by-products for collagen extraction enhances resource efficiency and aligns with circular economy principles.

Keywords: collagen; pepsin; osteoblast; zebrafish

Key Contribution: This study elucidates the successful extraction and characterization of type I collagen from the skin and fins of grouper with fin-derived collagen hydrolysates, demonstrating significant regulatory effects on cartilage biosynthesis in zebrafish.

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1. Introduction

Collagen, one of the most abundant proteins in the animal kingdom, serves as the principal component of extracellular matrices in tissues, including bone, skin, and various connective structures. Its primary function is to provide a structural scaffold that ensures mechanical integrity and resilience. Collagen is distinguished by its unique amino acid composition, being rich in glycine, proline, and hydroxyproline [1]. A hallmark of collagen is its triple-helical structure, where three parallel polypeptide chains adopt a left-handed polyproline II (PPII) helical conformation. These chains interlock with a one-residue offset, forming a right-handed triple helix, a structure essential for its biological function and stability [2]. The stability of this helix, with a molecular diameter of ~1.5 nm and a molecular weight of ~283 kDa, is maintained by strong hydrogen bonds between the polypeptide chains [3]. The repeating amino acid sequence, represented as -Gly–X–Y-, further strengthens its structure, with glycine accounting for 35% of the total amino acid content. Proline and hydroxyproline are also significant contributors, while cysteine, tyrosine, histidine, and methionine are present in only trace amounts, and tryptophan is entirely absent [1,4].

Collagen is predominantly sourced from animal tissues and broadly categorized into terrestrial and aquatic origins. Terrestrial collagen is primarily obtained from pigs and cows, offering the advantage of higher denaturation temperatures. In contrast, collagen from aquatic species is typically extracted from the skin of fish and cephalopods [5–8]. While 21 distinct collagen types have been identified, types I, II, and III are the most extensively studied [9]. Type I collagen, the most abundant, is found in load-bearing tissues like skin, tendons, and bones. Its molecular composition often involves two identical polypeptide chains, α1(I), and a third distinct α2(I) chain [10]. Types II and III collagen are present in specialized tissues such as cartilage and arteries, respectively, each fulfilling distinct physiological roles [11]. Of the various types of collagens identified, Type I is the most prevalent, comprising over 90% of the organic bone matrix, and it is widely applied [12].

Marine collagen, particularly from fish, is gaining attention due to its potential health benefits and environmental sustainability. Fish skin, bones, and scales, often regarded as by-products in seafood processing, are excellent sources of collagen. For instance, acid-extracted collagen from species like ayu, *Plecoglossus altivelis*, and Japanese seabass, *Lateolabrax japonicus* shows significant yield variability, with ayu bones yielding up to 54% collagen, while seabass fins yield only 5.2%, illustrating the variation in collagen content within fish tissues [6–8]. Additionally, fish bones, skins, and scales, which are often regarded as by-products of seafood processing, can be repurposed as valuable collagen sources.

Collagen extracted from the outer skin of cuttlefish, *Sepia Lycidas,* using acetic acid and pepsin, followed by SDS-PAGE analysis, revealed a heterotrimeric composition of $(\alpha1)_2\alpha2$, closely resembling type I collagen based on peptide mapping [5]. Similarly, collagen extracted from the pufferfish *Takifugu obscurus* was analyzed via SDS-PAGE, showing the presence of one β chain and at least two α chains (α1 and α2). The molecule weights of the α1 and β chains were approximately 130 and 200 kDa, respectively [7]. The subunit composition of all samples was similar to that of type I collagen from other fish species [13–15]. In the aquaculture sector, grouper (*Epinephelus* spp.) is a high-value species, particularly in Asia, where areas such as China mainland, Taiwan, and Indonesia account for 93% of global production [16]. Research has demonstrated that collagen extracted from the skin of giant grouper, *E. lanceolatus*, predominantly consists of type I collagen, characterized by its two α-chains (α1 and α2) [17]. The skin of the grouper is considered an exceptional source of collagen due to its thickness relative to other fish species, making it a promising material for collagen extraction.

Recent studies suggest that collagen supplementation may play a role in the management of degenerative bone and joint disorders, particularly when combined with physical exercise [18]. While research remains ongoing, evidence suggests that collagen peptides may inhibit bone collagen breakdown and alleviate symptoms of osteoarthritis and osteoporosis [19,20]. Collagen peptides are regarded as a safe and therapeutic supplement for managing symptoms related to osteoarthritis and osteoporosis [21,22]. Additionally, collagen-derived peptides have demonstrated therapeutic potential in promoting bone formation and musculoskeletal health [23].

Fish-derived collagen peptides hold significant potential not only for biomedical applications in humans, such as enhancing bone density and preventing osteoporosis but also in supporting sustainable agricultural practices through the recycling of fish by-products. Therefore, the present study aims to extract and hydrolyze collagen from the skin and fins of grouper and evaluate its bioactive properties. Specifically, collagen peptides will be administered to aged zebrafish to assess improvements in swimming performance, a proxy for musculoskeletal health. This research will also explore the potential of collagen peptides to enhance bone mass, laying the groundwork for future applications in human health.

2. Materials and Methods

2.1. Animals and Ethical Statement

Experimental procedures on animals were performed in accordance with methods approved by the Institutional Animal Care and Use Committee (IACUC) of the National Pingtung University of Science and Technology. The IACUC Approval No. is NPUST-110-138.

The wild-type AB strain of zebrafish (SL 3.5~4 cm) was obtained from Professor Hu Shao-Yang, National Pingtung University of Science and Technology, Pingtung, Taiwan, and had been reared in the zebrafish facility. In the facility, zebrafish were bred, raised, and maintained in a recirculating aquatic system with a 10/14 h dark/light cycle at 28 ± 1 °C, according to the standards [24]. During the acclimation, fish was fed with a control diet twice daily to satiation.

2.2. Preparation of Collagen

The collagen extraction was conducted following the protocol described by Nagai and Suzuki [8]. Skin and fin of grouper, *E. coioides* were sourced from a local seafood processing facility, promptly stored in a portable cooler, and immediately transported to the laboratory for experimentation. Skin or fins were cut into small pieces and weighed. The samples were then immersed in a 0.1 N NaOH solution at a ratio of 1:10 (w/v) for three days at room temperature to remove non-collagenous proteins, with the NaOH solution replaced twice daily. Afterward, the samples were thoroughly rinsed with deionized water, followed by immersion in a 10-fold volume of 0.5 M acetic acid for collagen extraction. This acid extraction was conducted over three days. Subsequently, the mixture was centrifuged at 9000× g for 20 min at 4 °C, and the supernatant was collected. Collagen was precipitated from the supernatant by adding NaCl to a final concentration of 0.7 M, followed by centrifugation at 9000× g for 20 min to obtain the collagen pellet. The pellet was redissolved in a minimal volume of 0.5 M acetic acid, dialyzed against deionized water for 3 days, with one change of solution per day. Finally, the sample was stored at −80 °C, followed by lyophilization to obtain collagen.

2.3. Characterization of Grouper Collagen

2.3.1. UV–Vis Measurement

The sample (0.2 g) was dissolved in 100 mL of deionized water for spectrophotometric analysis. UV–Vis full-wavelength scanning was conducted across the 190–400 nm range at a rate of 100 nm min^{-1}.

