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Effects of different modified diets on growth, digestive enzyme activities and muscle compositions in juvenile Siamese fighting fish (*Betta splendens* Regan, 1910)

Karun Thongprajukaew [a, b, #], Uthaiwan Kovitvadhi [b, c, *], Satit Kovitvadhi [d], Pisamai Somsueb [e], Krisna Rungruangsak-Torrissen [b, f, **]

[a] *Inter Departmental Multidisciplinary Graduate Program in Bioscience, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand*

[b] *Biochemical Research Unit for Feed Utilization Assessment, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand*

[c] *Department of Zoology, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand*

[d] *Department of Agriculture, Faculty of Science and Technology, Bansomdejchaopraya Rajabhat University, Bangkok 10600, Thailand*

[e] *Inland Fisheries Research and Development Bureau, Department of Fisheries, Bangkok 10900, Thailand*

[f] *Institute of Marine Research, Ecosystem Processes Research Group, Matre Research Station, N-5984 Matredal, Norway*

[*] Corresponding author: Tel.: +66 2562 5444x3250; fax: +66 2562 5444x3202.

E-mail address: fsciutk@ku.ac.th (U. Kovitvadhi)

[**] Corresponding author: Tel.: +47 56367539; fax: +47 56367585.

E-mail address: Krisnart@imr.no (K. Rungruangsak-Torrissen)

Present address: Department of Applied Science, Faculty of Science Prince of Songkla University, Songkhla, Thailand

Abstract

The effects of four modified diets (gamma-irradiated, microwave-irradiated, probiotic-supplemented and carbohydrases-supplemented diets) were studied on digestive enzyme specific activities and growth performance quality of juvenile Siamese fighting fish (*Betta splendens* Regan, 1910) during 2 weeks of critical and intensive rearing period. The modified procedures did not change biochemical compositions and gross energy of diets, but generally resulted in relatively higher *in vitro* digestibilities of protein and carbohydrate and fish survival rate, albeit insignificant. Only gamma irradiation significantly increased *in vitro* protein digestibility of the diet, and microwave irradiation increased starch gelatinization and water solubility ($P < 0.05$). Fish fed microwave-treated diet showed highest values in all studied growth indicators and digestive enzyme specific activities (except lipase), with significantly higher amylase specific activity and activity ratio of amylase to trypsin (A/T ratio). Correlation analysis indicated significant relationships ($P < 0.05$) among the levels of total protease, amylase and trypsin, and between SGR and A/T ratio. Muscle and body compositions of juveniles fed on microwave- or gamma- irradiated diets were similar to the control, while the juveniles fed on probiotic- or carbohydrases-supplemented diets showed lower protein depositions ($P < 0.05$). Similar levels of RNA, RNA/Protein ratio, and Protein/Lipid ratio in body and muscle in all dietary groups fed *ad libitum* suggested that the improved growth performance in juvenile Siamese fighting fish fed on microwave-irradiated diet may not be only due to improved physicochemical properties of the diet but also improved fish consumption rate.

Keywords: Digestive enzymes; *In vitro* digestibility; Modified diet; Muscle composition; Nutrient utilization; Siamese fighting fish

1. Introduction

Production of Siamese fighting fish (*Betta splendens* Regan, 1910) has been providing the highest income among exported ornamental fish in Thailand. During the fish life span, live diets such as rotifers, infusorians, water fleas (*Moina* sp.) and mosquito larvae are mainly used. Propagation of the live diets mostly uses the wastes from avian and porcine farms that cause the incidence of diseases and environmental impacts. These have contributed to the decrease in survival rate of juveniles and slow growth rate in maturing fish. In order to increase successive growth and survival of juvenile fish, artificial diets with improved nutrient utilization are important. Many methodologies were used to increase nutrient utilization, such as microwave cooking (Negi et al., 2001; Alajaji and El-Adawy, 2006; Khatoon and Prakash, 2006; Sadeghi and Shawrang, 2006; Hu and Wen, 2008; Ma et al., 2009), gamma irradiation (Al-Masri and Guenther, 1999; Fombang et al., 2005; El-Niely, 2007; Ebrahimi et al., 2009; Chung et al., 2010; Yoon et al., 2010), probiotics (Yanbo and Zirong, 2006; Son et al., 2009) and digestive enzymes supplementation (Mohapatra et al., 2002; Kumar et al., 2006; Lin et al., 2007). Carnivorous fishes, including Siamese fighting fish, have limited ability for carbohydrate digestion, especially at juvenile stage, due to short intestine and low activity of carbohydrate digestive enzymes. However, dietary carbohydrate appears to be necessary for improving growth and protein utilization in many fish species (Wilson, 1994). Mohapatra et al. (2002) reported a significant increase in carbohydrate utilization in *Labeo rohita* fry with increasing the level of gelatinized carbohydrate or by supplementing carbohydrases.

The objective of this study was to select a suitable modified method for improving nutrient utilization, based on equal nutritional values, in juvenile Siamese fighting fish. The main feed ingredients were selected based on *in vitro* digestibility studies of protein and carbohydrate using trypsin activity and amylase activity for standardization, respectively

(Thongprajukaew, 2011). The formulated feed was then treated by different procedures for improving nutrient utilization. Digestive enzyme specific activities and muscle compositions were used for determining physiological alterations during the experiment. Digestive enzyme indicators were measured; activity ratio of amylase to trypsin (A/T ratio) for feeding habit and metabolic flexibility of carbohydrate-protein utilizations (Hofer and Schiemer, 1981) and activity ratio of trypsin to chymotrypsin (T/C ratio) for evaluating growth efficiency (Sunde et al., 2001; Sunde et al., 2004; Rungruangsak-Torrissen, 2007). The diet with high carbohydrate content was chosen to test the hypothesis. Two week experiments were conducted at intensive rearing period after juvenile digestive tract was completely developed. These studies could provide knowledge for improving diet quality for rearing juvenile Siamese fighting fish.

2. Materials and methods

2.1. Experimental diets

2.1.1. Preliminary study

In vitro digestibility was performed for screening appropriate dose for gamma irradiation and appropriate time for microwave irradiation for modifying diets. Appropriate feedstuffs for culturing juvenile Siamese fighting fish were selected based on the *in vitro* protein and carbohydrate digestibilities, as described by Thongprajukaew (2011). The main feed mixture used for gamma or microwave irradiation contained fish meal (30%), soybean meal (20%), wheat gluten (12%), squid meal (5%) and wheat flour (20%), as shown in Table 1. For gamma irradiation, the main feed mixture was irradiated at the dose of 20, 40, 60 or 80 kGy using ^{60}Co as gamma irradiation source (Thailand Institute of Nuclear Technology, Thailand). For microwave irradiation, 100 g of the main feed mixture was placed in a plastic box (20 cm diameter × 10 cm height), mixed with distilled water (1:4 w/v) and then cooked

at 700 W in a microwave oven (SANYO, Model EM-700T, 2450 MHz) under agitation for 4, 8, 12, 16 or 20 min. The irradiated feed mixtures were kept at 4°C until used. They were freeze-dried, using Heto FD3 (Heto-Holten, Denmark), for 2 days before *in vitro* digestibility studies of protein and carbohydrate were performed by using enzyme extracts from 20 days old juvenile Siamese fighting fish.

