INTRODUCTION

Stressful conditions negatively affect the fish immune and antioxidant systems. Stress nature has significant effects on fish welfare and health (Tort, 2011). Among the stressors, high stocking density is common in aquaculture; moreover, intensive fish culture is an important issue in aquaculture, as it may increase farm income by boosting fish production per area unit or cause economical loss by growth and health deterioration (Yousefi, Abtahi, & Kenari, 2012). Fish differ in the case of tolerating high stocking density. Rainbow trout (Oncorhynchus mykiss) is an important aquaculture species in the world. Iran annual production of rainbow trout was more than 160,000 metric tons in 2016 according to the Annual Statistical Report of Iranian Fisheries Organization (Hoseini, Mirghaed, Iri, & Ghelichpour, 2018). The production of rainbow trout is about 30 kg/m³ using aeration, and aquaculturists are interested in intensification of rainbow trout. There are several reports on the effects of increased stocking density on rainbow trout (Oncorhynchus mykiss) immune and antioxidant systems. However, the effects of dietary tryptophan levels on these systems remain unclear. The present study aimed to investigate the effects of dietary tryptophan levels and fish stocking density on immunological and antioxidant responses and bactericidal activity against Aeromonas hydrophila in rainbow trout (Oncorhynchus mykiss).
density on rainbow trout health by monitoring the fish immune and antioxidant status. Yousefi, Paktnat, Mahmoudi, Pérez-Jiménez, and Hoseini (2016) and Naderi, Keyvanshokoo, Salati, and Ghaedi (2017) found that increase in stock density decreased plasma lysozyme activity and increased alternative complement (ACH50) activity of rainbow trout. Yarahmadi, Farahmand, Mandare, Mirvaghefi, and Hoseinifar (2014) found increase in the fish stocking density significantly decreased ACH50, bactericidal and total antioxidant activities, and IgM and leucocyte levels, but increased lysozyme activity. Sahin et al. (2014) and Taheri Mirghaed, Hoseini, and Ghelichpour (2018) found increased stocking density-induced oxidative stress, characterized by malondialdehyde (MDA) elevation and changes in antioxidant enzymes’ activities. Therefore, there is a need to find out methods mitigating adverse effects of high stocking density on rainbow trout immune and antioxidant system.

Tryptophan (Trp) is an essential amino acid in fish nutrition with many physiological roles, including modulation of stress response, immune response and antioxidant system [reviewed by Hoseini, Pérez-Jiménez, Costas, Azeredo, and Gesto (2019b)]. Modulation of immune system by Trp administration has been examined by different researchers, which found such a modulatory effects depend on the stress nature, Trp administration levels and duration, and fish species [reviewed by Hoseini et al. (2019b)]. However, it has been demonstrated that Trp potentiates to suppress stress in fish and thus may mitigate the stress-induced immunosuppression. There is only one study investigating the effects of Trp administration on immune responses of the fish under high stocking density. Azeredo et al. (2019) found that a moderate level of dietary Trp supplementation significantly improved the immune responses and disease resistance of Solea senegalensis, when reared under normal stocking density. A higher level of Trp administration had detrimental effects on fish under normal stocking density but improved the fish resistance against the disease, when they held under higher stocking density. Moreover, Trp may directly react with reactive oxygen species or improve the antioxidant enzymes’ activities; therefore, it is necessary for proper antioxidant system function [reviewed by Hoseini et al. (2019b)]. In this regard, previous studies have shown that deficient or excess dietary Trp administration led to oxidative stress, but optimum levels of the amino acid were necessary to prevent this in Ctenopharyngodon idella (Jiang et al., 2015; Wen et al., 2014) and Megalobrama amblycephala (Ji et al., 2019).

Considering the rare information about the effects of Trp administration on immune and antioxidant systems of fish under high stocking density, it was hypothesized that whether dietary Trp administration may protect rainbow trout against oxidative stress and immunosuppression under high stocking density. Thus, the present study aimed to investigate several immunological and antioxidant parameters of rainbow trout, reared under low and high stocking density for 70 days.

