



## DIETARY *CHLORELLA VULGARIS* MITIGATED THE ADVERSE EFFECTS OF IMIDACLOPRID ON THE GROWTH PERFORMANCE, ANTIOXIDANT, AND IMMUNE RESPONSES OF COMMON CARP (*CYPRINUS CARPIO*)

Andrés Alexis Ramírez-Coronel<sup>1,2,3</sup>, Saade Abdalkareem Jasim<sup>4</sup>, Ali Hasanpour Ardekani Zadeh<sup>5</sup>, Mohammed Abed Jawad<sup>6</sup>, Ghaidaa Raheem Lateef Al-Awsi<sup>7</sup>, Ali Hussein Adhab<sup>8</sup>, Gayrat Kodirov<sup>9</sup>, Zahra Soltanifar<sup>5</sup>, Yasser Fakri Mustafa<sup>10</sup>, Maryam Norbakhsh<sup>11\*</sup>

<sup>1</sup>Health and Behavior Research Group (HBR), Psychometry and Ethology Laboratory, Catholic University of Cuenca, Ecuador

<sup>2</sup>University of Palermo, Buenos Aires, Argentina

<sup>3</sup>CES University, Colombia

<sup>4</sup>Medical Laboratory Techniques Department, Al-maarif University College, Al-anbar-Ramadi, Iraq

<sup>5</sup>Department of Fisheries, Faculty of Natural Resources, University of Tehran, Karaj, Iran

<sup>6</sup>Al-Nisour University College, Baghdad, Iraq

<sup>7</sup>College of Science, University of Al-Qadisiyah, Iraq

<sup>8</sup>Department of Medical Laboratory Technics, Al-Zahrawi University College, Karbala, Iraq

<sup>9</sup>Jizzakh State Pedagogical Institute named after Abdullah Kadiri, Jizzakh, Uzbekistan

<sup>10</sup>Department of Pharmaceutical Chemistry, College of Pharmacy, University of Mosul, Mosul-41001, Iraq

<sup>11</sup>Department of Microbiology, Faculty of Biology, Islamic Azad University Science and Research Branch, Tehran, Iran

\*Corresponding author: microbiologymary2009@gmail.com

### Abstract

The use of pesticides to eliminate pests and weeds has raised concerns about water pollution and adverse effects on aquatic organisms, so many efforts have been made to increase the resistance of fish to these pesticides by using a proper nutrition strategy. Therefore, the aim of this study is to investigate the growth performance, antioxidant, and immune responses of fish exposed to Imidacloprid insecticide ( $C_9H_{10}ClN_3O_2$ ) by different doses of *Chlorella vulgaris* dry powder to the diet of common carp (*Cyprinus carpio*). In this study, 600 common carp with a medium weight ( $18.10 \pm 0.2$  g; mean  $\pm$  SE) were prepared and after adaptation and determination of lethal concentration of Imidacloprid, for 56 days in 6 treatments and each with 3 replications were classified and tested (Control (T1), 5% *Chlorella vulgaris* dry powder and no pollution (T2), 10% *Chlorella vulgaris* dry powder and no pollution (T3), No *Chlorella vulgaris* dry powder and 12.5%  $LC_{50}$  Imidacloprid (T4), 5% *Chlorella vulgaris* dry powder plus 12.5%  $LC_{50}$  Imidacloprid (T5) and 10% *Chlorella vulgaris* dry powder plus 12.5%  $LC_{50}$  Imidacloprid (T6)). After 96 hours of exposure to distinct concentrations of the insecticide, the total mortality was measured and the Imidacloprid median lethal concentration ( $LC_{50}$ ) over 96 hours was calculated (266.2 mg/l) using Probit analysis. According to the result, common carp fed T2 had the highest final weight (FW), weight gain (WG), and specific growth rate (SGR), and the lowest feed conversion ratio (FCR) among the groups ( $P < 0.05$ ). Fish in the T2 group had the highest total proteins, albumin and globulin ( $P < 0.05$ ). Fish in the group T4 had the highest cortisol, lactate dehydrogenase (LDH), alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) levels in the blood, while fish fed T2 and T3 had low values ( $P < 0.05$ ). The alternative complement pathway ( $ACH_{50}$ ) was significantly higher in T2 and T3 than other groups ( $P < 0.05$ ). Blood total immunoglobulin (Ig) and lysozyme activity were high in T2 and T3 groups, and had the lowest values in the T4 group ( $P < 0.05$ ). The superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) showed the highest activities in T2 ( $P < 0.05$ ). T4 group had the highest malondialdehyde (MDA) level, while T2 and T3 groups had the lowest MDA level ( $P < 0.05$ ). The highest amylase, protease and lipase were in the T2 group, while the lowest values were in the T4 group ( $P < 0.05$ ). In conclusion, dietary *Chlorella vulgaris* protects common carp from Imidacloprid insecticide, since it improved growth performance, antioxidant and immune responses of fish.