2.3.2. Amino Acid Composition

The amino acid composition of the hydrolysate was determined using reverse-phase high-performance liquid chromatography (RP-HPLC). A 19.5 mg sample was hydrolyzed under nitrogen gas in 1 mL of 6 N HCl containing 1% phenol at 110 °C for 24 h. After hydrolysis, the sample was neutralized to pH 7.0 with 4 N NaOH, followed by drying in a vacuum oven at 60 °C until fully desiccated. The residue was then dissolved in 1 mL of 0.001 N HCl, mixed, and filtered through a 0.22 μm PVDF syringe filter. Ortho-phthalaldehyde (OPA) was subsequently added at room temperature in equal volumes to facilitate the detection of amino acids by RP-HPLC, employing an autosampler and a Gemini-NX 5μ C18 110A column (25 cm × 4.6 mm, 5 μm particle size, Phenomenex, Torrance, CA, USA), coupled with a fluorescence detector (excitation at 340 nm; emission at 455 nm). Chromatographic conditions were based on the OPA reagent guidelines (P0532, Sigma, St. Lous, MO, USA) with minor adjustments. Methionine and cysteine were protected by pretreatment with a mixture of formic acid (9:1 ratio of 88% formic acid and 30% hydrogen peroxide) prior to acid hydrolysis with 6N HCl at 110 °C for 24 h. Tryptophan analysis followed the method of Çevikkalp et al. [25], utilizing hydrolysis in 5 N NaOH. OPA-derivatized amino acids were quantified by RP-HPLC (PU-2089 plus, JASCO, Tokyo, Japan), following the protocol described above.

2.3.3. Preparation of Collagen Hydrolysates and SDS-PAGE Analysis

The method for collagen hydrolysis was modified based on Li et al. [26]. A precise amount of 20 mg of extracted collagen was weighed, followed by the addition of 1 mL of pepsin solution (8 μg pepsin mL^{-1} in 10 mM HCl, pH 2). The mixture was incubated at 37 °C with shaking at 100 rpm for 24 h. After hydrolysis, the reaction was terminated by heating the sample in a dry bath at 100 °C for 3 min. The hydrolyzed collagen was used for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis or stored at −20 °C until further analysis.

Protein solutions were mixed with SDS-PAGE sample buffer (0.125 M Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, and 10% β-mercaptoethanol) at a 1:1 (v/v) ratio and subjected to boiling for 10 min. A 15 μL aliquot of each sample was loaded onto a gel composed of 5% stacking gel (5% acrylamide, 0.127 M Tris (pH 6.8), 0.1% SDS, 0.1% ammonium persulfate, 0.1% TEMED) and 12% separating gel (12% acrylamide, 0.38 M Tris (pH 8.8), 0.1% SDS, 0.1% ammonium persulfate, 0.04% TEMED), followed by electrophoresis at a constant current of 15 mA. Upon completion of electrophoresis, the gels were stained with 0.1% Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad, Hercules, CA, USA) in a solution of 40% methanol and 10% acetic acid, then destained using a solution containing the same proportions of methanol and acetic acid.

2.4. Analysis of the Effects of Collagen on Swimming Performance and the Expression of Cartilage Biosynthesis Genes in Zebrafish

To examine the impact of pepsin-hydrolyzed collagen derived from grouper fins on enhancing swimming performance and maintenance of cartilage in aged zebrafish, this collagen hydrolysate was incorporated into the diet of 1.5-year-old zebrafish.

2.4.1. Preparation of Experimental Diets and Fish Rearing

The formulation of the experimental diets was adapted based on Lin et al. [27], with a protein content of 23% and a lipid content of 7%. The experimental groups were divided into four distinct categories: a control group; a group supplemented with pepsin-hydrolyzed collagen (PHC); a glucosamine (GC) group; and a combined PHC-GC group. The basal diet composition is detailed in Table 1. Dosages for glucosamine and PHC were carefully calibrated to deliver 20 mg and 5.5 mg per kg of body weight per day, respectively. All ingredients were thoroughly mixed prior to the addition of fish oil and water to create a uniform dough, which was subsequently shaped into pellets. The pellets were dried at 50 °C until the moisture content was reduced to below 10%, and the dried feed was stored at 4 °C until further use.

Table 1. Ingredients used and proximate composition of the formulated diets. PHC: pepsin-hydrolyzed collagen; GC: glucosamine.

Ingredient	Experiment Diets (g kg^{-1})			
	Control	**PHC**	**GC**	**PHC + GC**
Fish meal	50	50	50	50
Soybean meal	360	360	360	360
Wheat middling	110	110	110	110
Rice bran	250	250	250	250
Soybean oil	54	54	54	54
cellulose	51	50.813	50.333	50.146
α-starch	90	90	90	90
PHC	0	0.187	0	0.187
GC	0	0	0.667	0.667
Vit. mix	15	15	15	15
Min. mix	20	20	20	20
	Proximate composition (%)			
Crude protein	22.75 ± 0.15	23.32 ± 0.35	22.98 ± 0.27	22.65 ± 0.47
Crude lipid	7.35 ± 0.08	7.49 ± 0.04	7.12 ± 0.08	7.23 ± 0.11
Moisture	7.25 ± 0.01	7.23 ± 0.04	6.98 ± 0.04	7.07 ± 0.05
Ash	11.2 ± 0.04	10.8 ± 0.06	11.4 ± 0.07	10.7 ± 0.06

The feeding trial was conducted in 100 L polyethylene tanks (66 cm × 43 cm × 30 cm) under a temperature of 28 °C, with a continuous water flow at a rate of 0.5 L h^{-1}. Aeration was continuously provided to ensure dissolved oxygen > 5 mg L^{-1}. Zebrafish (initial mean weight: 0.384 ± 0.05 g) were randomly allocated to four treatment groups, each with three replicates and 20 fish per replicate. During the 8-week trial, fish were fed twice daily at 3% of body weight. At the end of the feed trial, assessments included a swimming endurance test, analysis of cartilage biosynthesis-related gene expression, and Western blot analysis.

2.4.2. Zebrafish Swimming Endurance Test

The zebrafish swimming performance test was conducted using a custom-built swimming apparatus (Figure 1). The device comprises a 1.5 m transparent tube with an inner diameter of 3 cm, designed to facilitate controlled swimming experiments. Nets with a mesh size of 830 μm were positioned at both ends of the tube to confine the fish within the swim lane, ensuring accurate and uninterrupted performance measurements. The front of the lane is connected to a flow meter, which is further attached to a variable-frequency submersible motor to regulate a consistent water flow. The motor is submerged in a tank where the water temperature is maintained at 28 °C. During this experiment, zebrafish

were initially placed in the swim lane for a 10-min acclimation period. After acclimation, the system was activated, and the water flow was adjusted to 25 m per minute. As soon as the system began, the time each fish swam against the current was recorded. Timing ceased when the fish could no longer resist the flow, eventually being swept to the back of the lane and trapped by the mesh. This measure was used to assess the improvement in swimming performance following the feeding of different experimental diets. A total of 24 fish were used for the evaluation, with 6 fish per group. The body weights of fish in the control, PHC, GC, and PHC+GC groups were 0.36 ± 0.03, 0.37 ± 0.04, 0.36 ± 0.04, and 0.38 ± 0.03, respectively. Statistical analysis revealed no significant differences among these groups, confirming a consistent baseline across all experimental conditions for subsequent comparative assessments.

