The Effects of Dietary Gracilaria Corticata (Marine Macroalgae, Rhodophyta) Extraction on Growth Performance, Antioxidant Defence, Plasma and Mucosal Immune Components and Immune-related Gene Expressions in Goldfish, Carassius Auratus

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Abstract

The present study examined the effects of the hydro-alcoholic extraction of the red seaweed *Gracilaria corticata* (GCE), as food additive on growth, antioxidant defence and immunity in the goldfish, *Carassius auratus*. Four experimental treatments in three replications were established and fed the experimental diets for 60 days. The groups were: a control (fish fed only a basal diet), GCE1: fish supplemented with 0.5 % GCE/kg diet, GCE2: fish supplemented with 1 % GCE/kg diet, GCE3: fish supplemented with 1.5 % GCE/kg diet. After feeding period, the antioxidant [superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT)] and immune responses were evaluated. Dietary GCE had no effect on growth performance ($P>0.01$). The plasma immune responses including alternative complement ($ACH_{50}$) and lysozyme activities elevated in fish supplemented with 1 % and 1.5 % GCE compared to those fed 0.5 % GCE and basal diet ($P<0.01$). The plasma and mucosal immunoglobulin (Ig) significantly elevated in all GCE supplemented fish ($P<0.01$). The plasma peroxidase activity significantly increased only in the fish receiving GCE at dietary level of 1.5 % GCE compared to control and those supplemented with 0.5 % and 1 % GCE ($P<0.01$). The mucosal lysozyme, protease activity and alkaline phosphatase significantly increased in fish supplemented with 1 % GCE compared to other experimental diets ($P<0.01$). The activity of antioxidant enzymes (SOD, CAT, GPx) showed significant increases in fish supplemented with 1 % and 1.5 % GCE ($P<0.01$). Furthermore, the expression of the immune-related genes, complement and lysozyme significantly elevated in the treatments of 1 % and 1.5 % GCE compared to other experimental diets ($P<0.01$). The results of this study revealed that the use of GCE as a food additive in the diet of goldfish diet can improve the fish immunity without negative impacts on growth.

Highlights

- Dietary *Gracilaria corticata* extraction (GCE) had no effect on growth performance of gold fish.
- Dietary GCE at 1 % and 1.5 % levels stimulated plasma and mucosal immune responses in the gold fish.
- Dietary GCE at 1 % and 1.5 % levels up-regulated the expression of immune-related in the goldfish.
- Dietary GCE at 1 % and 1.5 % levels elevated liver antioxidant enzymes in the gold fish.

1. Introduction

Seaweeds (SWs) contain valuable bioactive compounds, making them a good choice for food and pharmaceutical usages for human and husbandry animals (Kolanjinathan et al., 2014; Kasimala et al., 2015; Khalil et al., 2017). During last decade, use of plant-based materials has considerably increased in aquaculture to enhance fish and shellfish immune system (Van Hai, 2015; Awad and Awaad, 2017). However, using SWs as food additive is a relatively new approach in fish nutrition and needs more attentions. In recent years, the potentials of SWs in fish nutrition have been studied by many studies. It was recognized that SWs improve growth (Valente et al., 2006; Xu et al., 2011; Ragaza et al., 2015;
Kamunde et al., 2019), immunity and antioxidant capacity (Peixoto et al., 2016; Hoseinifar et al., 2018; Sotoudeh and Mardani, 2018; Vazirzadeh et al., 2020) in fish. However, there are some studies, reporting the reducing effects of dietary SWs on fish growth and immune system (Yıldırım et al., 2009). In the study of Déléris et al. (2016), the SWs-related decreases in growth were related to the antinutritional factors in the algae and their adverse effects on digestive enzymes (Déléris et al. 2016). Similar results were observed in the African catfish *Clarias gariepinus* (Al-Asgah et al. 2016) and in the European sea bass, *Dicentrarchus labrax* (Valente et al. 2006), where fish fed diets containing 9-13.5% *Clarias gariepinus* and 5% *Gracilaria bursa-pastoris*, respectively. These studies clearly indicated that the effects of dietary SWs could be different depending on species and their dietary levels. Therefore, it is necessary to optimize the dietary levels of SWs, when used as food additive in the diet of fish.