**Key words:** microalgae, immunity, growth promoter, insecticide, fish

The use of a wide range of chemicals to destroy pests and weeds is an important aspect of agricultural practice in both developed and developing countries (Tudi et al., 2021). Undoubtedly, this has increased crop yield and reduced postharvest losses. However, the expanded use of such pesticides expectedly results in residues in nature, which has led to widespread concern over the potential adverse effects of these chemicals on the environment and wildlife, aquaculture as well as human food (Neamat-Allah et al., 2020; Farag et al.,

2021; Neamat-Allah et al., 2021). Water contamination by pesticides is known to induce harmful impacts on the production, reproduction, and survivability of living aquatic organisms, such as algae, aquatic plants, and fish (shellfish and finfish species) (Jabeen et al., 2015; Albano et al., 2021).

Numerous strategies have been undertaken to reduce these negative effects. Some have included less dangerous chemical replacements, others would either reduce the amount and dose of consumption or apply

natural pesticides; yet none of these methods are effective (Liu et al., 2016; De Souza et al., 2020; Khafaga et al., 2020). There is a huge demand for chemical pesticides each year worldwide. Some recent studies have been focusing on increasing the physical resistance of target animals to these toxins (Özkara et al., 2016; Dawood et al., 2020; Abd El-Hameed et al., 2021; Ismael et al., 2021).

Fishponds, which are usually built outdoors and sometimes on farmland, can be very vulnerable, and improper use of pesticides can result in the loss of entire pond production (Rani et al., 2021). Dawood et al. reported in 2020 that various aquatic pollutants derived from industrial effluents, overuse of some agricultural pesticides, herbicides, and insecticides may cause devastating toxicological aspects of aquatic organisms and deteriorate their health and growth and consequently threaten human health.

Today, many studies have focused on using dietary immunostimulants as an effective solution to prevent and treat toxin-related diseases in aquatic species (Gannam and Schrock, 1999; Mehana et al., 2015; Abdel-Tawwab et al., 2018; Adeshina et al., 2019; Abdel-Tawwab and El-Araby, 2021; Ghafarifarsani et al., 2022; Yousefi et al., 2022). One of the most economically beneficial methods to increase the resistance of fish to toxins has been improving the quality of their diet (Hua and Relyea, 2014; Khafaga et al., 2020; Ismael et al., 2021). A balanced diet in terms of protein, carbohydrate, vitamin, mineral, etc composition along with extra super-nutrient added compounds (superfoods) can act positively and effectively on the physical and immune resistance of fish against environmental toxins (Leung and Bates, 2013).

Microalgae are one of the best natural sources of nutrients. Microalgae, namely *Chlorella* sp., have recently received a great deal of attention for their high nutritional value, antioxidant capacity, and health benefits (Pina-Pérez et al., 2019). Adding a certain dose of microalgae dry powder to fish diet can result in a significant increase in their immune resistance to toxins (Nayak et al., 2020; Chen et al., 2021; Nagappan et al., 2021).

The insecticide Imidacloprid ( $C_9H_{10}ClN_5O_2$ ) is widely used against sucking insects. This compound has a high absorption capacity and is absorbed in large quantities by plant roots and stored in plant tissues (Contardo-Jara and Gessner, 2020). The insecticide can be transmitted to fish through oral, climatic, and intermediate organism pathways (Crayton et al., 2020), forming a big network to transfer these pesticides to the fishpond and it is almost impossible to prevent toxin penetration into the fish body (Roche et al., 2018). This is where scientists concluded to investigate more about how to increase fish immunity against such chemicals through safe, natural, oral bioproducts (Abdulrahman et al., 2019; Serradell et al., 2020; Khanjani et al., 2021).

The present study focuses on the effects of adding different doses of dry *Chlorella vulgaris* powder to the

common carp diet. Parameters included the effects of the microalgae diet on growth and nutrition indices, immune indices of blood serum and mucus, intestinal microbial population, and activity of digestive enzymes.

## Material and methods

### Fish reservoir and raising treatments

Six hundred common carp juveniles weighing about  $18.10 \pm 0.2$  g (mean  $\pm$  SE) on average were obtained from a private farm and kept at 24–25°C for two weeks to adapt to laboratory conditions. Feeding was applied using basal dietary (BD) food (Table 1). After the adaptation period and in order to design an experiment, 6 treatments, and 3 triplications were defined resulting in a total of 540 fish which were randomly distributed in 18 fiberglass 300-L tanks (150 L water: 30 fish per each ( $20.38 \pm 0.44$  g; mean  $\pm$  SE)).

