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Research Article

The effect of using hydrolyzed protein prepared from the viscera of rainbow trout in the fish diet on its shelf life at ambient temperature

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Abstract

The aim of this study was to investigate the effect of using hydrolyzed protein (HP) prepared from rainbow trout viscera in the fish diet on its shelf life in terms of chemical spoilage, bacterial load, and chemical composition at ambient temperature. HP was prepared from rainbow trout viscera using Alcalase (1.5% v/w, 55°C, pH 8.5). Five experimental diets were prepared to contain different levels of HP (0, 5, 10, and 20 g of HP/kg) and one diet containing 200 mg/kg of butyl hydroxytoluene (BHT). The prepared treatments were kept at 25±3°C for 18 weeks. The lowest mean TBA index was observed in the feeds with 20 g/kg of HP and Butylated hydroxytoluene (BHT) treatment (p<0.05). The lowest Total volatile basic nitrogen (TVB-N) index was measured in HP-containing treatments until the 14th week (p<0.05). The lowest bacterial count was measured in the HP-containing treatments from the 4th week to the end of the experiment (p<0.05). The highest total protein content belonged to the HP-containing treatments. The highest fat content was recorded in HP-containing and BHT treatments (p<0.05). According to the results, the addition of HP (20 g/kg) is recommended to maintain the quality of fish feed.

Keywords: Chemical composition, Fish feed, Hydrolyzed protein, Total volatile basic nitrogen, Shelf life, Spoilage

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Introduction

Chemical and enzymatic reactions occur naturally in the diet production process. Hence, the oxidation of unsaturated fatty acids, high moisture content, and other factors reduce the diet acceptability by the fish and its shelf life, and the nutritional value also changes during storage (Hossen *et al*., 2011). Fats are used to provide energy in the diet are prone to oxidative damage (Hamre *et al*., 2010). Extrusion, which is common in many food factories due to important benefits such as gelatinization of starch, inactivation of enzymes, and elimination of anti-nutritional agents (Cheng and Hardy, 2003), causes lipid oxidation during processing and subsequent storage (Lin *et al*., 1998). The use of oxidized fish oil in fish diets impairs the response of fish to stress and affects fish immunity (Obach and Laurencin, 1992), resulting in skeletal deformity because of damage to the osteoblast membrane and osteoclasts (Lall and Lewis-McCrea, 2007). These adverse effects have been prevented using synthetic antioxidants, including butyl hydroxytoluene and butyl hydroxyanisole in fish oil and ethoxyquinone in fishmeal (Hamre *et al*., 2010), but these are not desirable due to the adverse effects on consumer health caused by their residues in the fillet (Lundebyea *et al*., 2010). Consequently, natural antioxidants are gaining ground, including tocopherols, and herbal extracts (Hamre *et al*., 2010; Hernández *et al*., 2014).

The growing rate of aquatic animal production will also produce large volumes of low value by-products, including scales, skin, viscera, bones, and spine (Benjakul and Morrissey, 1997). Enzymatic hydrolysis is an ideal technology for converting low-value by-products of fish processing into functional bioactive products.

Hydrolysis is the chemical or enzymatic breakdown of proteins into peptides of different molecular weights (He *et al*., 2013). HPs perform biological activities, including antioxidant activity (Ktari *et al*., 2012), cholesterol-lowering ability (Khaled *et al*., 2012), and anticoagulant activity (Nasri *et al*., 2012). HPs have also been shown to have antibacterial effects (Zmysłowska and Lewandowska, 1999). These properties, together with the easier digestibility of HPs (Bui *et al*., 2014), have been the incentives to investigate the effect of using HPs in fish diets as a protein source substitute, or as a growth stimulant additive as well as the antioxidant and immunity properties in some studies, including the study of HPs from shrimp (*Litopenaeus vannamei*), krill (*Euphausia superba*), and tilapia (unknown genus and species) in the diet of *Pagrus major* (Bui *et al*., 2014), HP of *Sardina pilchardus*, in the diet of *Dicentrarchus labrax* (Kotzamanis *et al*., 2007), HP of rainbow trout (*Oncorhynchus mykiss*) in the diet of rainbow trout (Javaherdoust *et al*., 2020).

Considering the above and the no use of HP as an antioxidant in the fish diet, the aim of this study was to utilize fish waste to produce a natural antioxidant for use in the fish diet and to evaluate its

effect on chemical and microbial changes and the consequent shelf life of the diet at ambient temperature.

Materials and methods

This experiment was performed in the aquaculture propagation laboratory at Sari Agricultural Sciences and Natural Resources University (SANRU).