2.1.2. Preparation of experimental diets

The ingredients of experimental diets are shown in Table 1. The unmodified diet (control) was produced by mixing the main feed mixture with additives and vitamin-mineral premixes, and then water (30%) was added to make appropriate moisture. The glutinous mixture was passed through a hand pelletizer, then dried at 60°C for 3 h, and stored at 4°C until used. The modified diets were prepared by four different processes. 1) Gamma-irradiated diet and 2) Microwave-irradiated diet were prepared by irradiating the main feed mixture using gamma source from ^{60}Co or microwave oven, respectively, at the best dose and time obtained by highest *in vitro* digestibility values from the preliminary study in 2.1.1. The irradiated main feed mixtures were then mixed with the minor ingredients (see Table 1). 3) Probiotic-supplemented diet was freshly prepared by spraying the unmodified diet with probiotic, *Lactobacillus plantarum* KKU CRIT5 (Premer CO., LTD, Thailand) before used. The population level of *L. plantarum* in the diet was 2.7×10^8 CFU per g diet. 4) Carbohydrases-supplemented diet was prepared by spraying the unmodified diet with a mixture of carbohydrases (100 µl kg diet^{-1}), then dried at ambient temperature, and stored at 4°C until used. The mixture of the enzymes was from *Bacillus lentus* (Behn Meyer Chemical Co., Ltd., Thailand) containing the main mannan-digesting enzymes, β-mannanase, and the minor enzymes of amylase, β-glucanase, xylanase, cellulase and α-galactosidase. The

121 required amount of the carbohydrases was dissolved in distilled water before used. All
122 modified diets were pelleted and kept in the same way as the control diet.

123 *2.1.3. Biochemical composition study*

124 The diets were dried at 105°C for 24 h before analyzing protein, lipid, fiber, and ash, as
125 described by the AOAC (2005). The values were expressed as % on dry matter basis.
126 Carbohydrate values or nitrogen free extract (NFE) were calculated by the difference.

127

128 *2.1.4. Evaluation of gelatinization degree and water solubility*

129 The diets were freeze-dried for two days before analysis. The dried mass of the diets
130 was determined for degree of starch gelatinization according to Guraya and Toledo (1993).
131 Water solubility of all nutrients was measured according to the method of Chung et al. (2010).

132

133 *2.2. Fish husbandry and sample collection*

134 Juvenile fish were obtained from a private farm in Nakhon Pathom Province, the most
135 important area for producing exported Siamese fighting fish in Thailand. The fish were
136 acclimatized indoors, in tanks (60 cm diameter × 30 cm height) with water temperature of
137 28.5 ± 0.3°C, and fed with the control (unmodified) diet for 7 days before starting the
138 experiments. The fish of 72.73 ± 2.14 mg initial weight and 19.70 ± 0.04 mm initial length
139 were randomly distributed into 15 aquaria (18×19×34 cm), 30 fish per aquarium with a
140 porous white cubic box (6×16×22 cm) for reducing aggressive stress between fish members.
141 The experiment was conducted for 2 weeks with 12-h light/12-h dark and performed in
142 triplicate with five dietary groups (one control and four modified diets) comprised of 90 fish
143 each group. The fish were fed *ad libitum*, twice daily at 08:00 and 18:00 h. At the end of the
144 experiment, the fish were sacrificed by chilling in ice according to "Ethical Principles and
145 Guidelines for the Use of Animals for Scientific Purposes", National Research Council,

Thailand. The fish were not fed on the sampling day. Body weight and total length were measured before white muscle and digestive tracts were carefully collected. The tissues were then kept at –80°C until analyses.

Weight and length of the juvenile fish were measured individually. Growth performance parameters were calculated as the following formulae.

$$\text{Condition factor (g cm}^{-3}) = 100 \times (W/L^3),$$

where W = live body weight (g) and L = total body length (cm).

Specific growth rate (SGR) was calculated according to Houde and Schekter (1981).

$$\text{SGR (\% day}^{-1}) = 100[e^g - 1]$$

where $g = (\ln W_t - \ln W_0)/(t - t_0)$, W_t = mean weight at month t, W_0 = mean weight at month t_0.

$$\text{Net weight gain (NWG)} = \text{final body weight} - \text{initial body weight}$$

$$\text{Average daily growth (ADG, g day}^{-1}) = \text{net weight gain / rearing period}$$

$$\text{Digestosomatic index (DSI, \%)} = 100 \times [\text{gastrointestinal weight / body weight}]$$

2.3. Water quality management

The experiments were conducted at Kasetsart University in an indoor recirculating aquaculture system with a flow-rate of 280 ml min^{-1}. The recirculating aquaculture system was modified from Kovitvadhi et al. (2008). This system consisted of particulate filter cabinet (L×W×H = 35×22×51 cm), macrophytes filter cabinet (35×85×51 cm), biological filter cabinet (35×72×51 cm), water resting cabinet (35×35×51 cm), and five culture units (34×19×26 cm). All cabinets had the water level of 45 cm while the culture units had the water level of 18 cm. The water parameters were analyzed twice weekly. Water temperature, pH, conductivity and dissolved oxygen were analyzed using water analyzer (Multi probe system, 556 MPS, YSI Incorporated, USA). Other parameters including total alkalinity (phenolphthalein methyl orange indicator), free carbon dioxide (titration), total hardness (EDTA titration), total ammonia nitrogen (phenate method), nitrite (colorimetry), nitrate

(cadmium reduction) and orthophosphate (ascorbic acid method) were analyzed according to the method of APHA, AWWA, WPCF (1998).

The water quality during the experimental period had the temperature of 28.49 ± 0.28 °C, pH 7.52 ± 0.05, dissolved oxygen 3.95 ± 0.06 mg L^{-1}, conductivity 0.40 ± 0.01 mS cm^{-1}, total alkalinity 94.74 ± 1.13 ppm $CaCO_3$, total hardness 114.75 ± 0.81 ppm $CaCO_3$, free carbon dioxide 1.38 ± 0.05 ppm, nitrate 0.045 ± 0.003 ppm, nitrite 0.0033 ± 0.0001 ppm, total ammonia nitrogen 0.027 ± 0.004 ppm, and phosphorous 0.028 ± 0.002 ppm.