The red alga, *Gracilaria corticata*, belonging to the family Gracilariacea and order Gracilariales and is one of the most predominant seaweeds of the world including Oman sea and Persian Gulf coasts (Andriamanantoanina et al., 2007; Zandi et al., 2010; Teimouri et al., 2016). Many studies have reported the enhancing effects of *Gracilaria* sp. on the antioxidant and immune system of fish. However, there is no information about the immunogenic potentials of the *Gracilaria corticata* as, as one of the predominant seaweeds of Iran. Therefore, in this study, we used goldfish, *Carassius auratus* as a model fish (Li et al., 2016; Blanco et al., 2018; Ferrão et al., 2020) for evaluation of the *in vivo* effects of the dietary *G. corticata* extracts on fish growth, antioxidant and immune system.

2. Materials And Methods

2.1. Preparation of hydro-alcoholic algae extract

The *Gracilaria corticata* (GC) were collected during spring from intertidal regions of the Chabahar bay, Oman Sea, Iran. The collected algae were transferred to lab on ice, dried at 35°C for 48 h, pulverized and homogenized by a mill. The hydro-alcoholic extractions were prepared according to Mora et al. (2005) by a rotary (Buchi, Switzerland) at 80°C. The concentrated GC extracts were finally dried at 40°C and then pulverized. The powders were finally stored in nylon bags at 0°C for further usages.

2.2. Fish and experimental design

A total number of 240 goldfish (initial weight: 31.1 ± 0.6 g; initial length: 12.5 ± 0.5 cm) were distributed into 12 tanks (300 l) (20 fish/tank) containing aerated and disinfected water. After 10-day's acclimation period, fish were fed diets containing different levels of *Gracilaria corticata* extract (GCE), as three experimental groups and one basal diet supplemented group as control in three replicates. The experimental groups were: control: non-GCE-supplemented fish, GCE1: 0.5% GCE/kg diet, GCE2: 1% GCE/kg diet, GCE3: 1.5% GCE/kg diet. A commercially basal diet (STARTER for Common carp: crude protein: 41.5%; lipid: 6%, Fiber: 4.5%; Ash: 9%, Faradaneh CO., Shahrekord, Iran) was used to make the experimental diets. To prepare the diets, the basal diet was mill-powdered, the dried GCE added and then pasted by adding 100 ml of distilled water. After that, the paste was passed through a sieve to form granule particles (mean diameter: in 3 ± 0.1 mm; mean length: 2.6 ± 0.3 mm). The foods were incubated
at 35°C to dry and then stored at 4°C for further usages. Feeding was carried out daily at a rate of 5% of total fish weight. Feeding size was adjusted every five days by weighing 5 fish from each tank. Throughout the experiment, the water quality parameters were checked daily, which were at normal ranges for temperature: 21.1 ± 0.15°C, ammonia: 0.03 ± 0.004 mg/L (colorimetrically at 670 nm) and dissolved oxygen: 7.1 ± 0.2 mg/L (OxyGouard) and pH: 7.1 ± 0.1 (APX15/C-WTW-330i).

2.3. Growth parameters

The growth parameters were measure after feeding experiment according to following formula:

Weight gain (WG) = Wf (g) – Wi (g).

Specific growth rate (SGR) = 100 (ln Wf – ln Wi)/T.

Survival rate (%SR) = (final amount of fish/initial amount of fish) × 100.

Where Wi is the initial weight, Wf is the final weight and T is the number of days in the feeding period.

2.4. Mucus, kidney, liver and blood sampling

After 60 days feeding, fish (n= 20 fish/tank) were starved for 24 h and placed in a nylon bag and the mucus samples were collected according to Subramanian et al. (2007). The liver and kidney tissues were taken by dissecting out the fish after sedation with 30 mg/l clove oil. The blood samples were taken by heparinized syringe from the caudal vein and then centrifuged at 13000 g for 5 min to separate the plasma. The plasma and liver samples were kept at liquid nitrogen (−196°C) for biochemical analysis. In this study, the samplings and manipulation of the fish were carried out according the ethical standards of the University of Tehran, Iran.