Preparation of hydrolyzed protein (HP)

The viscera of rainbow trout as the raw material were provided from the Sari fish market and transported to the laboratory in an ice box. HP was prepared according to Safari *et al*. (2012) with Alcalase (Novozyme, Denmark) concentration of 1.5% (v/w), at 55°C, and a pH of 8.5 for 24 h. The properties of HP were reported from a previous study (Javaherdoust *et al*., 2020).

Diet and treatment preparation

Experimental diets containing 0, 5, 10, and 20 g of HP/kg of diet (Leal *et al*., 2010), and a diet with synthetic antioxidant, butylhydroxytoluene (BHT), at 200 mg/kg diet (Hernández *et al*., 2014) formulation based on the requirements (45% protein, 22% fat) of carnivorous fish (Table 1). Due to the similarity of protein percentage in HP and fishmeal, the HP replaced fish meal in the diet. The resulting mixture was then cut into strands of 3 mm diameter using a meat grinder and air-dried evenly, crushed after drying, numbered, packaged, labeled in different plastic (polyethylene) bags and stored at room temperature (25±3°C). At different time points with intervals of once every 2 or 4 weeks, depending the parameter, including weeks 0, 2, 4, 6, 8, 10, 12, 14, 16, and 18, the package for a represented time was opened and used to perform the tests.

Measurement of diet and HP composition

Total protein, fat, moisture, and ash contents were measured using a Kjeldahl apparatus, a Soxhlet apparatus, an oven at 105°C, and an electric oven at 550°C, respectively (AOAC, 2000). The total caloric value (kcal) was calculated theoretically using Atwater factors (Atwater and Woods, 1896) for lipid (9 kcal/g), protein (4 kcal/g) and carbohydrate (4 kcal/g).

Measurement of thiobarbituric acid (TBA) index

The TBA level was measured based on the colorimetric method by a spectrophotometer (HACH model, DR/2000 USA) and expressed as mg of malondialdehyde per kg of feed (Natseba *et al*., 2005). 200 mg of sample was made into 25 mL volume with 1-butanol, 5 mL of the mixture was poured into capped dry tubes, followed by adding 5 mL of standard TBA reagent and water bath at 95°C for 2 h and then cooled to ambient temperature. The absorbance was read at 532 nm against the blank (1-butanol) and the standard solutions.

Table 1: Composition of diets used in different treatments.

Ingredients (%)	Treatments				
	HP 0 (Control)	HP 5 g/kg	HP 10 g/kg	HP 20 g/kg	BHT 200 mg/kg
Fish meal	40	39.5	39	38	40
Soybean meal	18	18	18	18	18
Wheat gluten	10	10	10	10	10
Wheat flour	10	10	10	10	10
Corn powder	4	4	4	4	4
Canola oil	15	15	15	15	15
Vitamin premix[1]	1.5	1.5	1.5	1.5	1.5
Mineral premix[2]	1.5	1.5	1.5	1.5	1.5
HP	0	0.5	1	2	0
BHT	0	0	0	0	200
Proximate composition (% dry basis)					**HP**
Crude Protein	46.49±0.05	46.65±0.24	46.84±0.15	46.96±0.24	46.52±0.13	79.23
Crude Lipid	21.66±0.52	21.06±0.58	22.29±0.54	22.10±0.37	21.60±0.67	2.1
Ash	7.81±0.04	6.62±0.26	7.20±0.23	7.47±0.15	7.31±0.14	3.38
Moisture	4.38±0.05	4.94±0.48	5.09±0.30	4.63±0.25	4.42±0.05	
Gross energy (Kcal/g)	4.77	4.79	4.82	4.81	4.79	

[1] Vitamin supplement (per kg of premix): Vitamin A 10,000 IU; Vitamin D3 2000 IU; Vitamin E 100 mg; Vitamin K 20 mg; Vitamin B1 400 mg; Vitamin B2 40 mg; Vitamin B6 20 mg; Vitamin B12 0.04 mg; Biotin 0.2 mg; Choline chloride 1200 mg, Folic acid 10mg; Inositol 200 mg; Niacin 200 mg; Pantothenic calcium 100 mg.

[2] Mineral supplement (mg/kg premix): MgSO4.2H2O 127.5; KCl 50.0; NaCl 60; CaHPO4.2H2O 727.8; FeSO4.7H2O; 25.5; ZnSO4.7H2O, 5.5; CuSO4.5H2O, 0.785; MnSO4.4H2O 2.54; CoSO4.4H2O, 0.478; Ca(IO3)2.6H2O 0.295; CrCl3.6H2O 0.128.