2.4. Digestive enzyme studies

2.4.1. Enzyme extraction

The enzyme extractions were performed according to Rungruangsak-Torrissen (2007). Digestive tracts of juvenile fish were extracted in 50 mM Tris-HCl buffer pH 8 containing 200 mM NaCl (1:3 w/v) using micro-homogenizer (THP-220, OMNI International, USA). The homogenate was centrifuged at 10,000 × g for 20 min at 4°C. The supernatant was then collected and kept at –80°C in small portions for later determinations. Protein concentration in the crude enzyme extract was determined according to Lowry et al. (1951).

2.4.2. Digestive enzyme assays

The optimal conditions (pH and temperature) chosen for studying the main digestive enzymes in Siamese fighting fish were according to Thongprajukaew et al. (2010a, 2010b).

Amylase activity (at pH 8 and 50°C) was determined based on Areekijseree et al. (2004) modified from Bernfeld (1951) using starch solution as substrate. The enzyme digestion reaction was modified to 15 min. Amylase specific activity was expressed as µmol maltose produced h^{-1} mg $protein^{-1}$.

196 Total protease activity (at pH 8 and 50^{o}C) was assayed using azocasein as substrate
197 based on Areekijseree et al. (2004) modified from Garcia-Carreno (1992). The specific
198 activity of total protease was expressed as mU mg protein^{-1}. One unit (U) of total protease
199 activity was defined as the amount of enzyme giving an increase of 1.0 absorbance unit at
200 440 nm at the specified reaction condition.

201 Amidase activities of trypsin (at pH 8 and 50^{o}C) and chymotrypsin (at pH 8 and 50^{o}C)
202 were assayed by initial reactions based on Rungruangsak-Torrissen (2007) using BAPNA
203 (benzoyl-*L*-arginine-*p*-nitroanilide) and SAPNA (*N*-succinyl-ala-ala-pro-phe-*p*-nitroanilide)
204 as specific substrates, respectively. The specific activities of trypsin and chymotrypsin were
205 expressed as µmol *p*-nitroaniline produced h^{-1} mg protein^{-1}.

206 Esterase activity of lipase (at pH 8 and 40^{o}C) was analyzed based on Winkler and
207 Stuckmann (1979) using *p*-nitrophenyl palmitate as substrate. The specific activity of lipase
208 was expressed as µmol *p*-nitrophenol produced h^{-1} mg protein^{-1}.

209

210 *2.5. In vitro digestibility studies*

211 Crude enzyme extracts were dialyzed overnight against 50 mM Tris-HCl buffer pH
212 8.2 before used for determining *in vitro* digestibility. Freeze-dried diets were used as
213 substrate. Protein and carbohydrate digestibilities of the experimental diets using fish crude
214 enzyme extracts were determined using the method modified from Rungruangsak-Torrissen
215 et al. (2002) and Areekijseree et al. (2006). The reaction mixture containing 5 mg dried feed,
216 10 ml 50 mM phosphate buffer pH 8.2, 50 µl 0.5 % chloramphenical, and 125 µl dialyzed
217 crude enzyme extract, was incubated at 25^{o}C for 24 h.

218 Protein digestibility was determined by measuring the increase in liberated reactive
219 amino groups of cleaved peptides. The reaction mixture, containing 200 µl digested solution,
220 2 ml 50 mM phosphate buffer pH 8.2, and 1 ml 0.1% trinitrobenzene sulphonic acid (TNBS),

was heated in the dark at 60 ^{o}C for 1 h, and stopped by adding 1 ml 1 M HCl before measuring absorbance at 420 nm and comparison with *DL*-alanine standard curve.

Carbohydrate digestibility was determined by measuring the increase in reducing sugar. The reaction mixture containing 1 ml digested solution and 500 µl DNS, was heated in boiling water for 5 min and cooled to room temperature before measuring absorbance at 540 nm and comparison with maltose standard curve.

The blanks (without dialyzed crude enzyme extracts) were used to deduct liberated amino acids and reducing sugars. For comparison, the calculated values were standardized by trypsin activity for protein digestibility and by amylase activity for carbohydrate digestibility (Thongprajukaew, 2011). The *in vitro* digestibility of protein was expressed as µmol *DL*-alanine equivalent g dried feed^{-1} trypsin activity^{-1}. The *in vitro* digestibility of carbohydrate was expressed as µmol maltose g dried feed^{-1} amylase activity^{-1}.

2.6. White muscle and body compositions

Scale and skin of the fish were carefully removed, and the epaxial white muscle was dissected. RNA and protein concentrations in the muscle and body were determined as described in Rungruangsak-Torrissen (2007) modified from Sunde et al. (2001). The extinction coefficient for RNA is E_{260} = 40 µg RNA ml^{-1}, and for protein is E_{280} = 2.1 mg protein ml^{-1}. Lipids were extracted using ethyl acetate as described by Supannapong et al. (2008) and Rungruangsak-Torrissen et al. (2009). All values were expressed on wet weight basis.

2.7. Statistical analysis

Data were expressed as mean ± standard error of mean in triplicate observations. One-Way Analysis of Variance was used for evaluating growth performance parameters, digestive

enzyme specific activities, muscle compositions and body compositions. Significant differences between means were ranked using Duncan's multiple range test (DMRT) at 95% significance level. Pearson correlation coefficients (*r*) between the parameters were calculated.

3. Results

3.1. Preliminary study for screening irradiation conditions

In vitro digestibilities of protein and carbohydrate in the main feed mixtures treated with different irradiation procedures are shown in Fig.1. Protein digestibility values between treated and untreated feed mixtures were not different ($P > 0.05$). The value was relatively highest in microwave irradiation for 8 min cooking time. Carbohydrate digestibility values, on the other hand, showed some differences between the modified procedures ($P < 0.05$), with highest value also in 8 min microwave cooking time. Among gamma irradiation doses, carbohydrate digestibility value was relatively highest at 20 kGy. Therefore, the 8 min microwave cooking and 20 kGy gamma irradiation were chosen as the appropriate doses for modifying diets by the two irradiation techniques.

3.2. Biochemical compositions and some physical properties of experimental diets

No differences were observed in proximate compositions and gross energy among the experimental diets (Table 2). All modified diets showed relatively higher values of *in vitro* digestibilities and physical properties than the control (Table 2). Gamma irradiation increased protein digestibility significantly compared to the control ($P < 0.05$), but the increase was not different from the other techniques ($P > 0.05$). Microwave irradiation, on the other hand, increased carbohydrate digestibility (albeit insignificant), with significantly increased degree

of starch gelatinization and water solubility ($P < 0.05$), compared to the control and the other techniques.

