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# EFFECTS OF DIETARY *GRACILARIA PERSICA* ON THE INTESTINAL MICROFLORA, THYROID HORMONES, AND RESISTANCE AGAINST *AEROMONAS HYDROPHILA* IN PERSIAN STURGEON (*ACIPENSER PERSICUS*)

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**Abstract**

**Red seaweeds have several biomedical derivatives making them healthy additives for the aquaculture industry. Previously we reported enhanced growth performance, feed utilization, and immunity of Persian sturgeon treated with *Gracilaria gracilis*. Herein, we investigated the effects of *G. gracilis* on the intestinal microflora, thyroid hormones, and resistance against *Aeromonas hydrophila* in Persian sturgeon. Fish were fed *G. gracilis* at 0, 2.5, 5, and 10 g/kg for eight weeks, then challenged with *A. hydrophila* for ten days. The results showed that the total bacterial count in the fish intestines had no meaningful differences among the groups of fish fed varying levels of *G. persica* (P>0.05). Fish fed 10 g/kg of *G. persica* had a higher lactic acid bacteria (LAB) count than fish fed 0, 2.5, and 5 g/kg (P<0.05). Serum thyroid-stimulating hormone (TSH) showed higher levels in fish treated with 2.5 and 5 g/kg of *G. persica* than the other groups (P<0.05). Besides, fish fed 2.5 g/kg *G. persica* had higher thyroxine (T4) and triiodothyronine (T3) levels than the other groups (P<0.05). At the end of the challenge test, the highest mortality was seen in the fish fed the *G. persica* free diet. In summary, Persian sturgeon treated with *G. gracilis* had improved intestinal microflora, thyroid hormones, and resistance against *A. hydrophila*.**

**Key words: antibacterial capacity, *Gracilaria persica, Acipenser persicus,* thyroid hormones, disease resistance**

The increasing population requires sustainable food resources to afford humanity with secure suitable food needs (FAO, 2020; Kari et al., 2022). Aquaculture activity is recognized as a reasonable food provider, but this sector is endangered with risky factors that reduce aquatic animals' productivity and well-being (Dawood and Koshio, 2020; Kari et al., 2021). The climatic change, the spread of infectious diseases, low water quality, high feeding cost, and intensification of production systems are the significant challenges that led to the lack of aquaculture production and massive economic loss (Galappaththi et al., 2020). Specifically, infectious diseases resulting from immunity weakness and impaired health status in aquatic animals are the main risk that threatens the aquaculture industry (Bouwmeester et al., 2021). Pathogenic invaders attack aquatic animals suffering from oxidative stress and immunosuppression induced by uncontrolled farming conditions and malnutrition (Kiadaliri et al., 2020). *Aeromonas hydrophila* infection is among the main bacterial outbreaks in aquaculture involved in dropsy, hemorrhagic septicemia, hemorrhagic enteritis, epizootic ulcerative syndrome, and red body disease (Mzula et al., 2019). Commonly, antibiotics and vaccines were used to overcome the infection with *Aeromonas* (Lulijwa et al., 2020). However, continuous usage reduces the natural host immunity and increases the resistance of pathogenic bacteria to chemotherapy (Dawood et al., 2021).

Environmentally friendly substances such as phytogenics and seaweeds are comprehensively applied in aquaculture to replace antibiotics usage (López-Pedrouso et al., 2020). Seaweeds and their extracts are illustrated as functional additives in aquaculture due to the high content of alginate, carrageenan, galactan, fucoidan, ulvan, laminarin, and agar (Thépot et al., 2021). In this context, seaweed extracts are known for their antibacterial, antioxidative, immunostimulant, and anti-inflammatory effects *in vivo* and *in vitro* (Sanjeewa et al., 2018; Mohan et al., 2019). The inclusion of seaweeds and their extracts in aquafeed showed increased growth performance, feed utilization, antioxidative status, immune response, and resistance to infectious diseases (Safavi et al., 2019; An and Anh, 2020). Gracilariopsis species are red algae grown in the Persian Gulf area, including Iran (Jeliani et al., 2018). *Gracilaria gracilis* contains many biomedical derivatives such as phenolic content, antioxidants, lipids, proteins, minerals, and vitamins (Hindu et al.,

2019; Francavilla et al., 2013). In aquaculture, dietary *G. gracilis* resulted in various potential roles in growth performance, immune response, and disease resistance (Wijnana et al., 2018; Adel et al., 2021 b; Hoseinifar et al., 2018).