2 | MATERIALS AND METHODS

2.1 | Experimental protocol

This study was conducted in accordance with the ‘Ethics in Use of Aquatic Animals in Researches' and consulting with scientific board of Iranian Fisheries Sciences Research Institute. Rainbow trout with average weight of 63.4 ± 1.23 g was used in this experiment. The fish were stocked, for 1 weeks, in a rectangular tank with 2 × 2 m dimensions and 0.3 m water level. Then, the fish were distributed in 18 plastic tanks (0.3 m³, filled with 0.12 m³ water), connected to an air pump and water flow system. The tanks were divided into two classes of stocking density (each included nine tanks): low density (LD; 15 kg/m³ initial density) and high density (HD; 25 kg/m³ initial density). The fish of each density class were fed with three different diets (Table 1): control, T5 (control diet + 5 g Trp per kg) and T10 (control + 10 g Trp per kg) for 70 days. The fish were fed 20 g per kg biomass every day to have the highest feed efficiency (Hardy, 2002). The fish were weighed every 10 days to adjust feed amount. Water flow rate was 1.3 L/min per tank with the following physicochemical properties: temperature = 13.8 ± 0.68°C, pH = 8.00 ± 0.02, dissolved oxygen = 6.03 ± 0.23 mg/L, unionized ammonia = 0.02 ± 0.005 mg N/L.

The amino acid compositions of the three diets were determined by HPLC according to Hoseini, Hosseini, Eskandari, and Amirrahmani (2016). The samples were digested for 22 hr at HCl (6 M); derivatization was performed using O-phthalaldehyde, and the samples were injected to HPLC, equipped to fluorescent detector (Agilent 1090 system). Each amino acid levels were determined based on its corresponding standard peak.

The proximate compositions of the diets and fish carcasses were determined according to AOAC (2005). The samples' moisture percentage was determined by heating at 105°C for 24 hr in an oven. Crude protein and crude lipid percentages were determined by Kjeldahl and soxhlet apparatus. Crude ash percentage was determined by burning (550°C) the samples in furnace for 8 hr.

2.2 | Blood sampling and analysis

At the end of the experiment, two fish were sampled from each tank and anesthetized by eugenol (100 mg/L). Then, the fish blood samples were taken by heparinized syringes from the caudal vein. The blood samples were divided into two portions: one for haematological study and the other for plasma separation. White blood cells (WBC) were diluted in Dacie’s solution and counted by a Neubauer chamber. White blood cells differential counts were performed after preparing the blood smears and staining with May–Giemsa solution (Blaxhall, 1972). Plasma were separated after centrifugation for 7 min (1200 g) and used for determination of Trp, globulin, total immunoglobulin (Ig), malondialdehyde (MDA) levels, total antioxidant capacity (TAC), lysozyme, alternative complement (ACH50), superoxide dismutase (SOD), catalase (CAT) and bactericidal activities.

Plasma Trp levels were determined using HPLC (Younglin 9300), after derivatization with O-phthalaldehyde. The apparatus was equipped with a fluorescent detector and C18 column (GL Sciences) (Hoseini et al., 2016).

Plasma globulin levels were determined by subtracting the plasma total protein and albumin. The plasma total protein and
albumin were determined spectrophotometrically using commercial kits (Pars Azmun). Plasma total Ig levels were determined after precipitation with polyethylene glycol, according to Siwicki and Anderson (1993) method.

Plasma MDA was measured based on reaction with thiobarbituric acid using a commercial kit (ZellBio, GmbH). Plasma TAC was determined based on reducing ferric ions by the plasma using a commercial kit (ZellBio, GmbH). Superoxide dismutase activity was measured in the plasma based on the plasma ability to convert superoxide anion to hydrogen peroxide and oxygen, using a commercial kit (ZellBio, GmbH). Plasma CAT activity was determined according to Goth (1991), based on the decomposition rate of hydrogen peroxide.