3.3. Survival rate and growth performance of juveniles

No significant differences ($P > 0.05$) were found in survival rate of the juveniles from all dietary treatments (Fig. 2 and Table 3). Nevertheless, the levels of fish survival were relatively higher in fish fed the experimental diets (pooled data) when compared with the control ($P > 0.05$). Survival rate of juvenile Siamese fighting fish in this experiment was ranged from 72–79%.

The results of growth performance are shown in Table 3. No significant differences were observed in total fish length between the dietary groups ($P > 0.05$). On the other hand, body weight and its related parameters were different between the dietary groups ($P < 0.05$). At the end of the experiment, fish fed on microwave cooking diet showed highest values in body weight, condition factor, specific growth rate (SGR), net weight gain (NWG), average daily gain (ADG), and gastrointestinal weight. Among the dietary groups, the levels of these parameters were observed as microwave-irradiated dietary group > carbohydrases-supplemented dietary group > gamma-irradiated dietary group > control group > probiotic-supplemented dietary group. The probiotic-supplemented dietary group also showed significantly lowest values of the digestosomatic index (DSI) ($P < 0.05$).

There was a relationship between body weight and gastrointestinal weight ($r = 0.824$, $P < 0.0001$), regardless of dietary groups (Fig. 3).

3.4. Digestive enzyme specific activities

The results of digestive enzyme specific activities are illustrated in Table 4. Amylase and total protease specific activities were different between fish groups; showing highest

levels in microwave-irradiated dietary group and lowest levels in carbohydrases-supplemented dietary group. Gamma irradiation significantly decreased total protease specific activity ($P < 0.05$), and carbohydrases supplementation significantly decreased specific activities of both amylase and total protease ($P < 0.05$), compared to the control. No differences were observed in the levels of trypsin, chymotrypsin, activity ratio of trypsin to chymotrypsin (T/C ratio), and lipase. However, the highest levels of these enzyme parameters were also observed in microwave-irradiated dietary group. These resulted in highest T/C ratio (albeit insignificant) and activity ratio of amylase to trypsin (A/T ratio, $P < 0.05$) in fish fed microwave-irradiated diet. No differences were observed in A/T ratios among the other fish groups ($P > 0.05$).

The relationships between digestive enzymes specific activities and growth, regardless of dietary groups, are shown in Table 5. Amylase specific activity of juveniles showed positive relationship with specific activities of total protease and trypsin, as well as T/C ratio. Total protease specific activity correlated with trypsin specific activity, and as usual, specific activities of trypsin and chymotrypsin are correlated. The A/T ratio correlated with amylase and total protease specific activities, as well as fish SGR. The T/C ratio correlated with trypsin specific activity, but not with chymotrypsin specific activity.

3.5. Muscle and body compositions

Muscle and body compositions of juveniles were mainly similar among fish groups, except for the levels of protein (Table 6). The levels of protein concentrations in either muscle or body were highest in the control group and lowest in the probiotic-supplemented dietary group. Muscle protein concentrations were significantly lower in carbohydrases- and probiotic- supplemented dietary groups, compared to the control ($P < 0.05$). However, their

319 body protein concentrations were significantly lower than the control and microwave-
320 irradiated dietary group ($P < 0.05$).

321 There were no correlations between muscle or body compositions and other
322 parameters (growth and digestive enzymes) during 2 weeks experiment.

323

324 ## 4. Discussion

325 *4.1. Digestibility of irradiated-main feed mixture and irradiated-diet*

326 Differences in gamma irradiation doses and microwave cooking times did not seem to
327 affect protein digestibility of the main feed mixture in the preliminary study, but could
328 possibly influence carbohydrate digestibility (Fig. 1). Effects of gamma irradiation on protein
329 digestibility have been shown to be depended on irradiation dose and type of material
330 (Fombang et al., 2005). High correlation coefficient between radiation doses (5, 7.5 and 10
331 kGy) and *in vitro* digestibility of proteins from peas, cowpeas, lentil, kidney bean and
332 chickpea has been reported (El-Niely, 2007). Higher doses of gamma irradiation tended to
333 decrease carbohydrate digestibility, but the observations were not statistically different ($P >$
334 0.05, Fig. 1). This might be due to total carbohydrate digestibility was presented, as
335 significant changes in carbohydrate digestibility have been shown in terms of starch
336 digestible rate (Yoon et al., 2010; Chung et al., 2010). Moreover, raw materials from most
337 studies were starch sources while the main feed mixture in this study were comprised of both
338 protein and carbohydrate from plants and animals. For microwave cooking, its use for
339 improving protein and carbohydrate digestibilities has been reported in various legume seeds,
340 such as moth bean (Negi et al., 2001), green gram, Bengal gram and hourse gram (Khatoon
341 and Prakash, 2006). Intensity and irradiation time of microwave process and material
342 concentration were main factors governing enzymatic hydrolysis in rice straw (Ma et al.,
343 2009). This is in agreement with the observation at a proper cooking time (Fig. 1).

Microwave irradiation affected protein degradation (Sadeghi and Shawrang, 2006), which was also observed in our study using electrophoresis (SDS-PAGE) technique (results not shown).

Only gamma irradiation gave a significant increase in protein digestibility compared to the control (Table 2). The increase might be influenced by the breaking of disulphide bonds in protein molecules, as the digestibility levels were related positively with free sulphydryl group levels and negatively with disulphide bond levels (Rungruangsak-Torrissen et al., 2002). Higher quality feeds had higher levels of free sulphydryl group affected by different processing conditions (Sunde et al., 2004). Increased protein digestion in seeds by gamma irradiation was occurred by protein subunit degradation (Ebrahimi et al., 2009) and change in cell wall constituents of some agricultural by-products (Al-Masri and Guenther, 1999), allowing better contact to proteolytic enzymes. The use of different techniques also increased carbohydrate digestibility (albeit insignificant), with the highest digestibility value by microwave irradiation (Table 2). Microwave processing has been reported to improve carbohydrate digestibility of moth bean (Negi et al., 2001) and chickpea (Alajaji et al., 2006). Fish fed on gelatinized corn based diet with different levels of α-amylase supplementation showed significant increases in dry matter digestibility (Kumar et al., 2006). Increased carbohydrate digestibility was associated with increased physicochemical properties of the diets, as also observed in microwave-irradiated diet showing higher starch gelatinization and water solubility than the other diets, without affecting proximate compositions and gross energy (Table 2).