The fish were divided into 6 experimental groups as follows:

| Experimental groups | Commercial basic diet | Contaminated environment (12.5% LC <sub>50</sub> Imidacloprid) | 5% <i>Chlorella vulgaris</i> | 10% <i>Chlorella vulgaris</i> |
|---------------------|-----------------------|----------------------------------------------------------------|------------------------------|-------------------------------|
| Control – T1        | *                     |                                                                |                              |                               |
| T2                  | *                     |                                                                | *                            |                               |
| T3                  | *                     |                                                                |                              | *                             |
| T4                  | *                     | *                                                              |                              |                               |
| T5                  | *                     | *                                                              | *                            |                               |
| T6                  | *                     | *                                                              |                              | *                             |

Table 1. Feedstuffs and compositions of the experimental diets (%) (Yousefi et al., 2020)

|                                              | Control | CH 5% | CH 10% |
|----------------------------------------------|---------|-------|--------|
| Fish meal (67%)                              | 10      | 10    | 10     |
| Soybean meal (46%)                           | 23      | 18    | 13     |
| <i>Chlorella vulgaris</i> (powder)           | 0       | 5     | 10     |
| Meat meal                                    | 21      | 21    | 21     |
| Wheat meal                                   | 42.3    | 42.3  | 42.3   |
| Fish oil                                     | 1       | 1     | 1      |
| Soybean oil                                  | 1       | 1     | 1      |
| Lysine                                       | 0.7     | 0.7   | 0.7    |
| Methionine                                   | 0.5     | 0.5   | 0.5    |
| Vitamin mix                                  | 0.25    | 0.25  | 0.25   |
| Mineral mix                                  | 0.25    | 0.25  | 0.25   |
| Biochemical status of experimental diets (%) |         |       |        |
| dry matter (%)                               | 90.6    | 90.81 | 90.98  |
| crude protein (%)                            | 38.9    | 40.01 | 40.10  |
| crude fat (%)                                | 8.65    | 8.90  | 9.1    |
| crude ash                                    | 6.11    | 6.20  | 6.41   |

Table 2. Lethal concentrations (LC<sub>10-90</sub>) of Imidacloprid depending on time (24–96 h) for *Cyprinus carpio*

| Point            | Concentration (mg/l) (95% of confidence limits) |                            |                         |                            |                         |                            |                         |                            |
|------------------|-------------------------------------------------|----------------------------|-------------------------|----------------------------|-------------------------|----------------------------|-------------------------|----------------------------|
|                  | LC <sub>50</sub><br>24h                         | Upper Bound<br>Lower Bound | LC <sub>50</sub><br>48h | Upper Bound<br>Lower Bound | LC <sub>50</sub><br>72h | Upper Bound<br>Lower Bound | LC <sub>50</sub><br>96h | Upper Bound<br>Lower Bound |
| LC <sub>10</sub> | 308.5                                           | 208.7                      | 203.3                   | 125.3                      | 179.7                   | 105.4                      | 156.0                   | 88.28                      |
|                  |                                                 | 308.5                      |                         | 203.3                      |                         | 179.7                      |                         | 156                        |
|                  |                                                 | 258.6                      |                         | 202.1                      |                         | 183.3                      |                         | 159.7                      |
| LC <sub>30</sub> | 405.9                                           | 405.9                      | 272.3                   | 272.3                      | 248.9                   | 248.9                      | 215.7                   | 215.7                      |
|                  |                                                 | 281.4                      |                         | 245.8                      |                         | 227.3                      |                         | 200.1                      |
|                  |                                                 | 485.1                      |                         | 329.7                      |                         | 306.8                      |                         | 266.2                      |
| LC <sub>50</sub> | 485.1                                           | 485.1                      | 329.7                   | 329.7                      | 306.8                   | 306.8                      | 266.2                   | 266.2                      |
|                  |                                                 | 301.1                      |                         | 284.6                      |                         | 266.3                      |                         | 235.4                      |
|                  |                                                 | 567.3                      |                         | 391.9                      |                         | 369.7                      |                         | 321.7                      |
| LC <sub>70</sub> | 567.3                                           | 567.3                      | 391.9                   | 391.9                      | 369.7                   | 369.7                      | 321.7                   | 321.7                      |
|                  |                                                 | 327.5                      |                         | 336.7                      |                         | 318.8                      |                         | 282.7                      |
|                  |                                                 | 688.3                      |                         | 485.6                      |                         | 464.3                      |                         | 405.5                      |
| LC <sub>90</sub> | 688.3                                           | 688.3                      | 485.6                   | 485.6                      | 464.3                   | 464.3                      | 405.5                   | 405.5                      |
|                  |                                                 |                            |                         |                            |                         |                            |                         |                            |
|                  |                                                 |                            |                         |                            |                         |                            |                         |                            |

75% of tank water was changed every day during the breeding period and the same rate of Imidacloprid solution was added to each tank along with fresh water. The tanks were continuously aerated, the suspended particles were siphoned daily and the biomass of each tank was weighed every two weeks to adjust the feed based on Yousefi et al. (2020) method. The specimens were fed based on 2% of the biomass, daily (8:00, 13:00 and 18:00) (Yousefi et al., 2020). Physicochemical factors of water including temperature (24.2±0.7°C), dissolved oxygen (6.6±0.68 mg/l), pH (7.4 ±0.6) and non-ionized ammonia (0.05±0.01) were measured. Feeding was done twice a day at 20 g/kg of biomass (Hoseini et al., 2020).