Measurement of total volatile basic nitrogen (TVB-N)

The homogenized feed sample (3 g), 2 g of magnesium oxide powder, 300 mL of distilled water, and one glass pearl were mixed in a special Kjeldahl balloon. In the recipient's vessel, 5-10 cm^3 of 2% boric acid was poured with a few drops of reagent. The distillate was titrated with 0.1 N sulfuric acid solution until forming a red color. TVB-N (mg/100 g of feed) was calculated using the following equation (Goulas and Kontominas, 2005):

TVB-N = Sample weight / (amount of acid consumed × normality of acid × 14)

Total viable bacterial count (TVC)

For TVC of the prepared samples, nutrient agar medium (Merck, Germany) was used according to the AOAC method (2000). First, 1.0 g of the sample was made to a volume of 10 mL with dilution water. After preparing the culture medium, 0.1 mL of the prepared samples were spread on the culture medium surface by a micro-sampler.

The cultured plates for total bacteria were counted after incubation at 25°C for 24 h under stereo microscope.

Statistical analysis

This experiment was performed in a completely randomized design with five treatments each with three replications. Data were statistically analyzed using SPSS 18 software. After verifying the normality of data with the Shapiro-Wilk Test, the data were normalized if necessary. Different data were analyzed using a two-way analysis of variance (ANOVA) based on treatment and time variables. The means in each group were compared by Duncan's test at 95% probability level.

Results

HP levels and time had significant effects (p<0.05) on TBA (Table 2), but there was no interaction between the two parameters (p>0.05). Comparison of mean TBA levels at different times points showed an increase in this parameter from the beginning of the experiment (time zero) to the 2nd week (p<0.05) except in 20 g/kg and BHT treatments, which gradually decreased and reached the lowest level in the 10th week and then increased significantly (p<0.05) until the end of the experiment, except in treatment 20 g/kg. In weeks 4, 6, 8, 12, 14, 16 and 18, no significant difference was observed between the treatments (p>0.05). However, the highest amount of TBA in the second week of the study was observed in treatments 0, which was not significantly different from treatments 5 and 10 (p>0.05), but was significantly more than treatments 20 and BHT (p<0.05). Also, in the 10th week, the amount of TBA in treatment 20 was significantly lower than all treatments (p<0.05).

Table 2: Changes in the TBA index (mg MDA/kg) in the diet produced with different levels of hydrolyzed protein during 18 weeks of storage at 25±3°C.

Time (week)	Treatments				
	HP 0 (Control)	HP 5 g/kg	HP 10 g/kg	HP 20 g/kg	BHT 200 mg/kg
0	3.07 ± 0.10^{ABa}	2.82 ± 0.08^{ABCa}	2.93 ± 0.26^{ABCa}	3.09 ± 0.07^{Aa}	2.97 ± 0.07^{ABa}
2	5.08 ± 0.29^{Dc}	4.54 ± 0.31^{Dbc}	4.25 ± 0.51^{Ebc}	2.40 ± 0.86^{Aa}	2.96 ± 0.27^{ABab}
4	3.23 ± 0.43^{ABCa}	3.03 ± 0.23^{ABCa}	3.30 ± 0.20^{BCDa}	2.54 ± 0.38^{Aa}	2.91 ± 0.32^{ABa}
6	3.97 ± 0.33^{BCa}	3.04 ± 0.26^{ABCa}	3.09 ± 0.08^{ABCa}	2.59 ± 0.41^{Aa}	2.89 ± 0.21^{ABa}
8	3.20 ± 0.50^{ABCa}	2.54 ± 0.26^{ABa}	2.24 ± 0.45^{Aa}	2.12 ± 0.46^{Aa}	2.29 ± 0.43^{Aa}
10	2.99 ± 0.21^{Ab}	2.45 ± 0.22^{Ab}	2.38 ± 0.25^{ABb}	1.74 ± 0.16^{Aa}	2.68 ± 0.04^{ABb}
12	3.21 ± 0.22^{ABCa}	3.32 ± 0.10^{Ca}	2.81 ± 0.23^{ABCa}	2.54 ± 0.34^{Aa}	3.08 ± 0.13^{Ba}
14	2.77 ± 0.19^{Aa}	2.43 ± 0.11^{Aa}	2.41 ± 0.23^{ABa}	2.90 ± 0.31^{Aa}	2.62 ± 0.13^{ABa}
16	3.48 ± 0.49^{ABCa}	3.24 ± 0.35^{BCa}	3.41 ± 0.35^{CDa}	2.56 ± 0.31^{ABa}	2.79 ± 0.16^{Aa}
18	4.13 ± 0.13^{Ca}	4.16 ± 0.07^{Da}	3.70 ± 0.20^{CDa}	3.57 ± 0.18^{ABa}	4.02 ± 0.27^{Ca}

Factor	*p*-Value
Treatment	0.000
Time	0.000
Treatment × Time	0.051

Note: Different lower-case letters indicate a significant difference in each row and different capital letters indicate a significant difference in each column (p<0.05).