Persian sturgeon (*Acipenser persicus*) is a highly valued fish species in the Caspian Sea area (Aramli et al., 2015). Persian sturgeon is encountered with several farming stressors, including high stocking density, low water quality, and infectious diseases. For these reasons, Adel et al. (2021 b) performed a feeding trial to investigate the role of *G. persica* on the growth performance, blood chemistry, and immune response of Persian sturgeon. The authors concluded that dietary *G. persica* is recommended at 5–10 g/kg to enhance the growth performance, feed utilization, and immunity of Persian sturgeon. Herein, the role of *G. persica* on the intestinal microflora and serum thyroid hormones is investigated to have a complete overview of the expected mode of action. Besides, the potential role of *G. persica* against A. *hydrophila* infection as a natural alternative for antibiotics is studied.

## Material and methods

### *Gracilaria persica* powder preparation, test diets, and experimental fish

*Gracilaria persica* red macroalgae were obtained from the Oman Sea, the coast of Chabahar, Iran. The collected *G. persica* was cleaned with tap water then rinsed three times with distilled water to eliminate extraneous materials (Jafari et al., 2020). Then, *G. persica* was sun-dried for two weeks and finely ground by 60 mesh. The chemical composition of the *G. persica* was checked and recorded 34.8 ± 0.2, 8.3 ± 0.1, 26.1 ± 0.4, and 7.1 ± 0.08% for crude protein, crude lipid, ash, and fiber contents, respectively. The basal diet was formulated by mixing the ingredients presented in Table 1, then the powder of *G. persica* was included at 0, 2.5, 5, 10 g/kg (Hoseinifar et al., 2020). The ingredients were mixed with soybean and fish oils and water, then pelleted, air dried, and kept at 4°C until use. A stock of Persian sturgeon (*Acipenser persicus*) juveniles was transported from a fish farm in Gilan province, Iran, and kept in Ahmadabad-e Mostofi farm (Tehran province, Iran) in a 5000 L fiberglass tank. Fish were adapted to the laboratory conditions for two weeks and fed the basal diet twice daily (08:00 and 15:00). Afterward, fish were allocated in twelve fiberglass tanks, and each tank was stocked with thirty fish. Fish were fed the test diets at 3–4% of body weight for eight weeks. The water was exchanged daily with new water to guarantee high-quality water for the growth of fish. The water quality was determined and averaged 18.2±1.0°C, 6.8 ± 0.2 mg/L, 7.8 ± 0.21, 0.02 ± 0.01 mg/L, and 5836.2 ± 414.3 MM/cm for temperature, dissolved oxygen, pH, ammonia, and electrical conductivity, respectively.

Table 1. Dietary formulation and proximate composition of basal diet

| Ingredient | Composition (g/kg) | Proximate composition | (%) |
|---|---|---|---|
| Kilka fish meal | 445 | Crude protein | 40.1 |
| Wheat flour | 221.5 | Crude lipid | 15.3 |
| Wheat gluten | 60 | Ash | 18.2 |
| Starch corn | 50 | Fiber | 2.7 |
| Soybean oil | 100 | Moisture | 8.1 |
| Fish oil | 80 | NFE[e] | 18.1 |
| Mineral premix[a] | 10 | Gross energy (MJ/kg)[f] | 21.3 |
| Vitamin premix[a] | 15 | | |
| Cellulose | 10 | | |
| Binder[b] | 2 | | |
| Antifungal[c] | 3 | | |
| Antioxidant[d] | 3.5 | | |

[a]Premix detailed by Adel et al. (2020 b).
[b]AmetbinderTM, MehrTaban-e-Yazd, Iran.
[c]ToxiBan antifungal (Vet-A-Mix, Shenan- doah, IA).
[d]Butylated hydroxytoluene (BHT) (Merck, Germany).
[e]Nitrogen-free extracts (NFE) = dry matter – (crude protein + crude lipid + ash + fibre).
[f]Gross energy (MJ/kg) calculated according to 23.6 for protein, 39.5 for lipid and 17.0 kJ/g for NFE.