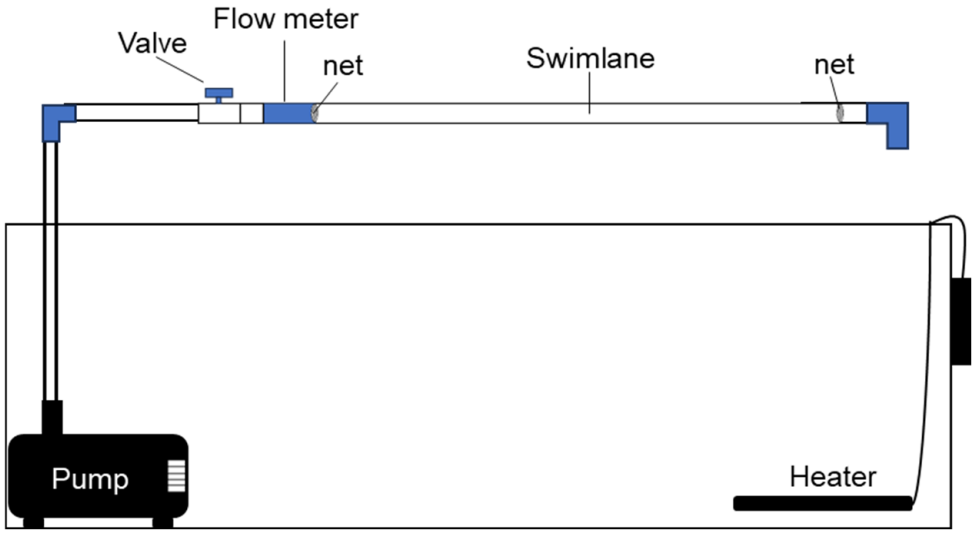

Figure 1. The apparatus used for the assessment of swimming endurance in this study.

2.4.3. Expression of Cartilage Biosynthesis Genes

The present study investigated gene expression profiles associated with cartilage biosynthesis, focusing on *sox9a* (*jef*), *acan* (*aggrecan*), *runx2α*, and *runx2β*. A total of six zebrafish from each experimental group were randomly selected for this analysis. Total RNA was extracted from the vertebrae using the Rezol reagent (AMRESCO, Solon, OH, USA), strictly adhering to the manufacturer's guidelines. The concentration of the extracted RNA was carefully normalized and quantified to ensure accuracy. For the synthesis of first-strand complementary DNA (cDNA), 1 μg of total RNA from each sample was reverse-transcribed using SuperScript II RNase H- reverse transcriptase (Promega, Madison, WI, USA) and oligo $d(T)_{18}$ as the primer, following the optimal conditions recommended by the manufacturer for poly(A)+ RNA synthesis.

Gene expression analysis was conducted using real-time polymerase chain reaction (PCR) in a 96-well plate format, employing the ABI StepOne Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA) and SYBR Green dye for detection. The primers utilized for amplifying the target genes were as follows: *sox9a*F: 5′-GCCAGGCAAAGCGGATCT-3′; *sox9a*R: 5′-GCGGGAGGTATTGGTCAAACT-3′; *acan*F: 5′-TGAGGTTGATGTATGCCATTCAA-3′; *acan*R: 5′-AACTTAGTCCAGCCCTTCTCACA-3′; *runx2α*F: 5′-GTGTACTGTAGCTTACCTTTGCTGACAT-3′; *runx2α*R: 5′-GGACGGCGGAC TGAACCT-3′; *runx2β*F: 5′-ACGCAAACGGAGGACATACG-3′; *runx2β*R: 5′-CCGGCGC TGGGATCTAC-3′. β-actin served as the reference control gene, with primers *β-actin*F: 5′-TCTGGCATCACACCTTCTACAAT-3′ and *β-actin*R: 5′-TGTTGGCTTTGGGATTC AGG-3′. The PCR reaction conditions were established in accordance with the meth-

ods outlined by Prabawati et al. [28]. Normalization of the target gene expression levels was performed relative to the internal control gene *β-actin*, which has been preliminarily validated for stable expression under the experimental conditions. The relative expression of the target genes was calculated using the $2^{-\Delta\Delta Ct}$ method [29]. The results are presented as mRNA expression levels relative to the control group.

2.4.4. Western Blot Analysis

The methodologies for protein separation and visualization were adapted from Tseng et al. [30] with slight modifications. For this study, three zebrafish were randomly selected from each group, and their vertebrae were homogenized in 50 mM Tris-HCl buffer (Sigma, St. Lous, MO, USA) (pH 7.6) until completely pulverized. The homogenate was subsequently centrifuged at 14,000 rpm for 20 min at 4 °C, and the resulting supernatant was transferred to new tubes, adjusting the protein concentration to 2.5 μg per sample. Proteins were separated using a 12% acrylamide SDS-PAGE gel and subsequently transferred to a polyvinylidene fluoride (PVDF) membrane (GE Healthcare, Amersham, UK). Hybridization was performed with an anti-Sox9 antibody (1:1000, Rabbit polyclonal anti-Sox9 antibody, GeneTex, Irvine, CA, USA) and an anti-actin antibody (1:10,000, Mouse monoclonal beta-actin antibody, GeneTex, USA), both diluted at a 1:1000 ratio and referred to as the primary antibodies. Following this, the membranes were incubated with alkaline phosphatase (AP)-conjugated goat anti-rabbit immunoglobulin G (Pierce, Appleton, WI, USA), diluted at a ratio of 1:10,000, to serve as the secondary antibody. The detection of protein bands was accomplished using Amersham ECL Plus Western Blotting Detection Reagents (GE Healthcare, Little Chalfont, Bucks, UK), and the resulting signals were visualized using a Multi-function Gel Imaging System (MultiGel-21, Topbio, Taipei, Taiwan). The relative intensity of each protein was normalized to the β-actin input in each lane. The experiment was performed in triplicate.

2.5. Statistical Analysis

Statistical analyses were performed using SAS software (version 9.4, Cary, NC, USA). Prior to conducting an analysis of variance (ANOVA), Levene's test was employed to assess the homogeneity of variance, and the Shapiro–Wilk test was utilized to evaluate the normality of the data. Differences among groups were assessed using one-way ANOVA, followed by Duncan's multiple range test as a post hoc analysis to identify specific group differences. A significance level of $p < 0.05$ was established for all statistical evaluations.

3. Results and Discussion

In this study, collagen was successfully extracted from the skin and fins of the grouper, yielding recovery rates of 4.45 ± 0.7% and 23.65 ± 1.18% (dry weight basis), respectively. Previous research has mainly focused on collagen extraction from various aquatic species using acetic acid. For instance, the yield of collagen from the skin of giant grouper was reported to be 39.51% [17], and from the skin of Nile tilapia, it was 39.4% [15]. However, collagen yields from different aquatic animals vary significantly; for example, cuttlefish yielded only 2% collagen [15], and threadfin bream skin yielded 2.3% [7]. Although the yield of collagen from grouper skin was relatively low in this study, the yield from the fins was considerably higher, indicating that fins represented an excellent potential source of collagen, particularly given that fins are not typically consumed as food.