4.2. Survival rate and growth performance of juveniles

Survivals of juvenile Siamese fighting fish at the end of experiment were similar among the dietary groups (Table 3). Generally, microwave irradiation is better than the other

techniques studied, as it improved physicochemical properties of the diets and growth of the juveniles (Tables 2 and 3). The diet may be more palatable than the other diets, as the fish were observed to take shorter time to ingest the microwave-irradiated diet. Carbohydrases supplementation did not improve fish growth performance (Table 3; Rungruangsak-Torrissen et al., 2010), similar to the observations of supplementations with hemicellulose digesting enzymes and α-galactosidase in lupin-based diets (Lin et al., 2007). Uses of probiotics for enhancing successive growth have been reported by Yanbo and Zirong (2006) and Son et al. (2009), however, it did not improve growth performance in our experiment (Table 3). Gastrointestinal weight and digestosomatic index (DSI) of juvenile fish fed on the modified diets were mainly similar to those of the control fish, except for supplementation with probiotic *L. plantarum* resulted in decreased DSI (Table 3). A decrease in the index was also reported in juvenile rohu (*Labeo rohita*) feeding on gelatinized corn based diet with or without α-amylase supplementation (Kumar et al., 2006).

4.3. Responses of digestive enzymes

The increased gelatinization and water solubility of the microwave-irradiated diet could have affected on the higher digestive enzyme specific activities, which contributed to the higher growth performance in this fish group (Tables 2–4). Increased gelatinization level of dietary carbohydrate resulting in increased amylase activity was also observed in rahu, *Labeo rohita* (Mohapatra et al., 2002). Up-regulations of enzymes involved in carbohydrate and protein digestions in microwave-irradiated dietary group could be due to greater utilization of both nutrients for higher energy requirement and growth performance, without changing muscle and body compositions (Table 6). However, down-regulated of these enzymes in carbohydrases-supplemented dietary group caused significantly lower protein levels in both body and muscle (Tables 4 and 6), because of lower in both energy and protein

394 utilizations than the control. Changes in dietary carbohydrate and protein structures (through
395 microwave or gamma irradiations) and supplementations of exogenous enzymes (from *L.*
396 *plantarum* or *B. lentus*), could influence endogenous enzymes productions for utilizing
397 energy and nutrients for fish growth performance. These are supported by the relationships
398 observed between the levels of amylase, total protease, trypsin, T/C ratio and A/T ratio
399 (Table 5). These parameters were higher in higher growth group (microwave-irradiated
400 dietary group), although some parameters may not show statistically different (Tables 3 and
401 4). The specific activities of the alkaline proteases trypsin and chymotrypsin, including T/C
402 ratio, are important for understanding growth performance quality and feed utilization
403 efficiency in different fish species (Sunde et al., 2001, 2004; Rungruangsak-Torrissen, 2007;
404 Rungruangsak-Torrissen and Fosseidengen, 2007; Rungruangsak-Torrissen et al., 2009, 2010)
405 including Siamese fighting fish, however, the A/T ratio was not found to associate with fish
406 growth (Thongprajukaew, 2011). The significantly higher growth performance and A/T ratio,
407 but not T/C ratio, in microwave-irradiated dietary group may indicate higher energy
408 requirement for protein utilization and growth in this group than the other groups. Lipid
409 utilization of the fish did not seem to be affected by the modified diets, as the specific activity
410 of lipase and lipid deposition in body and muscle were similar to the control (Tables 4 and 6).
411
412 *4.4. Muscle and carcass of juveniles*
413 Similarity of muscle and carcass compositions in microwave- and gamma- irradiated
414 dietary groups, compared to control group, indicated better growth of juvenile Siamese
415 fighting fish feeding on these irradiated diets. The uses of diets with probiotic- and
416 carbohydrases- supplementations, on the other hand, reduced protein depositions in body and
417 muscle of the fish, compared to the control. The modified diets did not affect capacities for
418 protein synthesis (RNA concentration) and turnover (RNA/Protein ratio), and protein growth

(Protein/Lipid ratio) in Siamese fighting fish. The experimental period of 2 weeks might be too short to observe the differences. However, 10 weeks feeding on pre-gelatinized starch had no effect on whole body and muscle compositions in juvenile European sea bass, *Dicentrarchus labrax* (Peres and Oliva-Teles, 2002) and juvenile yellowfin seabream (Wu et al., 2007). Increased *in vitro* protein digestibility of diet with fish meal as the whole protein source could increase feed efficiency and muscle protein synthesis capacity in fish (Rungruangsak-Torrissen et al., 2002; Sunde et al., 2004). On the other hand, increased *in vitro* protein digestibility of diets with high plant protein did not improve fish growth performance quality as well as diets with high animal protein did (Rungruangsak-Torrissen et al., 2010). The lower protein depositions in probiotic- and carbohydrases- supplemented dietary groups, and the lack of increased protein depositions in fish fed gamma-irradiated diet with increased *in vitro* protein digestibility value, compared to control, might be due to a high level of dietary plant proteins that could cause imbalance of amino acids in these diets.

5. Conclusion

Modifications of diets by irradiations (with 20 kGy gamma ray from ^{60}Co or 8 min microwave cooking at 700 W) or supplementations (with probiotic *L. plantarum* 2.7×10^8 CFU per g diet or enzymes mixture of commercial carbohydrases 100 µl per kg diet) could affect qualities of dietary nutrients. Gamma-irradiated diet showed higher *in vitro* protein digestibility than control diet, and it decreased total protease specific activity without changing fish growth performance quality. Microwave-irradiated diet, with insignificant improved *in vitro* digestibilities of protein and carbohydrate, showed significant improved starch gelatinization and water solubility that could improve fish growth performance through increased amylase specific activity and A/T ratio without changing muscle and body compositions of the fish. Probiotic-supplemented diet, with similar dietary qualities that

444 resulted in similar levels of digestive enzymes and fish growth as the control diet, decreased
445 protein depositions in body and muscle of the fish. Carbohydrases-supplemented diet, with
446 similar dietary qualities that resulted in similar fish growth as the control diet, decreased the
447 levels of amylase and total protease including protein depositions in body and muscle of the
448 fish. The modified diets did not show any effect on lipid utilization of the fish. There were
449 interactions between carbohydrate and protein utilizations, as shown by the relationships
450 among the levels of amylase, total protease, trypsin, T/C ratio and A/T ratio. All fish groups
451 showed similar capacity for protein synthesis and turnover including protein growth. Changes
452 in digestive enzymes specific activities and protein depositions in some dietary groups should
453 probably be due to consumption rate as the fish were fed *ad libitum*. Enhanced growth
454 performance in microwave-irradiated dietary group may not only be due to improved nutrient
455 quality in the diet but also improved fish consumption rate.
456

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Figure captions

Fig. 1 *In vitro* digestibilities of protein (μmol *DL*-alanine equivalent g dried feed^{-1} trypsin activity^{-1}) and carbohydrate (μmol maltose g dried feed^{-1} amylase activity^{-1}) of unmodified, gamma-irradiated (20–80 kGy) and microwave-irradiated (700 W for 4–20 min) main feed mixtures, using dialyzed crude enzyme extracts from 20-day-old Siamese fighting fish. Data was calculated from triplicate observations. The values with different superscripts are significantly different ($P < 0.05$).