2.5. Liver biochemical assays

The activity of antioxidant enzymes in liver were assayed using commercial assay kits (Sigma-Aldrich CO, USA) based on manufacturer's instructions.

The activity of SOD was measured colorimetrically at 440 nm through oxidation of xanthine to superoxide radicals (Marklund and Marklund 1974). The activity of GPx levels were spectrophotometrically assayed at 340 nm through enzymatic generation of oxidized glutathione from glutathione under GPx action. Catalase (CAT) activity was spectrophotometrically assayed at 240 nm through catalyzing hydrogen peroxide (H₂O₂) and production of water and oxygen (Claiborne, 1985). The lipid peroxidation was measured by thiobarbituric acid reaction method at 532 nm (Utley et al., 1967).

2.6. Plasma and mucosal immunological assays

The activity of plasma alternative complement activity (ACH₅₀) was assayed by haemolysis of rabbit red blood cells according to Karimi et al. (2020). The volume of plasma causing 50% haemolysis was considered to estimate the ACH₅₀ activity. The total immunoglobulin (Ig) of plasma and mucus was
measured according to Siwicki and Anderson (1993). To this end, the total protein was assayed and then the Ig molecules were precipitated by adding 12% polyethylene glycol solution. The difference in protein content before and after precipitation was estimated as plasma Ig concentration. The lysozyme activity in plasma and mucus was estimated using the turbidity assay according to Parry et al. (1965). Briefly, 50 µl of the plasma was added to 2 ml of the bacterial suspension [Chicken egg lysozyme (as the standard) + 0.2 mg/ml lyophilised Micrococcus lysodeikticus in 0.04 M sodium phosphate buffer (pH 5.8)] and the reduction in the absorbance at 540 nm was determined in two times of 0.5 and 4.5 min incubation at 22°C. A rate of 0.001 min⁻¹ reduction in absorbance was considered as one unit of lysozyme activity.

The plasma peroxidase activity was measured by a colorimetric method at 450 nm according to Quade and Roth (1997) through reaction of tetramethylbenzidine hydrochloride and 5 mM H₂O₂ as substrates and following calculation of decrease in absorbance. The mucosal protease activity was measured according to azocasein hydrolysis method (Ross et al., 2000). The mucosal alkaline phosphatase (ALP) activity was estimated by a commercial assay kit (Sigma-Aldrich Co., USA) based on manufacturers' protocol.

2.7. Gene expression assay

2.7.1. RNA extraction

The RNA extraction of kidney tissue was done was by acid guanidinium thiocyanate-phenolchloroform (Awad et al. 2011). The quantity of the extracted RNA (ERNA) was evaluated at 260 nm by a spectrophotometer (Ultraspec III, Pharmacia, Netherlands). In addition, the quality of ERNA was assayed using Nanodrop spectrophotometer at 260/280 and 260/230 absorbance ratios (Beheshti et al., 2018).

2.7.2. RT-PCR

1 µg of extracted RNA was subjected to synthesize first-strand cDNAs using a Fermentas cDNA synthesis Kit based on the manufacturer's protocol, followed by the reverse transcription polymerase chain reaction (RT-PCR). The real time PCR primers were produced on the basis of the sequences of DNA from Gen Bank, followed by the Gene Runner (version 6.) software. The sequences of the primers were: [for lysozyme gene: Forward: GTATCTTCAAGCGAGAGGGACT, Reverse: CCCTGTGGGTCTTATACTTACTC (accession number: KJ703111.1), for complement gene: Forward: CTGTGCTGGCTTATATCTTACTC, Reverse: ATCCTCCATAATGAGACTGGTTG (accession number: AM773828.1), for reference gene (β-actine gene): Forward: ACTGCACAGCCAAGAGAGTCTCA, Reverse: GTTTAAGACGCCGATATGC (accession number: AB039726)]. The real time PCR was done by an iCycler (BioRad, USA) using commercial kit (Bio flux-Bioer Technology Co., China) according to the manufacturer's protocol. The fold changes for complement and lysozyme relative mRNA expression was calculated by the 2^(-ΔΔCt) method (Livak and Schmittgen 2001) and an iQ5 optical system software version 2.1 (Bio-Rad) was used for data analysis. The expression of the β-actine gene was used as reference gene.