The UV–Vis spectrophotometric analysis of the extracted collagen revealed distinct absorbance peaks at wavelengths of 222 nm and 217 nm (Figure 2). These findings are consistent with the characteristic absorbance maxima around 220 nm reported for collagen derived from walleye pollock, *Theragra chalcogramma* [31], and Nile tilapia,

Oreochromis niloticus [15]. Similarly, in giant grouper, the UV–Vis spectra for both acid-solubilized collagen (ASC) and pepsin-solubilized collagen (PSC) exhibited high absorbance values between 210 and 230 nm, with minimal to no absorbance at 280 nm [17]. This absence of significant absorbance at 280 nm is attributable to the lack of tryptophan residues in the collagen molecule, further supporting the identification of the extracted protein as collagen. Additionally, collagen is known to contain only trace amounts of aromatic amino acids such as tyrosine, histidine, and methionine [13,32]. Notably, aromatic amino acids like tyrosine and phenylalanine typically exhibit absorbance maxima at 283 nm and 251 nm, respectively [33], which explains the relatively low absorbance observed in the 250–280 nm range. This pattern is in agreement with the absorbance profiles reported for collagen extracted from giant grouper [17], further corroborating the purity and structural characteristics of the extracted collagen.

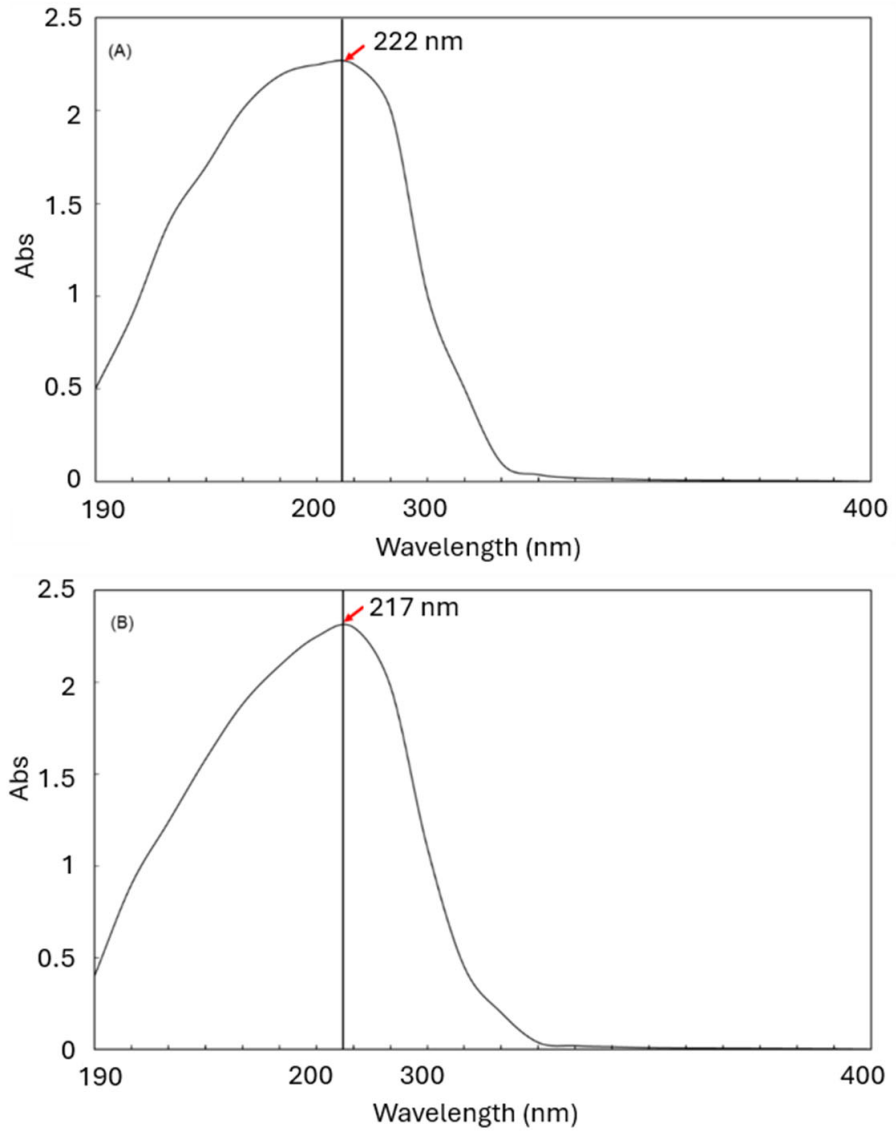

Figure 2. UV–Vis absorption spectrum of skin collagen (**A**) and fin collagen (**B**) of grouper.

Each collagen molecule is characterized by a triple-helix structure comprising three α-chains, which can be organized as either homotrimers (composed of a single type of α-chain, as in type II collagen) or heterotrimers (formed by two or more distinct α-chains, as seen in type I collagen), depending on the collagen subtype [34]. In the present investigation,

SDS-PAGE analysis was used to examine the protein composition of collagen extracted from grouper skin and fins (Figure 3).

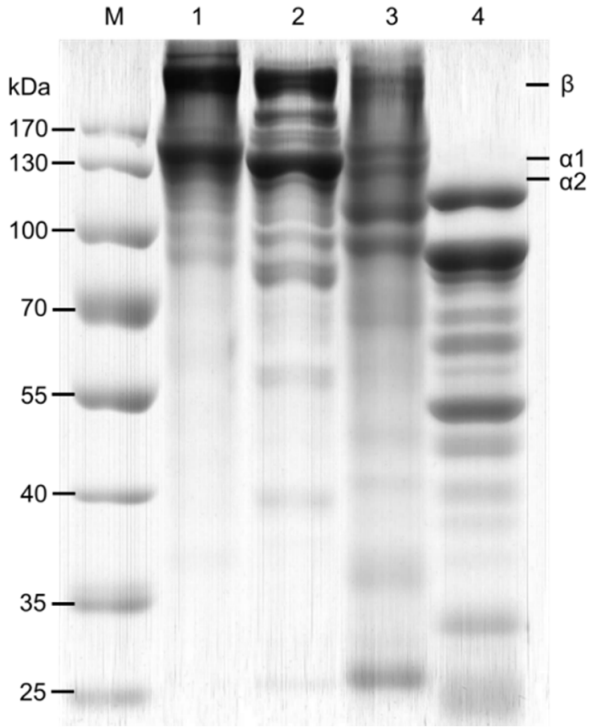

Figure 3. SDS-PAGE profiles of collagens extracted from the skin and fins of grouper. M: protein molecular weight marker; Lane 1: collagen derived from skin; Lane 2: collagen obtained from fins; Lane 3: skin collagen hydrolyzed with pepsin; Lane 4: fin collagen hydrolyzed with pepsin. Each lane was loaded with 2.5 μg of protein.

The findings revealed that both sources contained two distinct α-chains (α1 and α2) in addition to a β-chain. Notably, the molecular masses of the α1 and α2 chains were approximately 142 kDa and 126 kDa in skin collagen and 139 kDa and 124 kDa in fin collagen, respectively. These patterns were consistent with those observed in type I collagen from giant grouper [17], as well as in other species previously studied [7]. The β-chain was identified as the dominant component in both collagen sources, and both α1 and α2 chains were prominently displayed in the SDS-PAGE profiles. Based on the protein patterns, the extracted collagen was identified as type I collagen, which is predominant in fish [7], including species such as common carp, *Cyprinus carpio*, flatfish, *Paralichthys olivaceus*, and Nile tilapia. The SDS-PAGE profiles of these collagen extracts consistently demonstrated the presence of β, α1, and α2 chains, corroborating the findings from earlier studies [13–15]. This further supports the conclusion that the collagen extracted in this study aligns with the characteristics of type I collagen. Additionally, it is well-established that the α3-chain is widely distributed among teleost fish. However, the presence of the α3-chain could not be conclusively determined in this study due to its molecular weight being similar to that of the α1-chain, making it difficult to differentiate using electrophoresis.