Plasma bactericidal activity was determined according to Zargari, Mazandarani, and Hoseini (2018) with some modifications. *Aeromonas hydrophila* was cultured on nutrient agar and suspended in phosphate-buffered saline (PBS; pH 7) to have an optical density of 0.5 at 546 nm. Then, the suspension was diluted with PBS (pH 7) eight times. To 2 ml of the diluted suspension, 200 µl of plasma was added and the mixture incubated for 24 hr. Then, the suspension was cultured on nutrient agar and the colonies were counted after 24 hr. Bactericidal activity was calculated as follow:

\[ \text{Bactericidal activity} = 100,000 \times \left( \frac{1}{\text{colony number}} \right) \]

### 2.3 | Statistical analysis

The data normal distribution (Shapiro–Wilk) and the treatments variance homogeneity (Levene test) were confirmed before analysis. The percentile data were arc-sin transformed. The data were subjected to two-way ANOVA with stocking density and dietary Trp levels as the factors. Where there was an interaction effect of the stocking density and dietary Trp levels on the tested variables, the data were analysed by one-way ANOVA and Duncan test. All data were presented as mean ± SE. The statistical software, SPSS v.22, was used for the statistical analysis.

### 3 | RESULTS

There were no significant effects of dietary Trp on water physicochemical properties; however, stocking density significantly affected them. Dissolved oxygen (DO; \( p = .031 \)) and pH (\( p = .039 \)) significantly decreased, whereas unionized ammonia significantly (\( p = .008 \)) increased along with increase in the fish stocking density (Table 2).

There was an interaction effect of dietary Trp and stocking density on plasma Trp levels. Under the LD condition, the Trp-treated groups had similar plasma Trp levels, being significantly higher than the control group. Under the HD condition, the control and T5 groups had

<p>| TABLE 1 | Ingredients, chemical composition and amino acid profile of the diets |</p>
<table>
<thead>
<tr>
<th>Ingredients (g/kg)</th>
<th>Diets</th>
<th>Amino acid composition (% of dietary protein)</th>
<th>Diets</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soybean meal</td>
<td>Control</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>Fish meal</td>
<td>Control</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>Wheat gluten</td>
<td>Control</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Poultry by-product</td>
<td>Control</td>
<td>230</td>
<td>230</td>
</tr>
<tr>
<td>Wheat meal</td>
<td>Control</td>
<td>166</td>
<td>161</td>
</tr>
<tr>
<td>Fish oil</td>
<td>Control</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>Control</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Phytase</td>
<td>Control</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Lysine</td>
<td>Control</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Methionine</td>
<td>Control</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Mineral premix</td>
<td>Control</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Vitamin premix</td>
<td>Control</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>Control</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Proximate composition (%)</td>
<td></td>
<td>met 2.67</td>
<td>2.71</td>
</tr>
<tr>
<td>Moisture</td>
<td>Control</td>
<td>8.74</td>
<td>8.75</td>
</tr>
<tr>
<td>Crude protein</td>
<td>Control</td>
<td>40.6</td>
<td>40.9</td>
</tr>
<tr>
<td>Crude fat</td>
<td>Control</td>
<td>18.4</td>
<td>18.3</td>
</tr>
<tr>
<td>Crude fibre</td>
<td>Control</td>
<td>1.04</td>
<td>1.04</td>
</tr>
<tr>
<td>Crude ash</td>
<td>Control</td>
<td>7.23</td>
<td>7.21</td>
</tr>
</tbody>
</table>
Increase in stocking density led to significant increase and decrease in plasma Trp levels in the control and T10 groups, respectively, and, however, had no significant effects on T5 groups (Figure 1).

Dietary Trp and stocking density had interaction effects on plasma globulin levels and lysozyme activity; however, there were no significant effects of dietary Trp, stocking density and their interaction on plasma ACH50 activity and total Ig levels. There was no significant difference in plasma globulin levels among the control, T5 and T10 groups under the LD condition; however, the control and T5 groups had similar globulin levels under the HD condition, and both were significantly higher than that of the T10 group. The control and T10 groups had similar plasma lysozyme activities under the LD condition, being significantly lower than the T5 groups. However, under the HD condition, the Trp-treated fish had similar plasma lysozyme activities, significantly higher than the control group (Figure 2).