Fig. 2 Change in survival rate (%) of juvenile Siamese fighting fish fed the different diets. Data was expressed as the mean of triplicate aquaria. There was no significant difference ($P > 0.05$) among the dietary groups at the end of rearing period.

Fig. 3 General relationship between body weight and gastrointestinal weight of juvenile Siamese fighting fish in all dietary groups.

Fig. 1

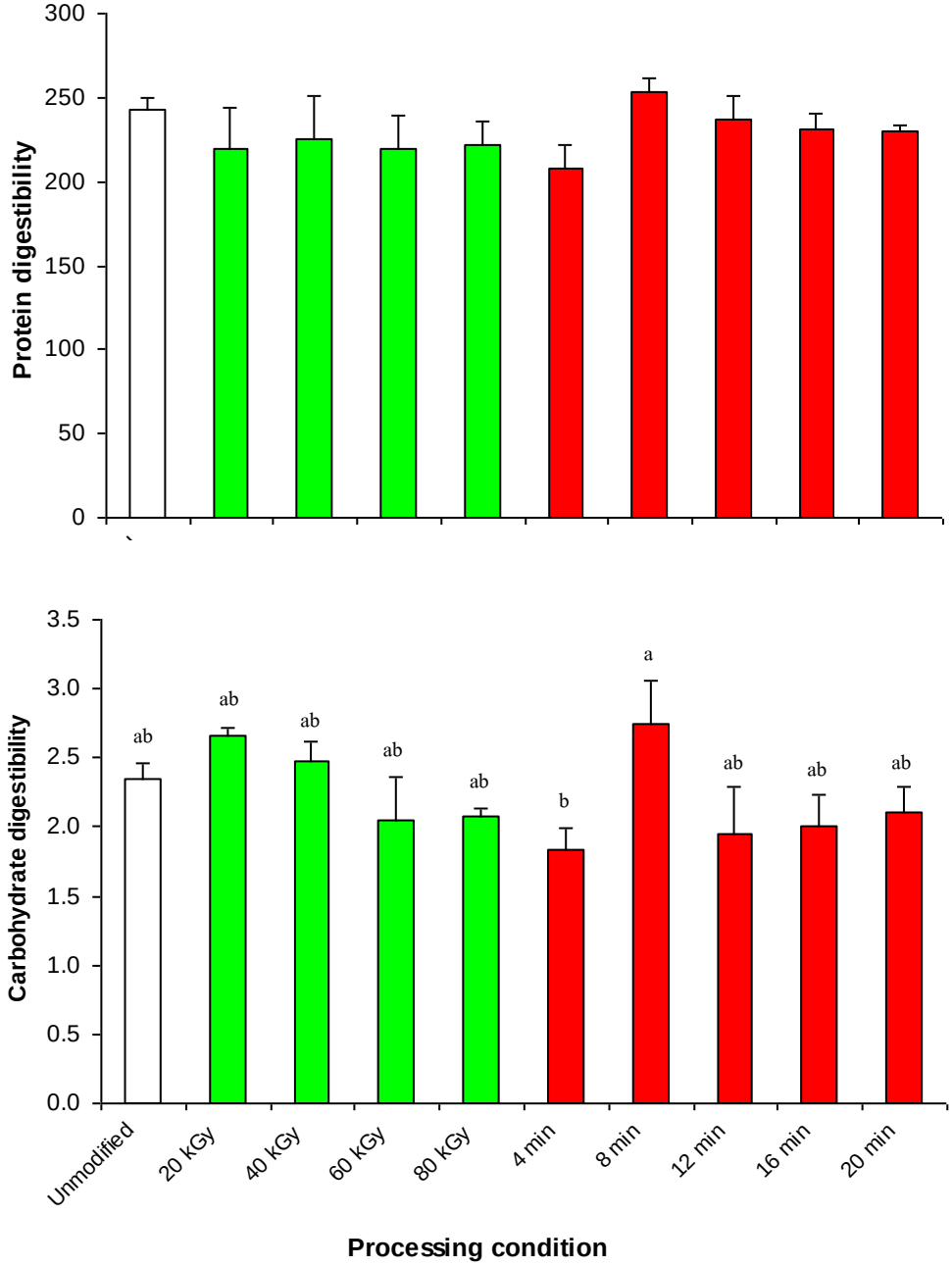
Protein digestibility
300
250
200
150
100
50
0
Carbohydrate digestibility
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
ab
ab
ab
ab
ab
b
a
ab
ab
ab
Unmodified
20 kGy
40 kGy
60 kGy
80 kGy
4 min
8 min
12 min
16 min
20 min
Processing condition

Fig. 2

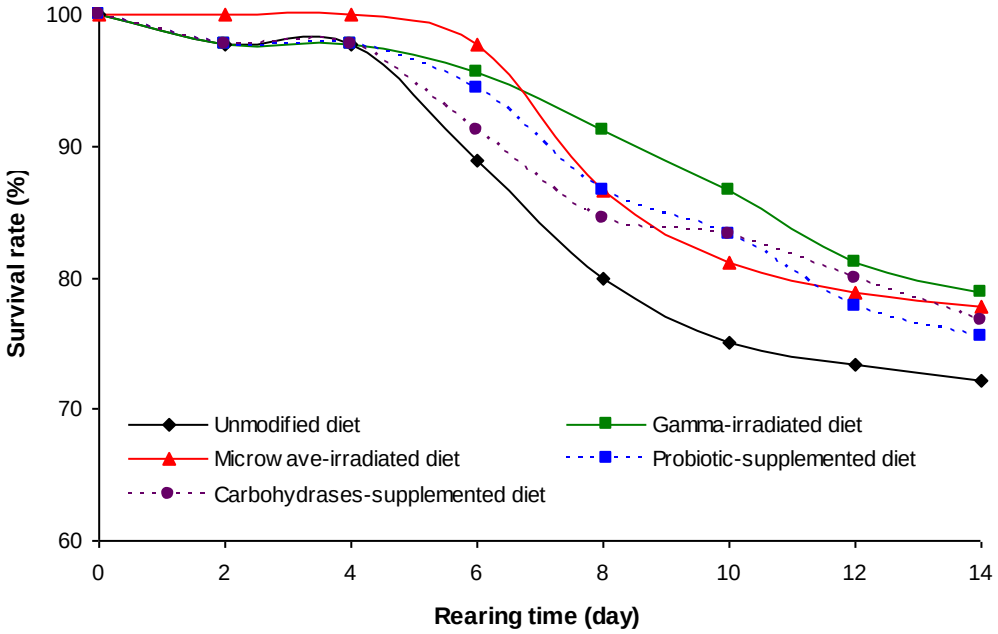
100
90
80
70
60
Survival rate (%)
0
2
4
6
8
10
12
14
Rearing time (day)
Unmodified diet
Gamma-irradiated diet
Microw ave-irradiated diet
Probiotic-supplemented diet
Carbohydrases-supplemented diet