HP levels, time, and their interaction had a significant effect (p<0.05) on TVB-N (Table 3). From the beginning of the experiment until the 2nd week, there was no significant difference in levels of TVB-N in the treatments. However, this parameter increased in the experimental treatments over time and the control treatment contained a significantly higher TVB-N (p<0.05) in the 6th week. In the HP-containing treatments, the TVB-N did not increase in the 10 and 20 g treatments until the 6th week of the experiment. Besides, TVB-N was significantly lower than the control treatment (p<0.05) until the 14th week of the experiment. From the 14thto the 18thweeks of the experiment, TVB-N levels increased in HP-containing treatments, with a significant increase in 10 g/kg treatment (p<0.05).

Table 3: Changes in the TVB-N levels (mg per 100 g of feed) in the diet produced with different levels of hydrolyzed protein during 18 weeks of storage at 25±3°C.

Time (week)	Treatments				
	HP 0 (Control)	HP 5 g/kg	HP 10 g/kg	HP 20 g/kg	BHT 200 mg/kg
0	40.13 ± 2.33^{Aa}	43.40 ± 0.81^{Aa}	46.20 ± 0.81^{Aa}	45.51 ± 3.59^{ABa}	39.89 ± 5.25^{Aa}
2	49.00 ± 2.42^{Aa}	39.27 ± 5.62^{Aa}	48.53 ± 0.47^{Aa}	45.03 ± 5.46^{ABa}	44.10 ± 2.02^{Aa}
6	66.53 ± 0.40^{Bc}	49.70 ± 1.21^{ABb}	44.10 ± 1.21^{Aa}	41.31 ± 2.02^{Aa}	58.11 ± 1.21^{Bb}
10	66.52 ± 2.02^{Bc}	53.43 ± 5.46^{ABabc}	51.33 ± 6.17^{Aab}	46.20 ± 0.81^{ABa}	63.93 ± 3.27^{BCbc}
14	74.88 ± 5.25^{Bc}	53.90 ± 5.11^{ABab}	46.90 ± 1.21^{Aa}	54.83 ± 2.37^{BCab}	63.03 ± 5.66^{BCbc}
18	74.20 ± 5.30^{Ba}	63.23 ± 7.02^{Ba}	60.43 ± 1.30^{Ba}	59.03 ± 3.24^{Ca}	72.33 ± 2.83^{Ca}

Factor	*p*-value
Treatment	0.000
Time	0.000
Treatment × Time	0.002

Note: Different lower-case letters indicate a significant difference in each row and different capital letters indicate a significant difference in each column (p<0.05).

The HP levels, time, and their interaction significantly affected (p<0.05) the TVC (Table 4). The TVC of the HP-containing treatments decreased during the experiment and reached the lowest level in the 20 g/kg treatment at the end of the experiment (p<0.05). The bacterial load at the beginning of the experiment was significantly higher in the control and 5 treatments than in the other treatments (p<0.05). From the second week of experiment, the bacterial load in the treatments containing FPH and BHT was lower than the control treatment. In weeks 4, 6, 10, and 14, a significant difference was found between the treatments containing FPH, so that in the 4th and 14th weeks, the lowest bacterial load was observed in treatment 20 (p<0.05), but In the 6th week, the lowest bacterial load was observed in treatment 10 (p<0.05). Also, in the 10th week, the bacterial load in treatment 20 was significantly lower than treatment 5 (p<0.05), but it was not significantly different from treatment 10 (p>0.05).

HP levels and time had significant effects (p<0.05) on the total protein and the fat contents (Tables 5 and 6).

Table 4: Changes in the TVC (Log CFU/g) in feed produced with different levels of hydrolyzed protein during 18 weeks of storage at 25±3°C.