The collagen samples underwent enzymatic hydrolysis using pepsin, which revealed that the molecular weights of the hydrolyzed proteins were significantly lower than those of the untreated collagen samples (Figure 3). This finding indicates that the covalent cross-linking at the telopeptide regions of collagen molecules, resulting from the condensation of aldehyde groups, as well as intermolecular cross-links, were not readily solubilized through acid extraction [35]. Such cross-linking generally contributes to a reduction in collagen solubility [36,37]. Pepsin effectively cleaved the cross-linked molecules at the telopeptide

regions, facilitating the extraction of collagens that were localized in the loosened structures resulting from this cleavage. Notably, the molecular weights of the β- and α-chains of collagen subjected to pepsin hydrolysis were found to be lower than those of collagen solubilized by acid extraction alone (without pepsin treatment). This observation suggests that small peptide fragments at the telopeptide regions were cleaved and removed, leading to the formation of collagens with reduced molecular weights.

The amino acid composition of collagen extracted from the skin and fins of grouper is detailed in Table 2. Glycine (Gly) was found to be the most abundant amino acid in both extracts, consistent with its role as every third residue in the repeating Gly–X–Y motif of collagen, except in the telopeptide regions [1]. Glycine is critical for maintaining the alpha-helix structure of collagen due to its lack of a bulky side chain, which allows for the free rotational flexibility necessary for the stability of the triple helix [1]. In this study, glycine represented 34.49 ± 5.63% and 21.93 ± 5.81% of the total amino acids in collagen derived from grouper skin and fin, respectively. These results are consistent with those of Duan et al. [13], who identified glycine as the most abundant amino acid in carp collagen, and Hsieh et al. [17], who similarly reported glycine as the primary amino acid in giant grouper collagen. Additionally, Zhang et al. [32] and Akita et al. [38] corroborated that glycine was the predominant amino acid across various collagen sources. Positions X and Y in the Gly–X–Y sequence are frequently occupied by hydroxyproline and proline, contributing to the unique properties of collagen, particularly its thermal and structural stability [3,4]. In the present study, proline and hydroxyproline accounted for 6.54 ± 3% and 5.18 ± 0.86% in skin-derived collagen and 3.2 ± 0.45% and 4.84 ± 0.36% in fin-derived collagen, respectively. These amino acids, characteristic of collagen, play a pivotal role in stabilizing the triple-helix structure [39,40], with their imino rings conferring rigidity to the helix [41]. The concentration of hydroxyproline is particularly noteworthy, as it is influenced by the temperature at which the fish are reared, affecting the collagen's thermal denaturation point [17,42]. Thus, proline and hydroxyproline content are key determinants of collagen's thermal stability and overall structural integrity.

Table 2. Amino acid composition of collagen isolated from skin and fin of grouper.

Amino Acids (%)	Skin	Fin
Aspartic acid	3.92 ± 0.71	3.72 ± 1.49
Glutamic acid	11.79 ± 1.19	9.82 ± 4.47
Serine	1.75 ± 0.19	1.84 ± 0.5
Histidine	0.3 ± 0.18	0.04 ± 0.01
Arginine	12.53 ± 0.75	10.09 ± 5.19
Glycine	34.49 ± 5.63	21.93 ± 5.81
Threonine	2.98 ± 0.32	2.84 ± 1.33
Alanine	8.77 ± 0.88	6.44 ± 1.35
Tyrosine	4.21 ± 0.35	3.96 ± 2.2
Methionine	3.52 ± 0.17	3.25 ± 1.64
Valine	0.6 ± 0.1	0.87 ± 0.37
Phenylalanine	2.08 ± 0.42	1.12 ± 0.56
Isoleucine	0.26 ± 0.12	0.56 ± 0.25
Leucine	2.39 ± 0.3	2.6 ± 1.22
Lysine	3.1 ± 0.69	3.1 ± 1.72
Proline	6.54 ± 3	3.2 ± 0.45
Hydroxyproline	5.18 ± 0.86	4.84 ± 0.36

Collagens are recognized as a significant source of bioactive peptides, as noted by Yaghoubzadeh et al. [43]. Research indicates that low molecular weight (LMW) peptides demonstrate enhanced bioactivity and functional properties, particularly those enriched

with proline and hydroxyproline. This enhancement is attributed to their increased resistance to enzymatic degradation by digestive peptidases [44]. Consequently, LMW peptides offer distinct advantages, including a well-balanced amino acid profile, superior digestibility, and rapid absorption. Such attributes, alongside their favorable functional and physicochemical properties, broaden their potential applications in various fields [45]. In this study, we incorporated PHC derived from grouper into the diet of 1.5-year-old zebrafish to evaluate its effects on swimming performance and the expression of cartilage biosynthesis-related genes. The results presented in Figure 4 indicate that the swimming durations for the control, PHC, GC, and combined PHC + GC groups were 192 ± 26.1 min, 247.4 ± 42.3 min, 204.9 ± 7.6 min, and 239.2 ± 19.6 min, respectively. Although no statistically significant differences were identified among the groups, the fish that received diets containing PHC or the combined PHC+GC demonstrated longer swimming durations than those on the control diet. The swimming duration did not show a statistically significant difference, and it may be attributed to individual variations. Future studies should include larger sample sizes to ensure a more comprehensive understanding of the effects of low-molecular-weight collagen peptides (PHC).