Fig. 3

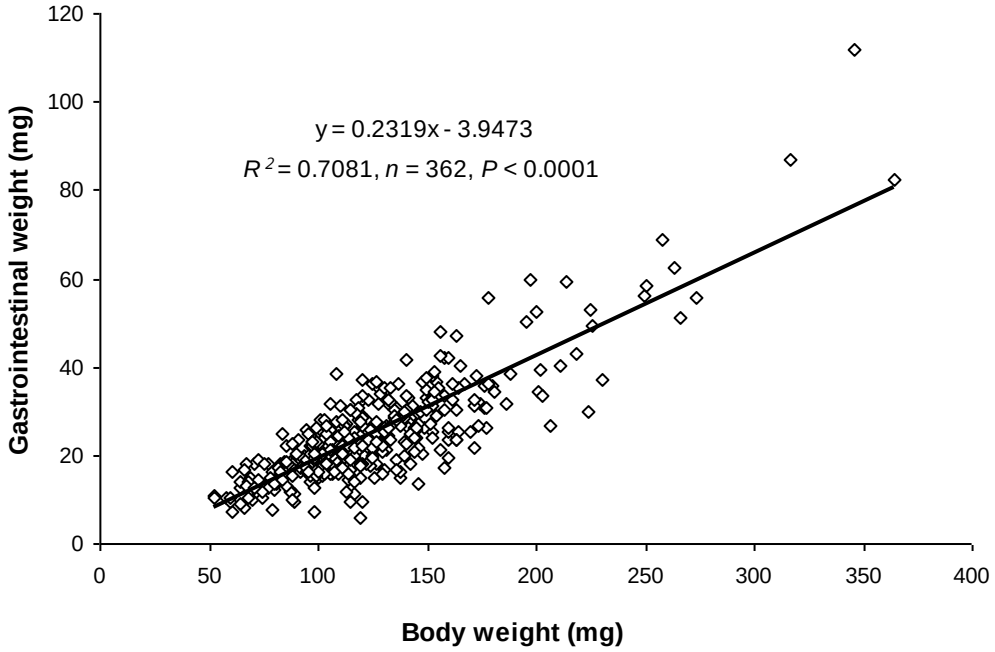
y = 0.2319x - 3.9473
R 2 = 0.7081, n = 362, P < 0.0001
Gastrointestinal weight (mg)
Body weight (mg)
0
20
40
60
80
100
120
0
50
100
150
200
250
300
350
400

Table 1

Ingredients of the experimental diets for rearing juvenile Siamese fighting fish.

Ingredients	Inclusion (%)
Fish meal*	30
Soybean meal*	20
Wheat gluten*	12
Squid meal	5
Wheat flour*	20
Lecithin	2
Fish oil	1
Soybean oil	2.6
Mineral mixture**	0.05
Vitamin mixture***	0.25
Vitamin C	0.1
Fermented red rice	2
Cellulose	5

* Main ingredients selected from *in vitro* screening of suitable feedstuffs using the enzyme extracts from juvenile Siamese fighting fish, as described by Thongprajukaew (2011).

** Mineral mixtures, 1 kg of feed contained 30 mg iron, 20 mg zinc, 25 mg manganese, 5 mg copper, 5 mg iodine and 0.2 mg selenium.

*** Vitamin mixtures, 1 kg of feed contained 4,000 IU vitamin A, 2,000 IU vitamin D_3, 50 mg vitamin E, 10 mg vitamin K, 20 mg thiamine, 20 mg riboflavin, 20 mg pyridoxine, 200 mg calcium panthothenate, 150 mg niacin, 2 mg biotin, 5 mg folic acid, 0.2 mg vitamin B_{12}, 400 mg inositol and 200 mg ethoxyquin.

Table 2

Biochemical compositions (on dry matter basis), *in vitro* digestibilities of protein (µmol *DL*-alanine equivalent g dried feed^{-1} trypsin activity^{-1}) and carbohydrate (µmol maltose g dried feed^{-1} amylase activity^{-1}), starch gelatinization (%), and water solubility (%) of the experimental diets used for rearing juvenile Siamese fighting fish. Data were obtained from triplicate observations.

Dietary parameters	Experimental diets				
	Unmodified	Gamma irradiation	Microwave irradiation	Probiotic	Carbohydrases
Crude protein (%)	41.9	41.8	42.3	42.4	42.6
Crude lipid (%)	7.0	7.2	7.0	7.0	6.8
Nitrogen free extract (%)	32.9	32.1	32.0	31.6	31.1
Crude fiber (%)	4.1	4.5	4.0	4.5	4.9
Ash (%)	14.1	14.4	14.7	14.5	14.6
Gross energy (kJ g^{-1})	19.5	19.4	19.3	19.3	19.2
In vitro protein digestibility	200.23 ± 9.80^{b}	234.78 ± 8.88^{a}	221.24 ± 10.77^{ab}	216.26 ± 8.07^{ab}	222.61 ± 8.93^{ab}
In vitro carbohydrate digestibility	1.80 ± 0.01	1.82 ± 0.17	2.48 ± 0.20	2.13 ± 0.47	1.92 ± 0.11
Starch gelatinization	63.69 ± 0.92^{b}	66.34 ± 0.35^{b}	76.23 ± 0.13^{a}	64.01 ± 0.83^{b}	63.89 ± 0.41^{b}
Water solubility	19.32 ± 0.26^{b}	22.23 ± 0.25^{b}	30.76 ± 0.63^{a}	20.02 ± 1.02^{b}	19.97 ± 0.71^{b}

The values in the same row with different superscripts are significantly different ($P < 0.05$).

Table 3

Effects of the experimental diets on survival rate and growth performance of juvenile Siamese fighting fish at the end of experiment. Data were obtained from triplicate observations.