Time (week)	Treatments				
	HP 0 (Control)	HP 5 g/kg	HP 10 g/kg	HP 20 g/kg	BHT 200 mg/kg
0	5.69±0.28^{Ad}	5.73±0.03^{Ed}	5.43±.05^{Hc}	4.97±0.04^{Ea}	5.32±0.04^{Ab}
2	5.76±0.38^{Ab}	5.11±0.02^{Da}	5.24±0.12^{FGHa}	4.85±0.03^{DEa}	5.19±0.29^{Aa}
4	7.16±0.09^{Be}	4.38±0.12^{Bb}	5.10±0.06^{FGc}	4.02±0.07^{BCa}	6.59±0.03^{BCd}
6	7.30±0.15^{Be}	4.90±0.06^{CDc}	4.02±0.07^{CDa}	4.42±0.09^{CDb}	6.64±0.03^{Dd}
8	7.61±0.09^{Bc}	4.16±0.12^{ABa}	4.12±0.07^{Da}	3.86±0.38^{Ba}	6.35±0.10^{BCb}
10	8.64±0.05^{Cd}	4.89±0.30^{CDb}	4.39±0.08^{Eab}	4.03±0.05^{BCa}	6.47±0.34^{BCc}
12	8.92±0.02^{Cd}	4.06±0.23^{Aba}	3.77±0.23^{Ca}	3.73±0.07^{Ba}	6.60±0.57^{BCb}
14	7.57±0.03^{Be}	4.51±0.09^{BCb}	4.97±0.03^{Fc}	2.99±0.21^{Aa}	5.73±0.09^{ABd}
16	6.05±0.64^{Ab}	3.74±0.13^{Aa}	3.00±0.05^{Aa}	3.17±0.10^{Aa}	6.77±0.20^{Db}
18	8.54±0.38^{Cc}	3.76±0.09^{Aa}	3.34±0.06^{Ba}	3.07±0.17^{Aa}	7.03±0.35^{Db}

Factor	*p*-value
Treatment	0.000
Time	0.000
Treatment × Time	0.000

Note: Different lower-case letters indicate a significant difference in each row and different capital letters indicate a significant difference in each column (p<0.05).

Table 5: Changes in the protein content (based on dry weight) of the diet produced with different levels of hydrolyzed protein during 18 weeks of storage at 25±3°C.

Time (week)	Treatments				
	HP 0 (Control)	HP 5 g/kg	HP 10 g/kg	HP 20 g/kg	BHT 200 mg/kg
0	46.49±0.05^{Ca}	46.65±0.24^{Da}	46.84±0.15^{CDa}	46.98±0.24^{BCa}	46.52±0.13^{Ba}
2	46.65±0.12^{Ca}	46.80±0.24^{Da}	47.23±0.33^{Da}	47.51±0.15^{Ca}	46.23±0.40^{Ba}
6	47.07±0.22^{Ca}	46.15±0.27^{CDa}	46.36±0.24^{BCa}	46.37±0.26^{ABa}	45.55±1.03^{ABa}
10	44.69±0.26^{Ba}	46.24±0.12^{CDbc}	46.23±0.24^{BCbc}	46.69±0.15^{BCc}	45.74±0.16^{Bb}
14	43.53±0.34^{Aa}	45.64±0.25^{ABb}	45.80±0.24^{Bbc}	46.52±0.04^{BCc}	45.29±0.23^{ABb}
18	42.89±0.34^{Aa}	44.91±0.32^{Abc}	44.61±0.09^{Abc}	45.51±0.61^{Ac}	43.88±0.45^{Aab}

Factor	*p*-value
Treatment	0.000
Time	0.000
Treatment × Time	0.001

Note: Different lower-case letters indicate a significant difference in each row and different capital letters indicate a significant difference in each column (p<0.05).

There was an interaction between HP levels and time on protein content (p<0.05), but not on fat content (p>0.05). There were no significant differences in protein contents of the treatments in weeks 0, 2, and 6 (p>0.05), but the highest protein content was observed in HP-containing treatments from the 10th week (p<0.05). The fat content showed decreases from the beginning (time zero) to the end of the experiment. At the end of experiment, the lowest and the highest fat content observed in the control and BHT treatments, respectively (p<0.05). The highest fat content was measured in weeks 0 and 2, which was significantly higher than those in the 10th week onwards (p<0.05). At the beginning of the experiment and in weeks 4 and 6, there was no significant difference between treatments (p>0.05). In the second week, the highest percentage of fat was observed in treatment 5 (p<0.05). From the 8th week, the amount of fat in

the treatments containing FPH was significantly higher than the control treatment (p<0.05). In the 12th and 18th week, the highest amount of fat was observed in the treatment of 20 and BHT (p<0.05).

In the 14th week, there was no difference between the fat content of treatments containing FPH and BHT (p>0.05). In the 16th week, the amount of fat in the treatment containing BHT was higher than the treatment containing FPH (p<0.05).