### Final sampling

At the end of the trial, all fish were anesthetized with clove oil (75 mg/L) and weighed to check their growth performance. The growth performance data were presented in our published study (Adel et al., 2021 b). Then three fish from each tank (9 fish per group) were collected to obtain blood samples and dissect their intestines and head kidney. The blood samples were obtained from the caudal vein of fish and kept in 1 mL non-heparinized tubes to allow the serum clot. Sera samples were collected through centrifugation at 3000 × g for 15 min then kept at –80ºC until use. The collected serum was used for the detection of thyroid-stimulating hormone (TSH), thyroxine (T4), and triiodothyronine (T3) by the ELISA method using commercial kits (Pishtaz Teb, Tehran, Iran).

### Intestinal bacterial count

The intestines were mixed with saline solution (1:9) then homogenized for 5 min. The homogenates were serially diluted with sterile saline solution. Around 0.1 mL of the diluted homogenate was inoculated onto Tryptic Soy Agar plates (TSA, Merck, Darmstadt, Germany) surface spread technique and after that incubated for 48 h at 36ºC. DeMan Rogosa and Sharpe (MRS) agar was used to detect the lactic acid bacteria at 36ºC for 72 h (Sallam, 2007). Following their respective incubation times, colonies were counted, and the bacterial count was expressed as log CFU/g intestine. All plates were performed in triplicate.

### Challenge test

For the challenge test, a lyophilized stock of *Aeromonas hydrophila* was obtained from the Ecology Research Center of the Caspian Sea (Sari, Iran) and cultivated in tryptic soy broth (TSB, Merck, Darmstadt,

Germany) at 25°C for 48 h. At the end of the eight week-feeding trial, ten fish from each tank (30 fish per group) were challenged by intraperitoneal (IP) injection with 0.1 mL of *A. hydrophila* in 0.9% (w/v) saline containing $3 \times 10^5$ colony forming units (CFU)/mL. In the control group, instead, 0.1 mL of PBS was injected. All fish were fed with a basal diet and kept under observation for 10 days to daily mortality (Adel et al., 2021 a; Baharloei et al., 2020). Finally, relative percent survival (RPS) values were calculated using the following formula described by Baharloei et al. (2020): RPS= 100 – ((treatment mortality/control mortality) ×100).

**Statistical analysis**

The data were subjected to statistical analysis using the SPSS software version no. 24 (SPSS Inc., Chicago, IL, USA). The statistical analysis was done using one-way variance analysis (ANOVA) followed by Duncan's multiple range tests. A P-value of <0.05 was considered significant. All the tests were performed in triplicate.

## Results

**Intestinal microbiota**

The total bacterial count in the intestines of Persian sturgeon showed no meaningful differences among the groups of fish fed varying levels of *G. persica* (P>0.05; Table 2). Fish fed 10 g/kg of *G. persica* had a higher LAB count than fish fed 0, 2.5, and 5 g/kg (P<0.05; Table 2).

Table 2. Average total count of bacteria and lactic acid bacteria (LAB) in the intestine (log CFU/g intestine) of *Acipenser persicus* fed with diets supplemented with *Gracilaria persica* powder at varying inclusion levels for 8 weeks

| Index | Control | 2.5 | 5 | 10 |
|---|---|---|---|---|
| Total count of bacteria | 4.92 ±0.04 a | 4.98 ±0.02 a | 4.95 ±0.1 a | 4.94 ±0.03 a |
| LAB | 1.76 ±0.01 b | 1.79 ±0.01 b | 1.83±0.02 b | 1.94 ±0.01 a |

Data are presented as mean ± SD (n=5). Data in the same row with different letters are significantly different (P<0.05).

**Thyroid-stimulating hormone (TSH), thyroxine (T4), and triiodothyronine (T3) levels**

Serum TSH showed higher levels in fish treated with 2.5 and 5 g/kg of *G. persica* than the other groups (P<0.05; Figure 1). Besides, fish fed 2.5 g/kg *G. persica* had higher T3 and T4 levels than the other groups (P<0.05; Figure 1). Fish fed 10 g/kg had no significant differences with the remaining groups in the case of TSH levels. While fish fed 5 g/kg had no significant differences with the remaining groups in the case of T3 levels (P>0.05; Figure 1).