All the experimental groups had statistically similar percent - ages of blood neutrophil, lymphocyte, monocyte and eosinophil (Table 3); nevertheless, there were interaction effects of dietary Trp and stocking density on blood WBC and plasma bactericidal activity. The control and T5 groups had similar blood WBC under the LD condition, significantly lower than the T1. Under the HD condition, the T5 group had higher blood WBC compared to the control; however, the T10 group had no significant difference compared to the other groups. The highest and lowest plasma bactericidal activities were related to the T5 and control group, respectively. Increase in the stocking density led to significant increase in plasma bactericidal activity in the control and T5 groups, but significantly decreased the activity in the T10 group (Figure 3).

All the experimental groups had statistically similar plasma TAC levels and SOD activities; nevertheless, dietary Trp and stocking density effects on plasma and liver had significant effects. However, there were no significant effects of dietary Trp, stocking density and their interaction on plasma Trp concentrations of rainbow trout fed with Trp-supplemented diets and reared under low and high stocking densities for 70 days. Different letters above the bars show significant differences among the treatments (mean ± SE; Duncan test; n = 6).

**FIGURE 1** Plasma Trp concentrations of rainbow trout fed with Trp-supplemented diets and reared under low and high stocking densities for 70 days. Different letters above the bars show significant differences among the treatments (mean ± SE; Duncan test; n = 6).

**TABLE 2** Effects of dietary Trp supplementation and rearing density of rainbow trout on water physiochemical parameters

<table>
<thead>
<tr>
<th></th>
<th>LD</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DO (mg/L)</td>
<td>pH</td>
<td>Unionized ammonia (mg N/L)</td>
</tr>
<tr>
<td>LD Control</td>
<td>6.66 ± 0.85</td>
<td>8.04 ± 0.03</td>
<td>0.013 ± 0.001</td>
</tr>
<tr>
<td>LD T5</td>
<td>6.40 ± 0.96</td>
<td>8.10 ± 0.02</td>
<td>0.011 ± 0.001</td>
</tr>
<tr>
<td>LD T10</td>
<td>6.45 ± 0.90</td>
<td>8.02 ± 0.04</td>
<td>0.012 ± 0.001</td>
</tr>
<tr>
<td>HD Control</td>
<td>5.03 ± 0.75</td>
<td>7.91 ± 0.05</td>
<td>0.033 ± 0.001</td>
</tr>
<tr>
<td>HD T5</td>
<td>5.20 ± 0.89</td>
<td>8.02 ± 0.04</td>
<td>0.028 ± 0.002</td>
</tr>
<tr>
<td>HD T10</td>
<td>6.40 ± 0.88</td>
<td>7.98 ± 0.03</td>
<td>0.030 ± 0.001</td>
</tr>
<tr>
<td>LD 0</td>
<td>5.67 ± 0.89</td>
<td>7.97 ± 0.06</td>
<td>0.023 ± 0.009</td>
</tr>
<tr>
<td>LD 0.5</td>
<td>5.80 ± 0.91</td>
<td>8.04 ± 0.07</td>
<td>0.020 ± 0.007</td>
</tr>
<tr>
<td>LD 1</td>
<td>6.44 ± 0.83</td>
<td>8.00 ± 0.06</td>
<td>0.021 ± 0.007</td>
</tr>
<tr>
<td>HD</td>
<td>6.47 ± 1.02b</td>
<td>8.06 ± 0.07b</td>
<td>0.012 ± 0.001a</td>
</tr>
<tr>
<td>HD</td>
<td>5.18 ± 1.04a</td>
<td>7.96 ± 0.06a</td>
<td>0.030 ± 0.001b</td>
</tr>
</tbody>
</table>

**Note:** Different letters show significant differences.

**TABLE 2** Effects of dietary Trp supplementation and rearing density of rainbow trout on water physiochemical parameters

- **Trp effects:**
  - LD Trp: 0.350
  - HD Trp: 2-Way ANOVA
- **Stocking density effects:**
  - LD Stocking density: 0.031
  - HD Stocking density: 0.039
- **Trp × Stocking density:**
  - 0.652
  - 0.797