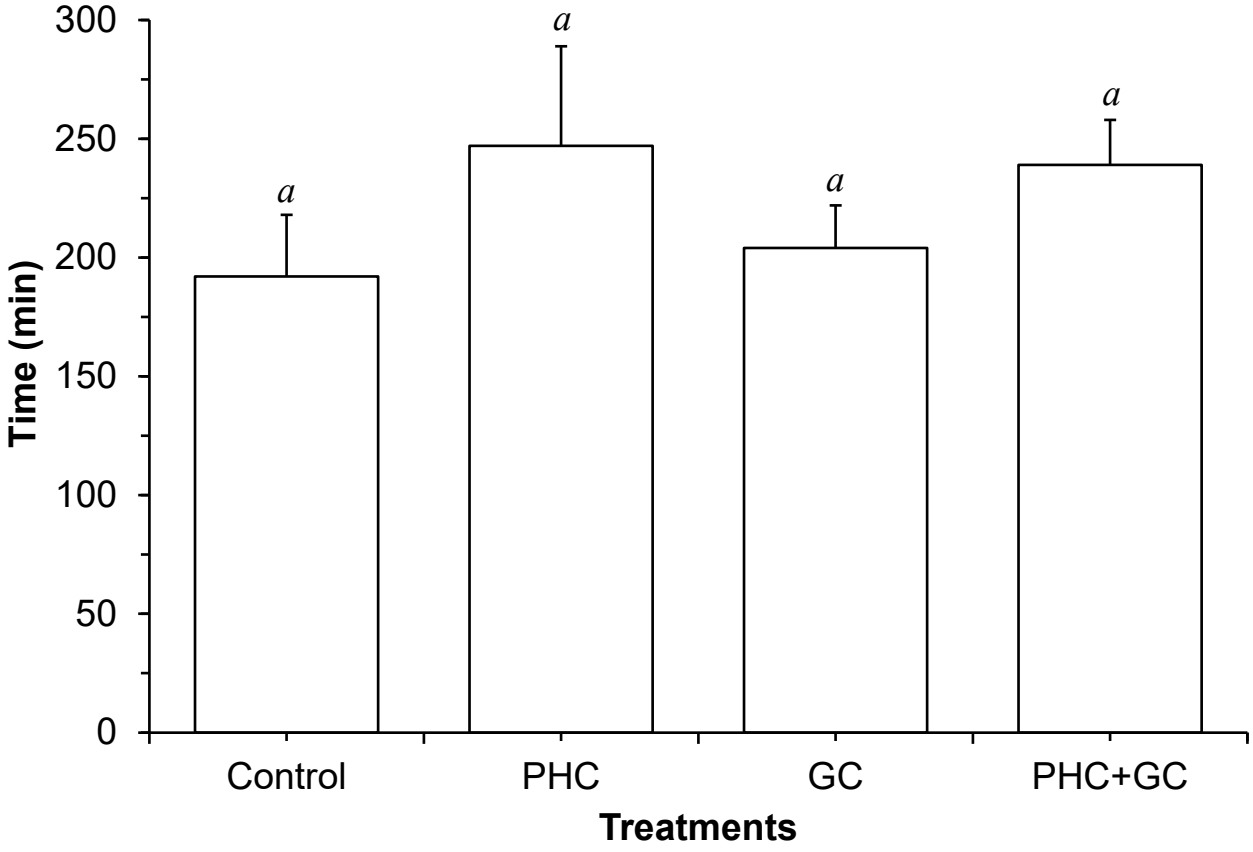

Figure 4. Assessment of swimming performance in 1.5-year-old zebrafish subjected to different dietary treatments for 2 months. The groups consisted of a control diet, a diet supplemented with pepsin hydrolyzed collagen (PHC) at a concentration of 0.187 g kg^{-1}, a diet enriched with glucosamine (GC) at 0.667 g kg^{-1}, and a combined diet containing both PHC (0.187 g kg^{-1}) and GC (0.667 g kg^{-1}). All groups were assessed under a water flow rate of 25 m min^{-1}. Results are presented as the mean ± standard deviation (S.D.). Data marked with the same letter "a" indicate that no significant differences were observed among the groups ($p > 0.05$). $n = 6$.

In this study, the glucosamine dosage was calibrated to deliver 20 mg per kilogram of body weight per day, aligning with the recommended daily intake for individuals with osteoarthritis as outlined by Oegema et al. [46]. The dosage for PHC was set at 5.5 mg per

kilogram of body weight per day based on preliminary findings suggesting its ability to enhance *sox9a* expression in fish (unpublished data). This dosage is consistent with previous studies demonstrating that oral administration of chicken collagen at doses ranging from 1 to 10 mg per kilogram alleviated joint pain, reduced plasma levels of inflammatory cytokines, and mitigated cartilage degradation [47]. Globally, dietary supplements containing chondroitin and glucosamine are marketed worldwide and have been evaluated in human osteoarthritis clinical trials with mixed results. Both in vitro and anecdotal animal and human data suggest that oral consumption of type I collagen could represent a strategy for supporting joint health [23]. Results reported by Dar et al. [48] suggest that daily consumption of type I collagen protects against cartilage loss and stimulates the production of proteoglycan by chondrocytes in injured joints of mice, suggesting chondrogenic and cartilage matrix anabolic effects of type I collagen in adipose tissue-derived stem cells [49] and the anabolic effects of type 1 collagen hydrolysate, prolyl-hydroxyproline [50,51], and collagen peptide fragments [52] on chondrocyte matrix production. These findings collectively support the potential of collagen supplementation as a therapeutic strategy for maintaining cartilage health and mitigating joint degeneration.

Chondroitin and glucosamine-based dietary supplements are widely marketed across the globe and have undergone evaluation in numerous clinical trials for human osteoarthritis, yielding variable outcomes. Evidence from both in vitro studies and anecdotal reports in animal and human models has suggested that oral administration of type I collagen may serve as a viable approach for promoting joint health [23]. Findings by Dar et al. [48] indicate that daily intake of type I collagen mitigates cartilage degradation while promoting proteoglycan synthesis by chondrocytes in the joints of injured mice, implying a chondrogenic and anabolic effect on cartilage matrix production. Moreover, studies have demonstrated the anabolic properties of type I collagen hydrolysate and prolyl-hydroxyproline on stem cells derived from adipose tissue [49], as well as the stimulatory effects of collagen peptides and specific peptide fragments [50–52] on the extracellular matrix production by chondrocytes. In this study, zebrafish that received a diet supplemented with type I collagen had an improvement in the cartilage biosynthesis-related gene expression.

Sox9 encodes a transcription factor of the *Sry*-related HMG box (*Sox*) family that binds to cis-regulatory DNA elements to control the transcription of downstream target genes and plays a pivotal role in regulating the transcription of downstream target genes, which is involved in chondrogenesis and the subsequent formation of cartilage-derived bones [53]. It serves as a powerful inducer of type II collagen, which is a hallmark of articular chondrocytes. In zebrafish, two co-orthologs of Sox9, *sox9a* and *sox9b*, have been identified. Among these, *sox9a* is essential for both the morphogenesis of chondrocyte condensations and the progression of cartilage differentiation. Studies by Lin et al. [54] demonstrated that *sox9a* mutant zebrafish larvae exhibited abnormal pectoral fins and lacked scapulocoracoid cartilage, while *sox9b* mutants developed normal cartilage similar to wild-type larvae. In this study, the effect of dietary PHC on the expression of *sox9a* was evaluated in aged zebrafish, as depicted in Figures 5 and 6. The relative expression levels of *sox9a* were recorded as 1.09 ± 0.38, 2.11 ± 0.31, 1.25 ± 0.36, and 2.06 ± 0.25 in the control, PHC, GC, and combined PHC+GC groups, respectively (Figure 5). Notably, the expression of *sox9a* was significantly elevated in the PHC and PHC+GC groups compared to the control. Western blot analysis further corroborated these findings, revealing a marked increase in Sox9 protein levels in zebrafish-fed diets enriched with PHC or the PHC+GC combination (Figure 6). These results suggest that dietary supplementation with PHC, derived from grouper, holds significant promise in enhancing chondrogenesis in aged zebrafish. Further research is warranted to explore the underlying mechanisms and broader applications of PHC in cartilage repair and regeneration.