#### Determination of acute toxicity of Imidacloprid

At first, in order to determine the lethal concentration of fifty percent of fish (LC<sub>50</sub>), before the main exposure test, it is necessary to determine the lethal range and the acute concentration of Imidacloprid on common carp, so that after this range determination, appropriate doses can be selected. For this purpose, after the adaptation period, the fish were exposed to different concentrations of this poison to obtain its lethal range. Therefore, fish (n=30, 10 fish per replicates) were exposed to concentrations of 0 (Control), 30, 60, 120, 240, and 480 mg/l Imidacloprid to determine the LC<sub>50</sub> according to the standard method (OECD, 1994) during 96 h. Finally, based on the Probit statistical analysis method, the values of LC<sub>10</sub>, LC<sub>30</sub>, LC<sub>50</sub>, LC<sub>70</sub>, and LC<sub>90</sub> were analyzed at 24, 48, 72 and 96 h (Table 2).

#### Diet preparation

*Chlorella vulgaris* dry powder was purchased from Persian Iran Microalgae Company (chemical composition including 51.49% protein, 7.75% lipid, 31.69% carbohydrates, 2.97% moisture, and 6.1% ash). In order to prepare experimental diets, each food ingredient was obtained separately from different companies in kilo-

grams and mixed after weighing in proper doses. Then, some water was added, and the paste was ground and cut into pellets. Afterward, pellets were dried at 37°C (Hoseini et al., 2020). It is worth mentioning that other experimental diets were prepared by adding 5 and 10% *Chlorella vulgaris* dry powder (Zahran et al., 2019; Mahmoud et al., 2020). The biochemical composition and feedstuff of experimental diets are presented in Table 1. Finally, the biochemical status of each diet was assessed by using the standard method of AOAC (Naeemi et al., 1995).

#### Sampling

After 56 days, feeding was stopped for 24 hours and all the fish in each tank were anesthetized by using eugenol (100 mg/l; Yousefi et al., 2022). Then, growth and nutrition indices were calculated by biometry of weight, and amount of eaten food using the following formulas (Houlihan et al., 1993; Raissy et al., 2022):

$$\text{Weight gain (WG, g)} = \frac{\text{final weight (FW)} - \text{initial weight (IW)}}{\text{day}} \quad (1)$$

$$\text{Feed conversion ratio (FCR)} = \frac{\text{feed intake}}{\text{FW-IW}} \quad (2)$$

$$\text{Specific growth rate (SGR, \% / day)} = 100 \times \frac{[\ln(\text{FW}) - \ln(\text{IW})]}{\text{day}} \quad (3)$$

$$\text{Survival rate (SR, \%)} = \frac{[\text{final number of fish (Nf)}]}{[\text{initial number of fish (Ni)}]} \times 100 \quad (4)$$

#### Sampling strategy

To evaluate serum immunity parameters, 3 sample fish from each replication were anesthetized by using eugenol (100 mg/l; Yousefi et al., 2022). Blood samples were taken from the caudal peduncle using a 2 ml syringe, samples were fractionated by using a centrifuge (3000 rpm for

10 minutes at 4°C). The obtained serum was stored at -70°C until the evaluation of immunity parameters. For mucus sampling, 3 sample fish were randomly caught from each tank and transferred to polyethylene bags containing 10 ml NaCl (50 mM); after 3 minutes the collected mucus was centrifuged (2500 g for 10 minutes at 4°C) and the upper phase was stored at -80°C (Ross et al., 2000). To evaluate the microbial population of the intestine, the fish skin surface area was disinfected using 70% ethanol and after splitting the abdominal cavity by using a disinfected instrument, and the intestine was separated. Digestive enzyme activity was measured as follows: 3 fish samples in each replicate were captured by using an overdose concentration of clove powder (300 mg/L). After the autopsy, the intestine was isolated, emptied, washed, homogenized using a 50 mM Tris buffer and mechanically. The homogenized samples were centrifuged ( $6000 \times g$  for 10 min, 4°C) and the upper phase was stored for analysis at -80°C.

#### *Intestinal microbial population*

To evaluate the microbial population of the intestine, the fish skin surface area was disinfected using 70% ethanol and after splitting the abdominal cavity by using a disinfected instrument, the intestine was washed with phosphate buffer saline (PBS) and homogenized by using PBS and a tissue homogenizer. The obtained homogenized sample was diluted in PBS. Then, 100 µl of the sample was transferred to deMan Rogosa Sharpe (MRS) medium (Merck, Germany) and tryptic soy agar (TSA; Merck, Germany) medium for lactic acid bacteria (LAB) and total bacteria count (TBC) assays; respectively. The pellets were stored at 30°C for 48 h, and LAB and TBC were counted in the common carp's intestine and reported in cfu/g of tissue (Merrifield et al., 2010).

#### *Digestive enzyme activity*

Digestive enzyme activity was measured as follows: 3 fish samples in each replicate were captured by using an overdose concentration of clove powder. After the autopsy, the intestine was isolated, emptied, washed, homogenized using a 50 mM Tris buffer and mechanically. The homogenized samples were centrifuged ( $6000 \times g$  for 10 min, 4°C) and the upper phase was stored for analysis at -80°C. Amylase activity was measured by Robyt and Whelan's (1972) methods using 2% starch as a substrate in 500 µl of 0.1 M phosphate buffer. Lipase enzyme activity was measured based on the method described by Iijima et al. (1998), by using p-nitrophenyl myristate in cholate buffer (0.25 mM Tris-HCl + 0.25 mM 2-methoxy ethanol + 5 mM sodium cholate, pH = 9). The reaction was incubated for 15 minutes at 30°C and stopped using acetone/n-heptane (5:2, v/v). The optical density (OD) for the upper phase was recorded spectrophotometrically (Biophotometer Eppendorf) at 405 nm. Total protease activity was also calculated using azo-casein in 0.5 ml Tris (Tris-HCl 0.1 M, pH = 8) (García-Carreño, 1992). The reaction was stopped using 5% trichloroacetic acid at 25°C for 1 hour. The mixture was

then centrifuged, and the light absorption of the upper phase was measured at 440 nm.