2.8. Statistical analysis
Data analysis was carried out by SPSS software. The data normality was evaluated by Kolmogorov–Smirnov test. After that, the one-way analysis of variance (ANOVA), followed by Tukey’s test were applied to find the statistical differences and to compare the means between the experimental groups, respectively. All data are presented as mean ± SD.

3. Results

3.1. Growth and survival rate

Use of GCE in the diet of the fish had no effects on growth parameters i.e. final weight (g), weight gain (g) (Fig. 1) and SGR (Fig. 2, P>0.01). Similarly, the survival rate (%) showed no changes between the experimental groups over the course of the experiment (Fig. 3, P>0.01).

3.2. Immunological parameters

The activity of plasma alternative complement (ACH50) (Fig. 4) and lysozyme (Fig. 5) was significantly increased in fish fed 1% and 1.5% GCE compared to those fed 0.5% GCE and basal diet (P<0.01). The Immunoglobulin (lg) levels (Fig. 4) significantly elevated in all GCE supplemented fish (P<0.01). The peroxidase activity (Fig. 5) significantly increased only in the fish receiving 1.5% GCE compared to control and those supplemented with 0.5% and 1% GCE (P<0.01).

The mucosal immunoglobulin (lg) significantly increased in all GCE supplemented fish (Fig. 6, P<0.01). The mucosal lysozyme and protease activity (Fig. 7) and alkaline phosphatase (Fig. 8) significantly increased in fish supplemented with 1% GCE compared to other experimental diets (P<0.01).

3.3. Liver antioxidant enzymes

The liver antioxidant enzymes, CAT (Fig. 9), GPx and SOD (Fig. 10) showed significant elevations in fish fed 1% and 1.5% GCE compared to control (P<0.01).

3.4. Malondialdehyde

Supplementation with GCE had no significant effect on MDA levels in liver (Fig. 11, P>0.01).

3.5. Gene expressions

The expression of immune-related genes, complement and lysozyme significantly increased in the fish fed 1% and 1.5% GCE compared to other experimental diets (Fig. 12, P<0.01).

4. Discussion

In recent years, many attentions have been paid to the use of algal resources and their derivatives as food additives in aquaculture (Fleurence et al., 2012; Rajauria, 2015; Thanigaivel et al., 2016). The
immunostimulatory properties of seaweeds in fish have been reported in various studies (Thépot et al., 2020). However, providing information about algae species, especially endemic species could be valuable. In the present study, the immunostimulatory and antioxidant effects of an endemic macroalgae, *Gracilaria corticata* extracts was examined in the goldfish by adding it in the diet. Based on our results, the growth performance the fish were not affected by dietary GCE. In general, the use of dietary *Gracilaria* sp. has resulted various results related to growth performance of fish. In Persian sturgeon, *Acipenser persicus*, dietary *Gracilaria* sp. (Adel et al., 2020) had no effects on growth performance. Similar results were observed in barramundi, *Lates calcarifer* and Meager, *Argyrosomus regius* supplemented with *Gracilaria pulvinata* and *Gracilaria* sp. respectively (Morshedi et al., 2018). In *Siganus canaliculatus*, use of *Gracilaria lemaneiformis* in the diet improved the fish growth (Xu et al., 2011). Contrary to these results, Younis et al. (2018) reported the reducing effects of dietary *Gracilaria arcuata* extracts in Nile tilapia, *Oreochromis niloticus*, which it was attributed to the high Nitrogen-free content of the extracts and its negative effects on digestibility and also to lower protein content of Gracilaria meal compared to fish meal. These results conclude that the growth response of fish to dietary *Gracilaria* sp. may be different depending on fish species, algal species and its chemical composition.