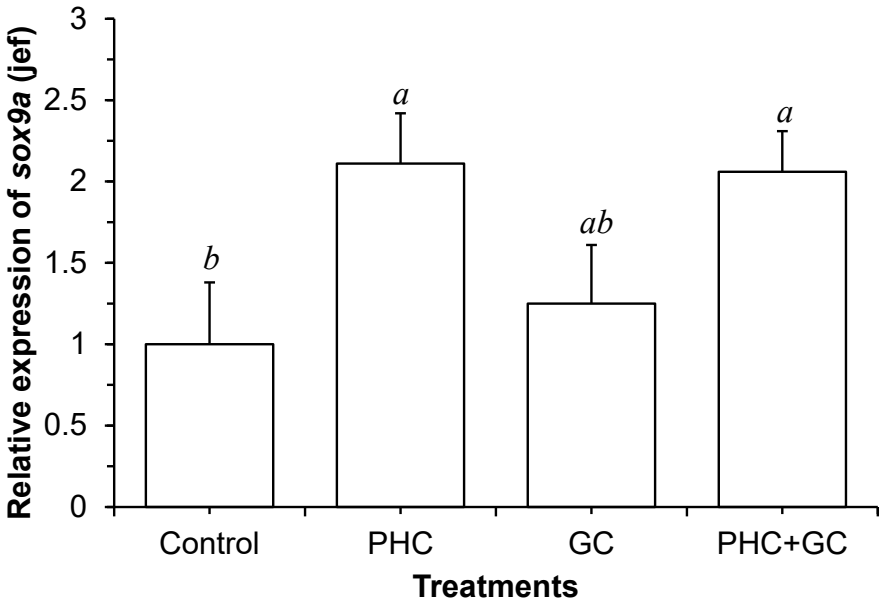

Figure 5. Relative expression levels of *sox9a* in the vertebrae of 1.5-year-old zebrafish subjected to different dietary treatments for 2 months. The experimental groups included a control diet, a diet supplemented with pepsin hydrolyzed collagen (PHC) at 0.187 g kg^{-1}, a diet enriched with glucosamine (GC) at 0.667 g kg^{-1}, and a combined diet containing both PHC (0.187 g kg^{-1}) and GC (0.667 g kg^{-1}). Results are presented as the mean ± standard deviation (S.D.). Data points annotated with entirely distinct letters represent significant differences among treatments ($p < 0.05$). Conversely, data points sharing identical letters or exhibiting overlapping annotations, such as "a" and "ab" or "ab" and "b", indicate no statistical differences among groups ($p > 0.05$). $n = 6$.

In addition to influencing *sox9a* expression, dietary supplementation with PHC and GC in zebrafish also modulated the expression of *runx2* (Figure 7). RUNX2 is a member of the Runx family that is widely regarded as a crucial modulator and master transcription factor in osteogenesis, as well as in the development of the prostate and skeleton. To date, RUNX2 has been implicated in a variety of physiological processes, such as the osteogenic differentiation of mesenchymal stem/stromal cells and the hypertrophy of chondrocytes [55]. The observed alterations in the expression of *runx2α* and *β* in aged fish fed with PHC and PHC plus GC suggest a role in the maintenance of osteoblast structure and function. No statistically significant differences were observed in the *acan* expression across the various treatments, including fish fed with PHC and/or GC diets. However, a slight elevation in the *acan* mRNA levels was found in these groups when compared to the control group (Figure 8). Bone morphogenetic proteins (BMPs) are pivotal in directing the differentiation of stem cells towards osteogenic or chondrogenic lineages. Specifically, Bmp-2 and Bmp-4 drive the upregulation of *sox9* during differentiation while concurrently suppressing *runx2* expression, thus promoting chondrogenesis. During the early stages of chondrogenic differentiation, stem cells undergo condensation and acquire a spindle-shaped morphology, followed by increased extracellular matrix synthesis during the later stages, leading to cell hypertrophy. Sox9 is primarily expressed in cartilage and condensed mesenchymal cells, where it plays a crucial role in upregulating *acan* gene, a major component of the cartilage extracellular matrix [56]. A related study by Kashiuchi et al. [21] evaluated the effects of dietary supplementation with glucosamine and collagen peptides derived from chicken cartilage on rheumatoid arthritis in a mouse model over a five-week period. Their study revealed that glucosamine supplementation led to decreased expression of *sox9*, *runx1*, and *runx2*, whereas collagen peptide supplementation resulted in an upregulation of *sox9* and a downregulation of *ruxn2*. These findings suggest

that dietary supplementation with collagen peptides or glucosamine can modulate gene expression linked to rheumatoid arthritis, with potential therapeutic implications for joint health and cartilage maintenance.

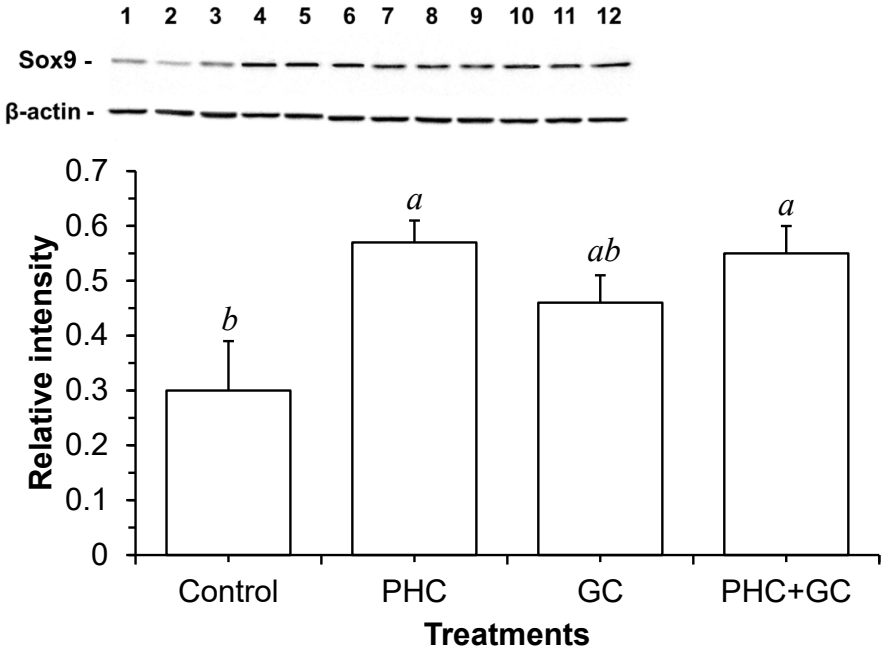

Figure 6. Western blot analysis of Sox9 in the vertebrae of 1.5-year-old zebrafish subjected to different dietary treatments for 2 months. The experimental groups included a control diet, a diet supplemented with pepsin hydrolyzed collagen (PHC) at 0.187 g kg^{-1}, a diet enriched with glucosamine (GC) at 0.667 g kg^{-1}, and a combined diet containing both PHC (0.187 g kg^{-1}) and GC (0.667 g kg^{-1}). Lanes 1–3: control; Lanes 4–6: PHC; Lanes 7–9: GC; Lanes 10–12: PHC+GC. Results are presented as the mean ± standard deviation (S.D.). Data points annotated with entirely distinct letters represent significant differences among treatments ($p < 0.05$). Conversely, data points sharing identical letters or exhibiting overlapping annotations, such as "a" and "ab" or "ab" and "b", indicate no statistical differences among groups ($p > 0.05$). $n = 3$.