#### *Analysis of biochemical and enzymatic parameters of the serum*

Serum cortisol level (ng/ml) was calculated by ELISA using a commercial kit (IBL Co., Gesellschaft für Immunchemie und Immunbiologie, Germany) and serum glucose level (mg/dl) was measured using a commercial kit (Pars Azmun Co., Tehran, Iran). Serum glutathione peroxidase (GPx) (u/ml) and superoxide dismutase (SOD) (u/ml) levels were measured by glutathione oxidation rate and cytochrome C reduction rate, respectively (ZellBio GmbH, Veltinerweg). Serum catalase (CAT) activity (u/ml) was calculated by reducing the amount of H<sub>2</sub>O<sub>2</sub> according to the method described by Goth (1991). The amount of malondialdehyde (MDA) (µM/l) was calculated by the thiobarbituric acid method and using a commercial kit (ZellBio GmbH, Veltinerweg). The activity of alkaline phosphatase (ALP), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) enzymes and the level of serum lactate dehydrogenase (LDH) (U/L) were measured using biochemical auto analysis (Beckman Coulter, Avanti J-26 XPI, CA, USA) and Pars Azmun Co. commercial kits.

#### *Serum and mucus immune responses*

Serum and mucus lysozyme activity was estimated based on the turbidity meter method described by Ellis (1990) using *Micrococcus lysodeikticus* as a target organism in phosphate buffer (0.2 mg/ml in a 0.05 M sodium phosphate buffer (pH = 6.2)). Total serum and mucus immunoglobulin (Total Ig) were calculated based on the amount of protein before and after adding polyethylene glycol. Concentrations of serum complement components (C3 and C4) (mg/dl) were estimated using ELISA (ELX800, BioTek, Vermont, USA) and based on the commercial kit (Pars Azmun Co., Tehran, Iran).

Serum complement alternative pathway (ACH<sub>50</sub>) activity was calculated based on the method described by Yano (1992). In this method, sheep erythrocytes in vernal tissue including egtazic acid (EGTA) and manganese are considered a target at OD 412 nm.

Mucus protease activity was measured based on the method of Hoseinifar et al. (2016). In this method, 100 µl of mucus was mixed with 100 µl of ammonium bicarbonate buffer containing 100 mM of 0.7% azocasein solution and then incubated at 30°C for 20 hours. The reaction was stopped by using trichloroacetic acid and the upper phase was collected by centrifugation (15000 g for 5 minutes). The upper phase was then mixed with 0.5 N hydroxide and the light absorption was measured at 450 nm. Mucus ALP activity was performed using Pars Azmun Co. commercial kit and according to manufacturer's protocol. Serum nitroblue tetrazolium test (NBT) was assayed using the method of Anderson and Siwicki (1995). Briefly, 100 µl of heparinized blood was added to 100 µL of 0.2% NBT. The mixture was incubated for 30 minutes

at 25°C. 50 µl of the obtained suspension was added to 1 ml of N, N-dimethyl formamide and centrifuged at 3000 g for 5 min. Finally, the OD of the upper phase was measured at 540 nm. Serum myeloperoxidase (MPO) activity was measured by Quade and Roth method (1997). In this method, 10 µl of serum with 90 µl of Hank's balanced salt solution (HBSS) without Na<sup>+</sup> and Mg<sup>+</sup> ions was added to 96 well plates, then 35 µl of tetramethylbenzidine (TMB) hydrochloride was added. The color change was stopped by adding 35 µl of 0.5 M sulfuric acid, and OD was measured at 450 nm. Esterase activity was measured based on Guardiola et al. (2017). In this method, an equal volume of skin mucus samples was incubated with 0.4 mM p-nitrophenyl myristate substrate in 100 mM ammonium bicarbonate buffer containing 0.5% Triton X-100 (pH 7.8, 30°C) and the OD was measured at 405 nm.

### Statistical analysis

In this study, all the statistical analyses were done using SPSS program version 21 (SPSS, Richmond, VA, USA). Before statistical analysis, the obtained data were tested for normality of distribution using the Kolmogorov–Smirnov test and for homogeneity of variances among different treatments using Bartlett's test. Then, data were subjected to one-way ANOVA, and differences between means were tested at the 5% probability level using the Tukey test as a post hoc test.