Table 6: Changes in the fat content (based on dry weight) of the diet produced with different levels of hydrolyzed protein during 18 weeks of storage at 25±3°C.

Time (week)	Treatments				
	HP 0 (Control)	HP 5 g/kg	HP 10 g/kg	HP 20 g/kg	BHT 200 mg/kg
0	21.60 ± 0.67^{Da}	22.10 ± 0.37^{Ea}	22.29 ± 0.54^{Ea}	21.06 ± 0.58^{CDa}	21.66±0.52^{Ca}
2	20.94 ± 0.21^{Da}	22.10 ± 0.37^{Eb}	21.38 ± 0.21^{DEab}	20.91 ± 0.10^{CDa}	21.95 ± 0.50^{BCab}
4	19.57 ± 0.35^{CDa}	20.01 ± 0.17^{Da}	20.61 ± 0.18^{CDa}	20.42 ± 0.64^{Ca}	20.62 ± 0.94^{ABCa}
6	18.13 ± 1.59^{CDa}	21.93 ± 0.33^{Ea}	21.64 ± 0.04^{DEa}	22.34 ± 0.65^{Da}	20.60 ± 1.60^{ABCa}
8	16.23 ± 0.62^{BCDa}	20.34 ± 0.24^{Db}	21.29 ± 0.36^{CDb}	21.40 ± 0.67^{CDb}	20.49 ± 0.55^{ABCb}
10	15.28 ± 0.55^{ABCa}	21.05 ± 0.12^{DEb}	19.20± 0.90^{Cb}	20.20 ± 0.76^{Cb}	20.74 ± 0.20^{ABCb}
12	13.00 ± 0.49^{ABa}	16.86 ± 0.48^{Cbc}	15.98 ± 0.29^{ABb}	17.37 ± 0.64^{ABbc}	17.81 ± 0.40^{ABCc}
14	14.35 ± 0.39^{ABCa}	16.03 ± 1.13^{BCab}	17.45 ± 0.75^{Bb}	18.09 ± 0.22^{Bb}	16.91 ± 0.90^{Ab}
16	12.60 ± 0.31^{ABa}	15.17 ± 0.38^{ABb}	15.28 ± 0.31^{Ab}	16.15 ± 0.60^{Ab}	17.62 ± 0.34^{ABc}
18	11.98 ± 0.53^{Aa}	14.08 ± 0.36^{Ab}	15.63 ± 0.68^{Abc}	15.83 ± 0.34^{Acd}	17.41 ± 0.64^{ABd}

Factor	*p*-value
Treatment	0.000
Time	0.000
Treatment × Time	0.065

Note: Different lower-case letters indicate a significant difference in each row and different capital letters indicate a significant difference in each column (p<0.05).

Discussion

TBA measures malondialdehyde (MDA) levels produced from hydroperoxides from the initial stage of oxidation (Sidwell *et al*., 1954). A high TBA index in the control treatment indicates the occurrence of more oxidation and the formation of more secondary metabolites. In the HP-containing treatments, however, the decreased lipid oxidation is probably due to HP antioxidant properties. The antioxidant properties of fish HP have been shown in previous studies (Chi *et al*., 2015; Lassoued *et al*., 2015). HPs are rich in hydrophobic amino acids, such as alanine, phenylalanine, isoleucine, leucine, valine, glycine, proline, methionine, tyrosine, histidine, lysine, and cysteine, have been shown to improve the antioxidant function of peptides. These amino acids act as a proton or electron donors, or as free radical scavengers (Wiriyaphan *et al*., 2012).

The allowable TBA limit for natural odor and taste has been determined at about 1-2 mg per kg of fish meat (Remya *et al*., 2017), but no standard has been defined for allowable TBA levels in aquatic animals feed. In the present study, the TBA levels measured in the treatments (1.74-5.08 mg MDA/kg) are much lower than that of 1.4-63 mg

MDA/kg feed reported by Ketola *et al*. (1989). In addition to the diet composition and the type of included oil, the observed difference can be attributed to the presence of antioxidants in the vitamin supplement (e.g., vitamins C and E) used in the present study, or the use of synthetic antioxidants in basic feed items available in factories, including fish meal, meat, and oil as well as vegetable oils, for quality maintenance.