**2-Way ANOVA**

- **Trp:**
  - 0.350
  - 0.749
  - 0.547
- **Stocking density:**
  - 0.031
  - 0.039
  - 0.008
- **Trp × Stocking density:**
  - 0.652
  - 0.797

**Note:** Different letters show significant differences.
density had interaction effects on plasma CAT activities and MDA levels. Under the LD condition, the Trp-treated groups had similar plasma CAT activities, significantly higher than the control group. Under the HD condition, the Trp-treated groups had similar plasma CAT activities, significantly lower compared to the control group. Plasma CAT activity showed significant decrease in the control, but significant increases in the Trp-treated groups, when the stocking density increased. Under both the LD and HD conditions, the control and T10 groups had similar plasma MDA levels, significantly higher than the T5 group. Increase in the stocking density led to significant increase in plasma MDA levels in all the experimental groups; the T10 groups had higher elevation in MDA levels, compared to the other groups (Figure 4).

**FIGURE 2** Plasma globulin, lysozyme, ACH50 and total Ig of rainbow trout fed with Trp-supplemented diets and reared under low and high stocking densities for 70 days. Different letters above the bars show significant differences among the treatments (mean ± SE; Duncan test; n = 6).

**TABLE 3** Effects of dietary Trp supplementation and rearing density on blood WBC differential count of rainbow trout

<table>
<thead>
<tr>
<th>Stocking density</th>
<th>Trp levels</th>
<th>Neutrophil (%)</th>
<th>Lymphocyte (%)</th>
<th>Monocyte (%)</th>
<th>Eosinophil (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LD</strong></td>
<td>Control</td>
<td>19.3 ± 1.28</td>
<td>74.3 ± 1.51</td>
<td>5.66 ± 0.61</td>
<td>0.66 ± 0.23</td>
</tr>
<tr>
<td></td>
<td>T5</td>
<td>19.3 ± 0.83</td>
<td>74.3 ± 0.61</td>
<td>4.66 ± 0.23</td>
<td>1.00 ± 0.40</td>
</tr>
<tr>
<td></td>
<td>T10</td>
<td>21.7 ± 0.61</td>
<td>73.0 ± 1.06</td>
<td>5.00 ± 0.40</td>
<td>0.33 ± 0.23</td>
</tr>
<tr>
<td><strong>HD</strong></td>
<td>Control</td>
<td>21.0 ± 0.40</td>
<td>73.0 ± 1.10</td>
<td>5.00 ± 0.40</td>
<td>1.00 ± 0.40</td>
</tr>
<tr>
<td></td>
<td>T5</td>
<td>20.6 ± 0.61</td>
<td>73.0 ± 0.69</td>
<td>5.66 ± 0.23</td>
<td>0.66 ± 0.46</td>
</tr>
<tr>
<td></td>
<td>T10</td>
<td>18.0 ± 0.40</td>
<td>77.0 ± 1.06</td>
<td>4.00 ± 0.40</td>
<td>1.00 ± 0.40</td>
</tr>
</tbody>
</table>

**DISCUSSION**

Serotonin and melatonin are products of Trp metabolism that modulate stress responses in fish. Trp availability for the fish brain determines serotonergic and melatonergic activities; thus, stressors may change circulating levels of Trp (Hoseini et al., 2019b). There are controversies in the results of different studies about the effects of dietary Trp and/or stress on fish plasma Trp levels. For example in *S. senegalensis*, repeated acute stresses for 9 weeks or chronic stress for 63 days led to significant decrease in plasma Trp levels (Aragao, Corte-Real, Costas, Dinis, & Conceição, 2008; Costas, Aragão, Mancera, Dinis, & Conceição, 2008); however, a short-term acute...
stress led to significant increase in plasma Trp levels (Costas et al., 2011). In rainbow trout, elevated dietary Trp intake significantly increased plasma Trp; however, short-term stressors had no significant effects on plasma Trp levels (Lepage, Tottmar, & Winberg, 2002; Lepage, Vilchez, Pottinger, & Winberg, 2003; Winberg, Øverli, & Lepage, 2001). The interaction effect of dietary Trp levels and stress on plasma Trp levels has not been reported previously. Further studies are necessary to illustrate this topic.