## Results

### The growth indices of common carp

It is noticed that fish growth and feed intake were adversely affected by Imidacloprid toxicity showing the

lowest performance (T4) as compared to other treatments ( $P < 0.05$ ; Table 3). The administration of *Chlorella vulgaris* dry powder to BD-fed fish (T2-T3-T5-T6) improved their growth significantly as presented by FW, WG, and SGR, over control treatment with BD fed alone. *Chlorella vulgaris* supplement improved fish growth and SGR over that of the control treatment but the percentage of *Chlorella vulgaris* was more efficient when using 10% versus 5% of dry powder/dry weight diet ( $P < 0.05$ ; control). Meanwhile, the addition of *Chlorella vulgaris* dry powder to BD-fed fish (T2-T3) made fish in those treatments have a better SGR than those fed with the presence of 12.5% LC<sub>50</sub> Imidacloprid and thus this insecticide can reduce the positive effects of adding *Chlorella vulgaris* supplement. Furthermore, the FCR value was the highest when 12.5% LC<sub>50</sub> Imidacloprid was used without any *Chlorella vulgaris* dry powder. No significant difference was observed between the survival rate among the different treatments ( $P > 0.05$ ; Table 3).

### Biochemical and enzymatic parameters of serum

The mean measured values of serum biochemical parameters are shown in Table 4. According to the results, there was no significant difference in albumin values between the fish fed with the dietary *Chlorella vulgaris* and those being deprived of it in both situations of presence and absence of 12.5% LC<sub>50</sub> Imidacloprid. Treatments of T2 and T4 are the only treatments with significant differences in total protein values. As is clear from the table using 5% of *Chlorella vulgaris* dry powder has mild effects and 10% dietary *Chlorella vulgaris* shows stronger results in total protein alteration.

Table 3. Growth performance of common carp, fed on diets with (T2, T3, T5, T6)/without (T1, T4) *Chlorella vulgaris* dry powder for 4 weeks in the presence (T4, T5, T6) and absence (T1, T2, T3) of 12.5% LC<sub>50</sub> Imidacloprid

| Parameters               | T1             | T2           | T3            | T4           | T5             | T6            |
|--------------------------|----------------|--------------|---------------|--------------|----------------|---------------|
| IW(g)                    | 20.50±0.28 a   | 20.10±0.20 a | 20.30±0.40 a  | 20.73±0.14 a | 20.33±0.35 a   | 20.36±0.06 a  |
| FW (g)                   | 40.50±1.32 abc | 45.00±1.15 a | 43.26±1.17 ab | 37.33±0.88 c | 40.66±0.88 abc | 39.96±0.65 bc |
| WG (g)                   | 20.00±1.44 ab  | 24.90±1.02 a | 22.96±1.57 a  | 16.60±0.97 b | 20.33±0.76 ab  | 19.60±0.72 ab |
| FCR                      | 2.02±0.12 ab   | 1.65±0.04 b  | 1.83±0.12 b   | 2.44±0.11 a  | 1.94±0.08 b    | 1.99±0.08 ab  |
| SGR (% d <sup>-1</sup> ) | 1.21±0.07 ab   | 1.43±0.03 a  | 1.35±0.08 a   | 1.04±0.05 b  | 1.23±0.03 ab   | 1.20±0.03 ab  |
| SR (%)                   | 94.42±1.09 a   | 96.63±1.93 a | 94.55±2.85 a  | 92.21±1.10 a | 95.33±1.01 a   | 93.31±1.90 a  |

IW: initial weight; FW: final weight; WG: weight gain; SGR: specific growth rate; FCR: feed conversion ratio; SR: survival rate. Data represented as mean ± SE. Means with different letters (a–d) in the same row indicate significant differences ( $P < 0.05$ ). Control (T1), 5% *Chlorella vulgaris* dry powder and No pollution with LC<sub>50</sub> Imidacloprid (T2), 10% *Chlorella vulgaris* dry powder and no pollution with LC<sub>50</sub> Imidacloprid (T3), no *Chlorella vulgaris* dry powder and 12.5% LC<sub>50</sub> imidacloprid (T4), 5% *Chlorella vulgaris* dry powder plus 12.5% LC<sub>50</sub> Imidacloprid (T5) and 10% *Chlorella vulgaris* dry powder plus 12.5% LC<sub>50</sub> Imidacloprid (T6).

Table 4. Serum biochemical parameters of common carp fed on diets with (T2, T3, T5, T6)/without (T1, T4) *Chlorella vulgaris* dry powder for 4 weeks in presence (T4, T5, T6) and absence (T1, T2, T3) of 12.5% LC<sub>50</sub> Imidacloprid

| Parameters           | T1             | T2           | T3           | T4            | T5             | T6             |
|----------------------|----------------|--------------|--------------|---------------|----------------|----------------|
| Total protein (g/dL) | 3.23±0.14 ab   | 4.00±0.26 a  | 3.61±0.19 ab | 2.98±0.07 b   | 3.18±0.14 ab   | 3.25±0.20 ab   |
| Albumin (g/dL)       | 1.36±0.14 a    | 1.70±0.15 a  | 1.41±0.04 a  | 1.28±0.06 a   | 1.51±0.06 a    | 1.38±0.07 a    |
| Globulin (g/dL)      | 1.86±0.14 a    | 2.30±0.11 a  | 2.20±0.15 a  | 1.70±0.05 a   | 1.66±0.20 a    | 1.86±0.18 a    |
| Glucose (g/dL)       | 65.43±1.83 b   | 57.50±1.44 b | 58.66±1.45 b | 75.40±1.81 a  | 63.80±1.90 b   | 65.00±1.52 b   |
| Cortisol (nmol/L)    | 109.66±5.48 ab | 95.00±4.61 b | 98.00±3.21 b | 121.66±3.28 a | 108.00±5.19 ab | 109.23±4.93 ab |

Data represented as mean ± SE. Treatments are the same as mentioned in Table 2. Means with different letters (a–d) in the same row indicate significant differences ( $P < 0.05$ ).