In this study, dietary GCE had enhancing effects on immune components and immune-related gene expressions in goldfish, as reported previously in other studies with *Gracilaria* sp. Hoseinifar et al. (2018) showed that dietary *Gracilaria gracilis* powder enhances the skin mucus immune components including total Ig and total protein, however the expression of immune (TNF-α, LYZ and IL) and antioxidant enzyme-related (SOD and CAT) genes showed no changes. Silva-Brito et al. (2020) reported a dose-dependent pattern for the effects of dietary *Gracilaria arcuata* extracts in Nile tilapia, *Oreochromis niloticus*, which it was attributed to the high Nitrogen-free content of the extracts and its negative effects on digestibility and also to lower protein content of Gracilaria meal compared to fish meal. These results conclude that the growth response of fish to dietary *Gracilaria* sp. may be different depending on fish species, algal species and its chemical composition. Use of *Gracilaria lemaneiformis* in the diet of rainbow trout enhanced serum lysozyme, SOD, peroxidase activities and also the expression of immune-related gens such as LyzII, TNFα and IL-1β (Vazirzadeh et al., 2020). The immune enhancing effects of *Gracilaria* sp. have been widely reported in shrimps (Hou and Chen, 2005; Yeh and Chen, 2009; Yeh et al., 2010; Lin et al., 2011; Sirirustananun et al., 2011; Chen et al., 2012). Nevertheless, some studies have also reported the reducing impacts of dietary *Gracilaria* sp. on fish immunity. In European sea bass, *Dicentrarchus labrax*, Gracilaria-supplemented fish showed lower ACH<sub>50</sub> activity compared to non-supplemented fish (Peixoto et al., 2016). Silva-Brito et al. (2020) reported a decrease in GPx activity in the gilthead seabream fed 2.5% and 5% *Gracilaria* sp. In the present study, GCE at dietary levels of 1% increased the activity of mucosal lysozyme, alkaline phosphatase and protease. Mucosal proteases play an essential role in the breakdown of proteins involved in inflammatory, coagulation, apoptotic and tissue regeneration processes (Ahmad et al., 2021). Skin mucus alkaline phosphatase is known as part of fish mucosal immune system, though its actual mode of action is still unknown. However, some antibacterial properties have been reported for skin mucus alkaline against water pathogens (Esteban et al., 2015).

Based on above results, it is recognized that the effects of dietary *Gracilaria* sp. on fish immune system may be different depending on fish species, algal species, the dose administrated and condition of
experiment.

MDA levels are known as biomarker of lipid peroxidation in organisms including fish (Avci et al., 2005; Grotto et al., 2009; Mendes et al., 2009; Rafieepour et al., 2018; Hajirezaee et al., 2019). In this study, the MDA levels did not show significant changes between the experimental groups, which could indicate that the GCE did not induce oxidative stress. However, GCE at high concentrations (1-1.5 %) enhanced the activity of liver antioxidant enzymes (CAT, SOD, GPx), which clearly shows the enhancing effects of GCE on antioxidant defense system of the fish.

5. Conclusion

In conclusion, the findings of the current study demonstrated the antioxidant and immunostimulatory effects of dietary GCE in the goldfish, without negative impacts on the fish growth.

Declarations

6.1. Ethical Approval

The welfare of fish was taken into consideration and all the experimental procedures followed the ARRIVE guidelines and were carried out in accordance with the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978) to minimize fish suffering.

6.2. Consent to participate

Not applicable

6.3. Consent to Publish

Not applicable

6.4. Availability of data and materials

The datasets used during the current study are available from the corresponding author on reasonable request.

6.5. Compliance with ethical standards

The authors declare that they have no competing interests.

6.6. Funding

There is no government or organizational fund for this work.