Plasma globulin level is an indicator of fish health, and inappropriate conditions lead to decline in plasma globulin levels (Ghelichpour, Taheri Mirghaed, Mirzargar, Joshaghani, & Ebrahimzadeh Mousavi, 2017). Plasma lysozyme is an important immune-related enzyme that increases under stressful conditions or immunostimulant administration (Hoseinifar et al., 2019; Saurabh & Sahoo, 2008). Stressful conditions increase blood WBC and bactericidal activity (Azeredo et al., 2019).

**Figure 3** Plasma bactericidal activity against A. hydrophila and blood WBC count of rainbow trout fed with Trp-supplemented diets and reared under low and high stocking densities for 70 days. Different letters above the bars show significant differences among the treatments (mean ± SE; Duncan test; n = 6).

**Figure 4** Plasma total antioxidant capacity, superoxide dismutase, catalase and malondialdehyde of rainbow trout fed with Trp-supplemented diets and reared under low and high stocking densities for 70 days. Different letters above the bars show significant differences among the treatments (mean ± SE; Duncan test; n = 6).
might, partly, contribute in immunosuppression in the HD treatments (Gonçalves et al., 2017; Ni et al., 2014; Xing et al., 2016). The present results showed Trp at 5 g per kg diet significantly improved the fish immune strength, but 10 g Trp per kg diet significantly suppressed the immune strength of the fish. Immunomodulatory effects of Trp have been assessed in several studies; however, the results have been variable and contradictory [reviewed by Hoseini et al. (2019b)]. Several studies have shown Trp administration significantly mitigated immunosuppression caused by chronic thermal (Akhtar, Pal, Sahu, & Ciji, 2013b; Akhtar et al., 2013a; Kumar et al., 2018), osmotic (Akhtar et al., 2013b, 2013a) and pollution (Ciji, Sahu, Pal, & Akhtar, 2015) stresses in different species. Interestingly, Azeredo et al. (2019) found that chronic stress caused by stocking density led to increased need for dietary Trp for elevation in S. senegalensis survival against a pathogen, but such a Trp level deteriorated the fish survival against the pathogen under normal stocking density. The present results suggest 10 g Trp per kg diet was surplus causing health deterioration and immunosuppression in the fish. These results are in line with Machado et al. (2019) showing that surplus dietary Trp significantly compromised immune responses in fish.

Oxidative stress is common when fish are reared under high stocking density (Pérez Jiménez et al., 2009; Yousefi, Vatnikov, Kulikov, & Ghelichpour, 2019). Moreover, increase in water ammonia leads to oxidative stress in fish (Hegazi, Attia, & Ashour, 2010; Hoseini, Yousefi, Hoseinfar, & Van Doan, 2019a). Similar to present results, Taheri Mirghaed et al. (2018) reported that high stocking density significantly increased oxidative stress characterized by MDA elevation. Trp has antioxidant ability by direct reaction with pro-oxidant molecules and/or stimulation of the antioxidant enzymes (Hoseini et al., 2019b). The present results showed that 5 g Trp per kg diet potentiated to suppress oxidative stress both under the LD and HD conditions. But, 10 g Trp per kg diet had no benefits on oxidative stress. The present results are in line with Jiang et al. (2015), Wen et al. (2014) and Ji et al. (2019) reporting an optimum levels of dietary Trp significantly suppressed oxidative stress in C. idella and M. amblycephala.

In conclusion, the present results show that dietary Trp supplementation at 5 g per kg diet is beneficial in improving rainbow trout immune and antioxidant defence both under LD and HD conditions. Dietary Trp levels and stocking density stress have interaction effects on the immunological and antioxidant responses, which complicate interpreting the mode of action of Trp on these responses.

**ACKNOWLEDGMENT**

This study was supported by Iran National Science Foundation (INSF).

**CONFLICT OF INTEREST**

There is no conflict of interest about this article

**DATA AVAILABILITY STATEMENT**

Research data are not shared.

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