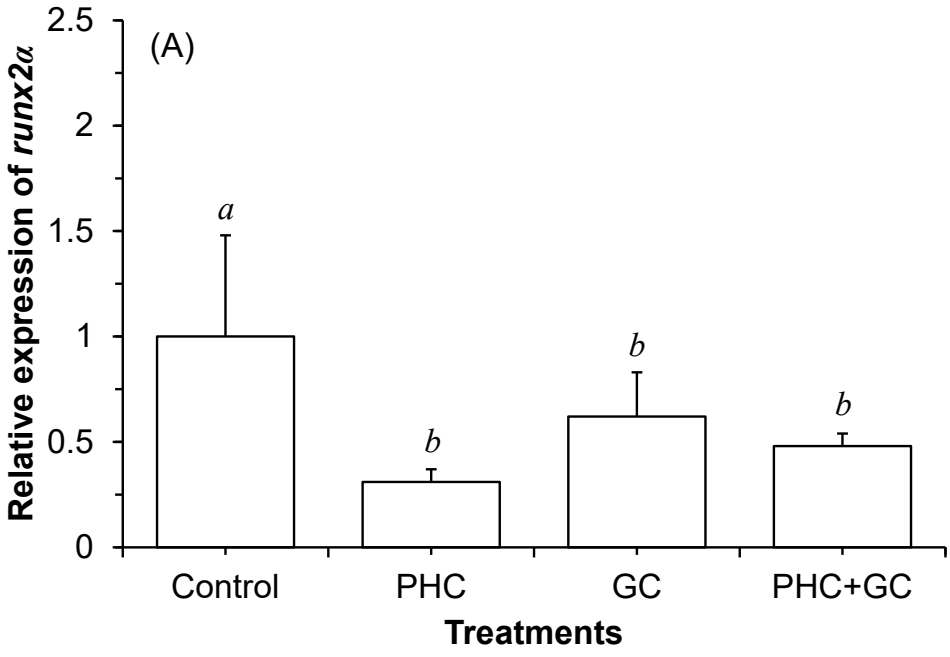

Figure 7. *Cont.*

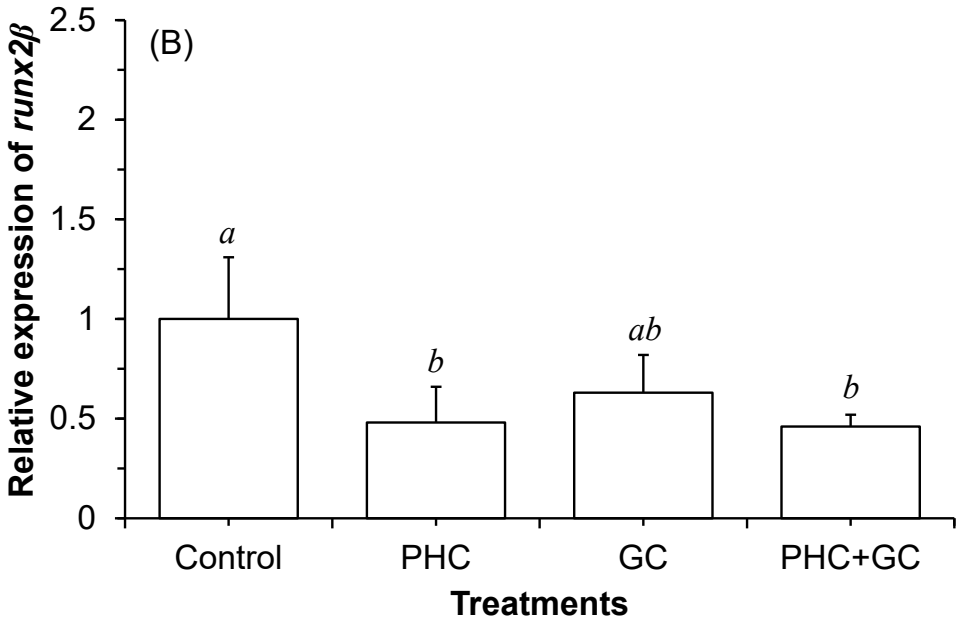

Figure 7. Relative expression levels of *runx2α* (**A**) and *runx2β* (**B**) in the vertebrae of 1.5-year-old zebrafish subjected to different dietary treatments for 2 months. The experimental groups included a control diet, a diet supplemented with pepsin hydrolyzed collagen (PHC) at 0.187 g kg^{-1}, a diet enriched with glucosamine (GC) at 0.667 g kg^{-1}, and a combined diet containing both PHC (0.187 g kg^{-1}) and GC (0.667 g kg^{-1}). Results are presented as the mean ± standard deviation (S.D.). Data points annotated with entirely distinct letters represent significant differences among treatments ($p < 0.05$). Conversely, data points sharing identical letters or exhibiting overlapping annotations, such as "a" and "ab" or "ab" and "b", indicate no statistical differences among groups ($p > 0.05$). $n = 6$.

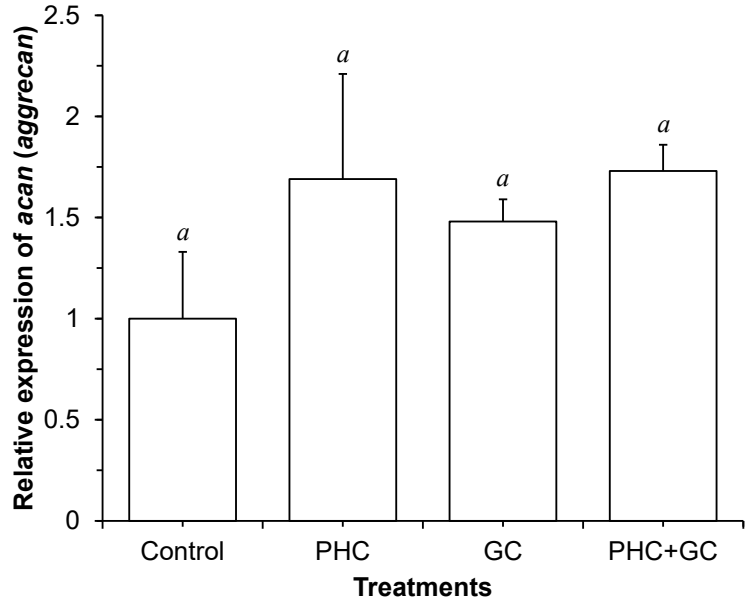

Figure 8. Relative expression levels of *acan* in the vertebrae of 1.5-year-old zebrafish subjected to different dietary treatments for 2 months. The experimental groups included a control diet, a diet supplemented with pepsin hydrolyzed collagen (PHC) at 0.187 g kg^{-1}, a diet enriched with glucosamine (GC) at 0.67 g kg^{-1}, and a combined diet containing both PHC (0.187 g kg^{-1}) and GC (0.67 g kg^{-1}). Results are presented as the mean ± standard deviation (S.D.). Data marked with the same letter "a" indicate that no significant differences were observed among the groups ($p > 0.05$). $n = 6$.

4. Conclusions

The findings of this study highlight the potential of pepsin-solubilized collagen (PHC) derived from grouper fins in supporting cartilage and joint health. Molecular characterization, including UV–Vis spectroscopy, SDS-PAGE, and amino acid profiling, confirmed that the extracted protein is type I collagen, a structurally significant collagen subtype that is widely distributed in fish. The observed increase in *sox9a* expression in zebrafish fed PHC or PHC+GC diets suggests a synergistic effect in promoting cartilage maintenance. Importantly, the upregulation of *sox9a*, coupled with the downregulation of *runx2*, provides robust evidence that PHC may actively facilitate cartilage formation by enhancing the expression of key chondrogenic markers. To comprehensively evaluate the protective effects of collagen derived from grouper fin on the joint health of aged zebrafish, it is essential to conduct detailed histological analyses to assess cartilage integrity and structure. Additionally, further investigation of key molecular markers associated with cartilage maintenance and chondrogenesis is warranted to substantiate these findings.

Author Contributions: S.-Y.H.: conceptualization, methodology, data curation, visualization, review, and editing; C.-H.T.: methodology, investigation, data curation, visualization; C.-C.T.: methodology, validation, resources; T.-W.H.: conceptualization; C.-H.L.: conceptualization, methodology, validation, resources, data curation, supervision, project administration, funding acquisition, writing—original draft, writing—review and editing. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The animal study protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the National Pingtung University of Science and Technology. The IACUC Approval No. is NPUST-110-138. The approval date is 28 December 2021.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data are contained within the article.

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

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