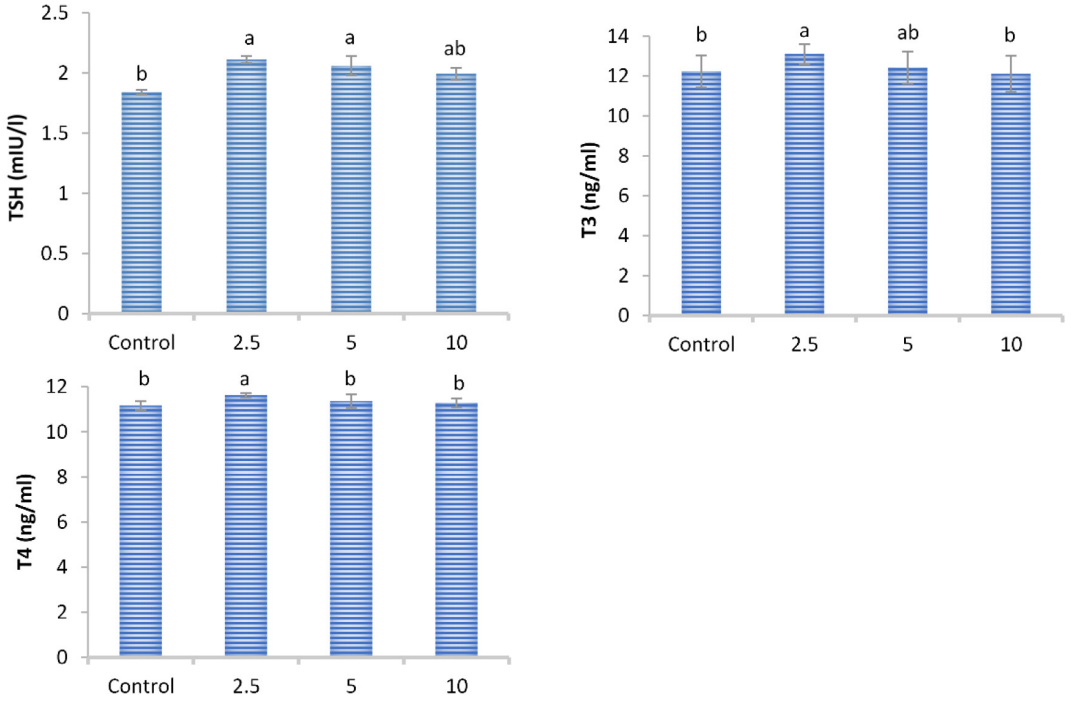



Figure 1. Serum thyroid-stimulating hormone (TSH), thyroxine (T4), and triiodothyronine (T3) levels in *A. persicus* fed diets enriched with different levels of *G. persica* powder for 8 weeks. Data are presented as mean ± SD (n=9). Bars with different superscript are significantly different (P<0.05)

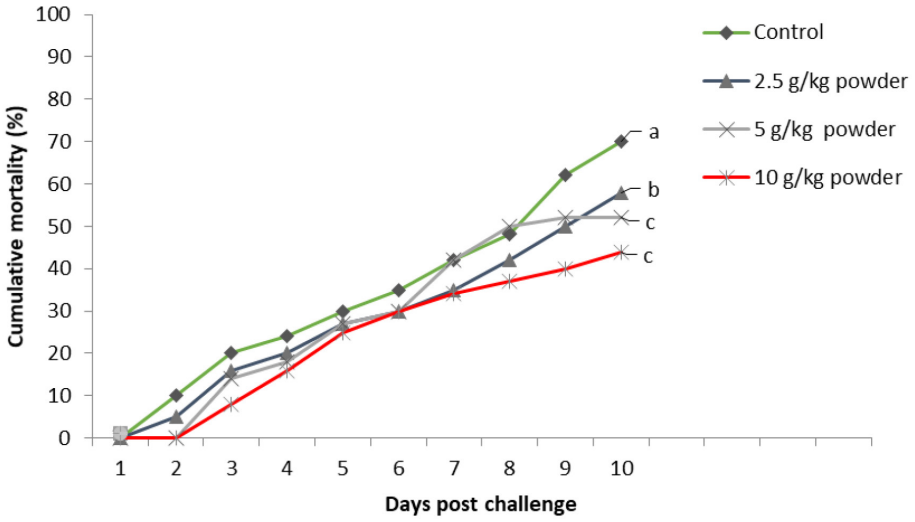



Figure 2. The percentage of cumulative mortality in *A. persicus* fed with diets supplemented with *G. persica* powder against *Aeromonas hydrophila*. Data are presented as mean ± SD (n=3). Bars with different superscript are significantly different (P<0.05)

**Challenge test**

At the end of the challenge test, the highest mortality was seen in the fish fed the *G. persica* free diet. However, fish fed *G. persica* showed a lower mortality rate than the control (P<0.05; Figure 2). Obviously, fish fed 5 and 10 g/kg *G. persica* had a lower mortality rate than fish fed 2.5 g/kg (P<0.05; Figure 2).