The highest values of total protein and globulin were observed where *Chlorella vulgaris* dry powder was used and there was no insecticide in the diet (T2, T3); surprisingly the treatment containing 5% of *Chlorella vulgaris* dry powder has the highest total protein value (T2) ( $P < 0.05$ ). However, 5% *Chlorella vulgaris* supplement represented the highest albumin values both with and without 12.5% LC<sub>50</sub> Imidacloprid (T2, T5). The results also revealed no significant decrease in the levels of LDH, glucose, and cortisol in the fish fed with *Chlorella vulgaris* dry powder containing 12.5% LC<sub>50</sub> Imidacloprid (T5) compared to the diet without this supplement ( $P < 0.05$ ). The maximum values of LDH, glucose and cortisol were observed in the T4 group where fish were fed by BD without *Chlorella vulgaris* dry powder, and the environment was polluted with 12.5% LC<sub>50</sub> Imidacloprid. The use of 12.5% LC<sub>50</sub> imidacloprid has an obvious increasing effect on these three parameters while using *Chlorella vulgaris* dry powder has a significant reducing effect. The values of the parameters decrease to approximately the same value as those in the control group after using dietary *Chlorella vulgaris* in a 12.5% LC<sub>50</sub> Imidacloprid polluted environment. Cortisol was found to be the highest in the T4 group, while its lowest values were observed in T2 with 5% of *Chlorella vulgaris* dry powder in the diet and no pollution with LC<sub>50</sub> Imidacloprid.

#### Serum/mucus immune parameters

Determination of immunological parameters in serum and mucus showed that, in most cases, *Chlorella vulgaris* supplemented diets improved the immune system of the fish (Table 5 and Figures 1–5). The differences between the immune parameters were more recognizable in *Chlorella vulgaris* supplemented fish groups, both in LC<sub>50</sub> Imidacloprid polluted and non-polluted environments compared to non-fortified diets (control and T4). Although supplemented diet with *Chlorella vulgaris* dry powder has a clear increasing effect on immunity, the results of immune parameters such as ACH<sub>50</sub>, Ig and MPO are still a bit lower in T5 and T6 than those of control group which show a strong negative effect of 12.5% LC<sub>50</sub> Imidacloprid ( $P < 0.05$ ) on immunity which is softened by *Chlorella vulgaris* powder. Moreover, in serum, except for peroxidase and Ig which were the highest in T3 with 10% *Chlorella*

*vulgaris* dry powder dietary without LC<sub>50</sub> Imidacloprid, other serum immune parameters were maximum in the group fed by 5% *Chlorella vulgaris* dry powder without LC<sub>50</sub> Imidacloprid (T2). In this regard, the fish fed with 5% *Chlorella vulgaris* powder also represented the highest serum total lysozyme, ACH<sub>50</sub>, MPO, NBT, C3, and C4; while treatments containing 10% *Chlorella vulgaris* powder have the maximum peroxidase values (Table 5). In regard to mucus immunity, the ALP values change significantly in T2 and T5 (5% *Chlorella* sp. diet) but not in T3 and T6 (10% *Chlorella* sp. diet) compared to control (Figure 4). This can also confirm that the optimal dose of algae powder in diet is more likely to be around 5%. Also, protease activity in common carp mucus is highly affected by 12.5% LC<sub>50</sub> Imidacloprid pollution as the values decrease from about 20% in T3 to less than 15% in T6 while both T3 and T6 have the same amount of 10% *Chlorella vulgaris* dry powder in the diet (Figure 1). Yet total Ig does not change much and the Ig values are not significantly different in mucus (Figure 2). Mucus lysozyme values are the highest in T2 and T6. Interestingly, the values are not significantly different while the *Chlorella* dosage is different (Figure 3). Also, in T2 there is no 12.5% LC<sub>50</sub> Imidacloprid pollution while in T6 samples are taken from fishes in polluted environment. Maximum esterase value (about 2.25) is observed in T2 while other treatments show no significant change in esterase values compared to control. On the other hand, esterase levels show (Figure 5) a drastic significant increase in T2 after adding 5% dietary *Chlorella vulgaris* but the increasing tendency is not as strong in 10% of *Chlorella* supplemented diet (T3). Minimum esterase levels were detected in T4 expectedly and in T5 and T6 an increase in esterase values is visible but not significant.