Parameters	Unmodified	Gamma irradiation	Microwave irradiation	Probiotic	Carbohydrases
Survival rate (%)	72.22 ± 2.22	78.89 ± 1.11	77.78 ± 2.94	75.56 ± 2.94	76.67 ± 6.67
Total length (mm)	22.08 ± 0.14	22.33 ± 0.46	22.88 ± 0.40	21.81 ± 0.23	22.40 ± 0.34
Body weight (mg)	111.72 ± 6.88^{b}	118.13 ± 6.61^{ab}	134.75 ± 1.03^{a}	108.00 ± 5.15^{b}	123.28 ± 8.61^{ab}
Condition factor (g cm^{-3})	1.03 ± 0.03^{b}	1.04 ± 0.02^{b}	1.15 ± 0.03^{a}	1.01 ± 0.01^{b}	1.06 ± 0.05^{ab}
Specific growth rate (% day^{-1})	3.09 ± 0.45^{b}	3.50 ± 0.41^{ab}	4.50 ± 0.06^{a}	2.85 ± 0.36^{b}	3.81 ± 0.51^{ab}
Net weight gain (mg)	38.98 ± 6.88^{b}	45.40 ± 6.61^{ab}	62.02 ± 1.03^{a}	35.27 ± 5.15^{b}	50.55 ± 8.61^{ab}
Average daily gain (mg day^{-1})	2.78 ± 0.49^{b}	3.24 ± 0.47^{ab}	4.43 ± 0.07^{a}	2.51 ± 0.38^{b}	3.61 ± 0.61^{ab}
Gastrointestinal weight (mg)	22.08 ± 1.60^{ab}	22.28 ± 2.13^{ab}	27.03 ± 1.09^{a}	20.30 ± 2.26^{b}	25.17 ± 2.02^{ab}
Digestosomatic index (%)	20.64 ± 0.46^{a}	19.77 ± 0.32^{a}	20.40 ± 0.57^{a}	17.92 ± 0.46^{b}	20.53 ± 0.45^{a}

The values in the same row with different superscripts are significantly different ($P < 0.05$).

Table 4

Effects of the experimental diets on digestive enzyme specific activities of total protease (mU mg protein^{-1}), trypsin (µmol *p*-nitroaniline h^{-1} mg protein^{-1}), chymotrypsin (µmol *p*-nitroaniline h^{-1} mg protein^{-1}), amylase (µmol maltose h^{-1} mg protein^{-1}), and lipase (µmol *p*-nitrophenol h^{-1} mg protein^{-1}) in juvenile Siamese fighting fish at the end of experiment. Data were obtained from triplicate observations.

Parameters	Unmodified	Gamma irradiation	Microwave irradiation	Probiotic	Carbohydrases
Amylase (A)	113.26 ± 2.10^{b}	95.29 ± 7.88^{bc}	166.42 ± 1.48^{a}	105.75 ± 10.31^{b}	84.82 ± 1.39^{c}
Total protease	72.33 ± 1.50^{ab}	63.30 ± 6.74^{b}	87.11 ± 3.50^{a}	69.09 ± 9.66^{ab}	62.03 ± 6.61^{b}
Trypsin (T)	4.20 ± 0.30	3.66 ± 0.07	4.68 ± 0.40	3.91 ± 0.78	3.83 ± 0.34
Chymotrypsin (C)	5.94 ± 0.30	5.98 ± 0.60	6.23 ± 0.39	5.83 ± 0.46	5.96 ± 0.46
Lipase	4.81 ± 0.39	4.69 ± 0.12	3.92 ± 0.02	3.99 ± 0.08	4.39 ± 0.32
T/C ratio	0.71 ± 0.02	0.63 ± 0.07	0.75 ± 0.03	0.63 ± 0.08	0.63 ± 0.04
A/T ratio	27.20 ± 1.64^{b}	26.07 ± 2.28^{b}	36.03 ± 2.87^{a}	20.91 ± 2.82^{b}	24.52 ± 2.29^{b}

The values in the same row with different superscripts are significantly different ($P < 0.05$).

Table 5

Pearson correlation efficiency (*r*) among different digestive enzyme specific activities and specific growth rate (SGR) of juvenile Siamese fighting fish at the end of experiment. Data were calculated from fish in all treatments (*n* = 15).

	SGR	Amylase	Total protease	Trypsin	Chymotrypsin	Lipase	T/C ratio
SGR	1						
Amylase (A)	0.480	1					
Total protease	0.425	**0.851****	1				
Trypsin (T)	0.185	**0.635***	**0.686****	1			
Chymotrypsin (C)	–0.020	0.253	0.378	**0.584***	1		
Lipase	0.259	0.394	0.252	0.467	0.149	1	
T/C ratio	0.272	**0.547***	0.466	**0.777****	–0.050	0.462	1
A/T ratio	**0.535***	**0.786****	**0.563***	0.028	–0.084	0.142	0.088

* $P < 0.05$, ** $P < 0.01$

Table 6

Effects of the experimental diets on white muscle and body compositions (on wet weight basis) of juvenile Siamese fighting fish at the end of experiment. Data were obtained from triplicate observations.

Parameters	Unmodified	Gamma irradiation	Microwave irradiation	Probiotic	Carbohydrases
White muscle composition					
RNA (μg g^{-1})	4212 ± 118	4302 ± 121	4609 ± 171	4695 ± 18	4686 ± 127
Protein (mg g^{-1})	158.21 ± 3.01^{a}	145.32 ± 3.59^{ab}	150.71 ± 8.20^{ab}	136.68 ± 7.83^{b}	136.77 ± 3.25^{b}
Lipid (mg g^{-1})	9.82 ± 1.01	9.44 ± 0.35	11.92 ± 0.67	10.97 ± 0.89	9.71 ± 0.90
RNA/protein ratio (μg mg^{-1})	28.75 ± 1.94	29.62 ± 0.80	30.53 ± 0.43	32.81 ± 0.36	32.17 ± 2.76
Protein/lipid ratio (mg mg^{-1})	16.44 ± 1.63	15.46 ± 0.94	12.66 ± 0.32	12.53 ± 0.71	14.38 ± 1.66
Body composition					
RNA (μg g^{-1})	4349 ± 243	4269 ± 120	4565 ± 240	4445 ± 215	4357 ± 317
Protein (mg g^{-1})	151.98 ± 2.89^{a}	139.60 ± 3.45^{ab}	144.78 ± 7.88^{a}	131.30 ± 7.53^{b}	131.39 ± 3.12^{b}
Lipid (mg g^{-1})	10.14 ± 0.06	9.56 ± 1.21	12.28 ± 1.32	11.43 ± 0.78	10.63 ± 1.33
RNA/protein ratio (μg mg^{-1})	28.59 ± 1.22	30.60 ± 0.82	31.54 ± 0.44	33.89 ± 0.37	33.24 ± 2.85
Protein/lipid ratio (mg mg^{-1})	15.00 ± 0.32	14.79 ± 1.32	12.19 ± 1.94	11.52 ± 0.31	12.88 ± 1.66

The values in the same row with different superscripts are significantly different ($P < 0.05$).