Hernández *et al*. (2014) found a decreasing trend in the TBA index of the feed at ambient temperature until week 12, but it rose between weeks 12 and 24, due to the TBA instability. This instability is because MDAs react with various biological compounds, such as amino acids, nucleotides, nucleic acids, proteins, phospholipids, and other aldehydes that are the end products of oxidation (Aubourg *et al*., 2004). On the other hand, the increased TBA levels at the 18th week can also be due to the reduction or degradation of antioxidant compounds or declined antioxidant properties of HP, which eventually led to lipid oxidation (Hernández *et al*., 2014). The fluctuation of TBA was observed in the study of Pezeshk *et al*. (2017) when they used HP derived from Tuna waste as an antioxidant during the minced Silver carp (*Hypophthalmichthys molitrix*) refrigeration (12 days), more over the lowest TBA was observed in HP treatments. Although BHT is a potent antioxidant, high antioxidant properties have also been identified for FPH. This antioxidant property of FPH was comparable (Thiansilakul *et al*., 2007; Samaranayaka and Li-Chan, 2008; Sheriff *et al*., 2014) or even higher (Yang *et al*., 2011) than BHT.

TVB-N mostly consists of trimethylamine (TMA) and ammonia. This index is mostly used to control the quality of the fishmeal and raw materials for fishmeal production. Increased TVB-N during storage has been shown in various studies (Arancibia *et al*., 2014; Gómez-Estaca *et al*., 2010). Hossen *et al*. (2011) claimed that the total nitrogen content of two commercial feeds decreased after 60 days of storage at room temperature. They concluded that the nitrogen was lost in the form of ammonia or other volatile compounds. Similarly Misir and Koral (2019) observed Lower TVB-N production in bonito (*Sarda sarda*) fillets stored at 4±1°C coated by trout FPH. According to Ghanbarinia *et al.* (2022), the lowest TVB-N in hamburgers at the end of the storage time (day 16) was observed in the treatment 3% soy hydrolyzed protein, and the highest values were observed in the control treatment. They concluded that the lower amount of TVB-N is due to the lower activity of bacteria in these treatments or the reduced oxidative ability of bacteria to separate amines from non-volatile nitrogenous compounds or both, which is the result of the effect of hydrolyzed protein on bacteria.

The increase in TVB-N at room temperature could be due to microbial activity; therefore the low TVB-N in HP-containing treatments might have probably been caused by the HP antibacterial activity, as discussed

below. Due to the lack of a significant increase in bacterial load in these treatments after the 14th week, it seems that the prior increase resulted from chemical spoilage or degradation of peptides related to HPs, which are more prone to degradation due to their small size.

A permissible level of TVB-N in aquatic animal feed is not mentioned in the available literature and the studies have mostly focused on the permissible level of TVB-N in fish meal used in aquatic animal feed. The acceptable TVB-N levels are 25-35 mg/100 g in fresh fish for human consumption (Jinadasa, 2014) and less than 150 mg/100 g in the fish meal (Tacon *et al*., 2009). Compared to studies on fish fillets, TVB-N levels were low in the present study (39.89-74.20 mg/100 g of feed) (Ojagh *et al*., 2011). This may be due to the different mechanisms of protein breakdown in the feed compared to fish fillets that contain more moisture. By removing the carboxyl group from amino acids, bacteria produce biogenic amines, including histamine, cadaverine, and putrescine, which are known compounds without volatility, as in trimethylamine oxide. Hence, this portion of protein spoilage is not measured in the TVB-N index. If bacteria are given more time, they will eventually produce carbon dioxide and ammonia (Jinadasa, 2014).

The effect of HP was evident from the beginning of the experiment and the lowest TVB was observed in the HP treatment with 20 g/kg of feed at the beginning of the experiment and afterward decreased in the HP treatments during the study. The upper limit of bacterial load in freshwater and marine fish fillets is 7 log CFU/g (Pezeshk *et al*., 2011), but a common standard is not available for feed bacterial load. Zmysłowska and Lewandowska (1999) reported the permissible bacterial load of about 5.5 log CFU/g in aquatic animal feed. In the present study, the bacterial load was less than 6 log CFU/g at the beginning of the experiment.

Almost all antimicrobial peptides isolated from fish have shown antibacterial activity against a large number of Gram-negative and Gram-positive bacteria (Zamora-Sillero *et al*., 2018). Our results are in line with previous studies showing the antibacterial properties of HP. Mackerel (*Scomber scombrus*) HP showed antibacterial activity against Gram-positive (*Listeria immocua*) and Gram-negative (*Escherichia coli*) bacteria (Ennaas *et al.*, 2015). The HP of tilapia (*Oreochromis niloticus*) waste also showed antibacterial activity against *Edwardsiella tarda* and *Bacillus megaterium* (Robert *et al*., 2015). The Tuna waste HP showed the antibacterial effect during the refrigeration of minced Sliver carp (Pezeshk *et al*., 2017). In the study of Yeganeh and Reyhani Poul (2022) was also reported the antibacterial effect for bioactive peptides (weight less than 3 kDa) derived from shrimp waste hydrolysis and peptide nanoencapsulated with a combined coating of nanoliposome and chitosan against *E. coli*, *Bacillus cereus* and