6.7. Author's contributions
Alexei Valerievich Yumashev: Writing—original draft preparation; Rumi Iqbal Doewes: Conceptualization; Rustem Adamovich Shichiyakh: Supervision, Writing—review and editing; Shadia Faris Ahmad: Writing—review and editing; Wanich Suksatan: Methodology; Walid Kamal Abdelbasset: Supervision; Mazin A.A. Najm: Resources; Mohammed Abed Jawad: Formal analysis; Huynh Tan Hoi: Writing—review and editing; Fariborz Narimanizad: Methodology.

6.8. Conflict of interest

The authors declare that there is no conflict of interest regarding present data and article.

6.9. Acknowledgments

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Figures

Figure 1

Effects of different dietary levels of Gracilaria corticata extracts (GCE) on growth performance of the goldfish, Carassius auratus. Different superscripted letters indicate significant differences between the
dietary groups ($P<0.01$). Data are presented as mean ± SD.

Figure 2

Effects of different dietary levels of *Gracilaria corticata* extracts (GCE) on specific growth rate of the goldfish, *Carassius auratus*. Different superscripted letters indicate significant differences between the dietary groups ($P<0.01$). Data are presented as mean ± SD.

Figure 3
Effects of different dietary levels of *Gracilaria corticata* extracts (GCE) on survival rate of the goldfish, *Carassius auratus*. Different superscripted letters indicate significant differences between the dietary groups ($P<0.01$). Data are presented as mean ± SD.

**Figure 4**

Effects of different dietary levels of *Gracilaria corticata* extracts (GCE) on plasma immunoglobulin (Ig) concentrations and ACH$_{50}$ activity in the goldfish, *Carassius auratus*. Different superscripted letters indicate significant differences between the dietary groups ($P<0.01$). Data are presented as mean ± SD.

**Figure 5**
Effects of different dietary levels of *Gracilaria corticata* extracts (GCE) on plasma lysozyme and peroxidase activity in the goldfish, *Carassius auratus*. Different superscripted letters indicate significant differences between the dietary groups (*P*<0.01). Data are presented as mean ± SD.

Figure 6

Effects of different dietary levels of *Gracilaria corticata* extracts (GCE) on mucosal immunoglobulin in the goldfish, *Carassius auratus*. Different superscripted letters indicate significant differences between the dietary groups (*P*<0.01). Data are presented as mean ± SD.

Figure 7
Effects of different dietary levels of *Gracilaria corticata* extracts (GCE) on skin mucus lysozyme and protease activity in the goldfish, *Carassius auratus*. Different superscripted letters indicate significant differences between the dietary groups (*P*<0.01). Data are presented as mean ± SD.

![Figure 8](image)

**Figure 8**

Effects of different dietary levels of *Gracilaria corticata* extracts (GCE) on skin mucus alkaline phosphatase in the goldfish, *Carassius auratus*. Different superscripted letters indicate significant differences between the dietary groups (*P*<0.01). Data are presented as mean ± SD.

![Figure 9](image)

**Figure 9**
Effects of different dietary levels of *Gracilaria corticata* extracts (GCE) on liver antioxidant enzyme, catalase in the goldfish, *Carassius auratus*. Different superscripted letters indicate significant differences between the dietary groups (*P*<0.01). Data are presented as mean ± SD.

![Figure 10](image)

**Figure 10**

Effects of different dietary levels of *Gracilaria corticata* extracts (GCE) on liver antioxidant enzymes, GPx and SOD in the goldfish, *Carassius auratus*. Different superscripted letters indicate significant differences between the dietary groups (*P*<0.01). Data are presented as mean ± SD.

![Figure 11](image)

**Figure 11**
Effects of different dietary levels of *Gracilaria corticata* extracts (GCE) on liver malondialdehyde (MDA) levels in the goldfish, *Carassius auratus*. Different superscripted letters indicate significant differences between the dietary groups (*P*<0.01). Data are presented as mean ± SD.

Figure 12

Effects of different dietary levels of *Gracilaria corticata* extracts (GCE) on immune-related gene expressions in the goldfish, *Carassius auratus*. Different superscripted letters indicate significant differences between the dietary groups (*P*<0.01). Data are presented as mean ± SD.