## Discussion

Using of seaweed and its extract is markedly applied in aquafeed to replace antibiotics and chemotherapies (Lim et al., 2018). Several beneficial effects of using seaweeds were observed in aquatic animals (Thépot et al., 2021). The enhancement of the growth performance and feed utilization of finfish species are among the beneficial roles of seaweeds. Our earlier study reported enhanced growth performance and feed utilization in Persian sturgeon treated with *Gracilaria persica* (Adel et al., 2021 b). The dose of 5–10 g/kg of *G. persica* is recommended as the optimum level for enhancing the growth performance of Persian sturgeon. We hypothesized that the improvement of the growth performance is probably attributed to the improved feed utilization and digestibility of nutrients. *G. persica* has abundant micronutrients, vitamins, and minerals involved in regulating metabolic functions (Peixoto et al., 2016). Besides, seaweed extracts have marked roles in increasing the amount of beneficial intestinal biota involved in regulating the absorption of digested nutrients (Walsh et al., 2013). Longitudinal results are observed in this study where Persian sturgeon treated with *G. persica* had an increased intestinal LAB population. The increased LAB may be attributed to the role of *G. persica* in blocking the attachment of pathogenic bacteria to the intestinal layer (Kulshreshtha et al., 2017). This allows the beneficial microorganisms to show their beneficial role in digesting consumed feed (Dawood, 2021). Concurrently, local intestinal immunity is expected to enhance because of *G. persica* feeding. The enhanced resistance to *A. hydrophila* infection in the current study is also related to activated local intestinal immunity (Hindu et al., 2019).

Thyroid-stimulating hormone (TSH) is produced from the thyroid gland to regulate the production of thyroxine (T4) and triiodothyronine (T3) (Bhat et al., 2017). TSH is a glycoprotein hormone that regulates the metabolic function, development, and growth of body cells (Blanton and Specker, 2007). Interestingly, *G. persica* treatment resulted in increasing the secretion of TSH in the blood of Persian sturgeon. The increased TSH may explain the enhanced growth performance of Persian sturgeon, as reported earlier in our published study (Milad et al., 2021).

Pathogenic invaders accidentally attack fish, resulting in a high mortality rate and lack of production (Hayatgheib et al., 2020). *Aeromonas hydrophila* is one of the bacterial infections resulting from decreased fish immunity (Adel et al., 2021 a). Using antibiotics is the traditional method to lower *A. hydrophila* infection, but seaweed is an alternative friendly option (Dawood et al., 2016). The results showed a low mortality rate in Persian sturgeon fed dietary *G. persica* compared with fish fed the control diet. *G. persica* is a red seaweed with high polysaccharides and antibacterial substances, which may reduce the populations of *A. hydrophila* (Wijnana et al., 2018). These results indicate that fish treated with *G. persica* had stable health status without impaired immunity. The results indicate the immunostimulatory role of *G. persica*. In line with the current study, Adel et al. (2021 b) stated that Persian sturgeon fed dietary *G. persica* had an enhanced immune response. Additionally,

*G. persica* had an immunostimulant effect involved in increasing the resistance of fish against pathogenic bacterial infection. Seaweed contains several bioactive compounds and polysaccharides known for their immunomodulating roles (Thépot et al., 2021). Polysaccharides, vitamin C, carrageenan, β-carotenes, agar, and alginate are abundantly presented in seaweed and can activate the local intestinal immunity and thereby the entire body's immunity (Hindu et al., 2019).

## Conclusion

In conclusion, using dietary *G. persica* at 5–10 g/kg is recommended to enhance the antibacterial capacity and resistance against *A. hydrophila* infection in Persian sturgeon. Besides, fish treated with *G. persica* had high TSH levels in the blood, indicating increased metabolic function. Consequently, Persian sturgeon treated with *G. persica* had increased resistance against *A. hydrophila* infection. The obtained results in this study, along with those reported earlier in our published article (Adel et al., 2021 b), confirm that *G. persica* is recommended as a natural growth promoter for enhancing Persian sturgeon productivity.

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