Staphylococcus aureus. The exact mechanism for the antibacterial activity of peptides is not yet well understood. The presence of hydrophobic amino acids, such as alanine, valine, isoleucine, leucine, tyrosine, phenylalanine, proline, and methionine, in the peptide, increases the antibacterial property (He *et al*., 2013). In the present study, Alcalase was used for the hydrolysis of rainbow trout viscera. Previous studies have shown better retention of hydrophobic amino acids by the use of Alcalase (Intarasirisawat *et al*., 2013). Some researchers believe that the interaction of peptides with bacterial membranes through hydrophobic bonds can create holes in the membrane. In fact, the hydrophobic portions of HPs are used for their entry into the cell membranes of microorganisms, thereby disrupting cellular osmotic regulation leading to the destruction of cellular components (Hancock and Scott, 2000; He *et al*., 2013; Zamora-Sillero *et al*., 2018). Almost all studies that have studied hydrolyzed protein as an antibiotic have been in meat tissue or high-moisture materials that are prone to severe bacterial contamination in the short term. However, in a few of these studies, the use of protein hydrolyzed reduced the number of bacteria during the experiment period or until the end of the experiment to a lesser extent than at the beginning of the experiment (Vallejo-Cordoba *et al*., 1987; Nafei *et al*., 2018; Verma *et al*., 2018). Therefore, considering that the present study was performed with fish feed, which normally does not favor the growth conditions of bacteria due to low humidity, increasing the antibiotic effect of hydrolyzed protein and reducing the number of bacteria during the experiment is not illogical.

According to the literature, different bacterial species have shown different behaviors towards the antibacterial properties of HPs. Peptides with a molecular weight of less than 10 kDa have more antimicrobial properties (Beaulieu *et al*., 2013). In the present study, the mean total weight of peptides was 1.09×10^3 g/mol (Dalton) (Javaherdoust *et al*., 2019). Feed microorganisms can have different survival rates depending on the feed chemical composition and storage conditions. According to existing standards, the proteolytic, ammonifying, and saprophytic bacterial communities, as well as toxin-producing fungi must be controlled in the dry feed (Zmysłowska and Lewandowska, 1999).

In present study, the protein and fat contents of the feed decreased in all treatments over time. Our observed trend in feed chemical composition is similar to that of previous studies. Similarly, Nyong (2014) observed fat and protein contents decreased after 6-weeks storage of commercial feeds (Coppens and Vital). Likewise, Hossen *et al*. (2011) noticed that protein and fat contents decreased after a 2-month fish feed storage at 25-30°C. Mitchell and Beadles (1949) reported a decrease in the quality and nutritional value of wheat, corn, and soybeans during storage. In the study of Ghanbarinia *et al*. (2022), in hamburgers containing 3%

of hydrolyzed soy protein, after 16 days of storage at refrigerator temperature, the amount of protein was significantly higher than the control treatment, but the amount of fat decreased significantly. This reduction was attributed to the high percentage of replacing hydrolyzed soy protein with soy, which is high in fat.

From the aquaculture perspective, feed protein is of paramount importance as it comprises 70% of fish muscle dry weight and is the most expensive dietary ingredient. In addition, a minimal amount of fat is necessary to meet the fatty acid requirements of farmed species and the excess fat for energy supply (Davis, 2015). Feed storage at high temperatures can cause oxidative and hydrolytic degradation and thus reduce feed quality (Ramezanzadeh *et al*., 1999). Fats were also shown to be unstable at high temperatures (Ruiz *et al*., 2000).

The addition of HP to diets reduced feed protein and fat lose during 18 weeks. HP also showed similar performance at all three levels in preventing the reduction of feed fat content in comparison to the BHT treatment. According to the results obtained for TBA and TVB-N as the fat and protein spoilage indices, the decreases in these valuable feed components seem to be due to antioxidant and microbial activities. This hypothesis is also confirmed by the results of bacterial load, showing the strong activity of bacteria in the control and antioxidant treatments.

The addition of HP to the diet had a positive effect on the reduction of oxidative and microbial spoilage up to 18 weeks of storage, reduced the spoilage rate, and increased the shelf life and chemical quality of the feed compared to the control. The best recommended dose is 20 g of HP per kg feed, taking into account a combination of TVB-N and TBA indices during storage.

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