Generally, the levels of antioxidant enzymes in treatments with *Chlorella vulgaris* dry powder were not significantly ( $P < 0.05$ ) higher than those in the control group except for SOD (Table 6). The maximum values of CAT, SOD, and GP<sub>x</sub> were observed in treatment with 5% *Chlorella vulgaris* dry powder and without LC<sub>50</sub> Imidacloprid (T2). Compared to the control group, the MDA level was not significantly ( $P < 0.05$ ) lower in *Chlorella vulgaris* enriched treatments and its lowest value was observed at the treatment with 5% *Chlorella vulgaris* (T2) (Table 6).

Table 5. Serum immunological indices of common carp fed on diets with (T2, T3, T5, T6)/without (T1, T4) *Chlorella vulgaris* dry powder for 4 weeks in presence (T4, T5, T6) and absence (T1, T2, T3) of 12.5% LC<sub>50</sub> Imidacloprid

| Parameters               | T1 (control)   | T2            | T3            | T4            | T5             | T6             |
|--------------------------|----------------|---------------|---------------|---------------|----------------|----------------|
| Lysozyme (U/ml)          | 20.60±0.87 abc | 25.00±1.03 a  | 21.96±1.15 ab | 16.16±0.44 c  | 20.96±1.17 ab  | 20.06±0.92 bc  |
| ACH <sub>50</sub> (U/ml) | 111.50±2.84 ab | 128.16±5.34 a | 124.16±5.62 a | 103.40±4.62 b | 109.50±3.17 ab | 107.70±3.72 ab |
| Ig (mg/ml)               | 19.20±0.75 a   | 19.83±1.16 a  | 20.33±0.44 a  | 16.78±0.91 a  | 19.06±0.84 a   | 18.66±0.99 a   |
| MPO (OD at 450)          | 1.23±0.14 ab   | 2.03±0.18 a   | 1.96±0.20 a   | 0.96±0.20 b   | 1.20±0.15 ab   | 1.16±0.29 ab   |
| NBT (OD at 540)          | 0.53±0.05 a    | 0.74±0.07 a   | 0.68±0.06 a   | 0.47±0.08 a   | 0.51±0.07 a    | 0.52±0.07 a    |
| Peroxidase (U/ml)        | 6.83±0.46 bc   | 9.70±0.86 ab  | 10.16±0.61 a  | 6.06±0.63 c   | 7.10±0.49 bc   | 6.96±0.60 bc   |
| C3 (g/dL)                | 19.20±1.17 ab  | 24.60±1.30 a  | 22.26±2.33 a  | 14.60±1.13 b  | 18.43±1.31 ab  | 20.83±1.01 ab  |
| C4 (g/dL)                | 9.60±0.97 ab   | 12.53±0.54 a  | 12.46±1.12 a  | 8.36±0.44 b   | 10.36±0.80 ab  | 9.83±0.61 ab   |

ACH50: The alternative complement pathway; total Ig: total immunoglobulin; MPO: myeloperoxidase; NBT: nitro-blue-tetrazolium; C3 and C4: Concentrations of serum complement components. Treatments are the same as mentioned in Table 2. Data represented as mean ± SE. Means with different letters (a–d) in the same row indicate significant differences ( $P < 0.05$ ).

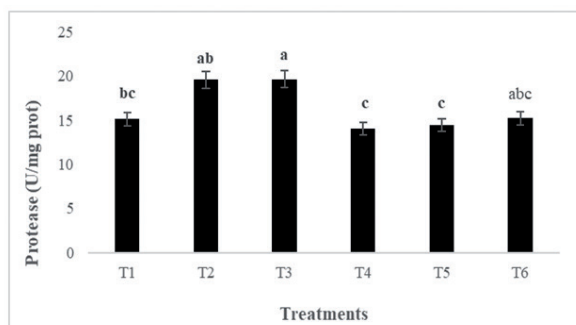


Figure 1. Protease activity in common carp mucus fed on diets with (T2, T3, T5, T6) and without (T1, T4) *Chlorella vulgaris* dry powder for 4 weeks in the presence (T4, T5, T6) and absence (T1, T2, T3) of 12.5% LC<sub>50</sub> Imidacloprid. Treatments are the same as mentioned in Table 2

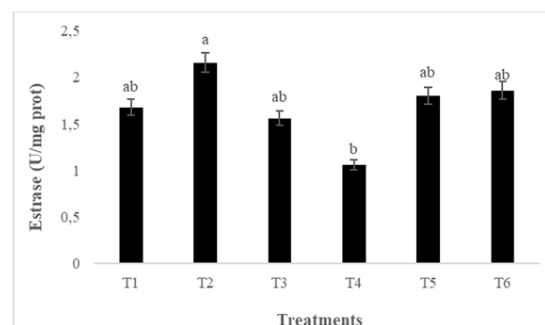


Figure 5. Esterase levels in common carp mucus fed on diets with (T2, T3, T5, T6) and without (T1, T4) *Chlorella vulgaris* dry powder for 4 weeks in the presence (T4, T5, T6) and absence (T1, T2, T3) of 12.5% LC<sub>50</sub> Imidacloprid. Treatments are the same as mentioned in Table 2

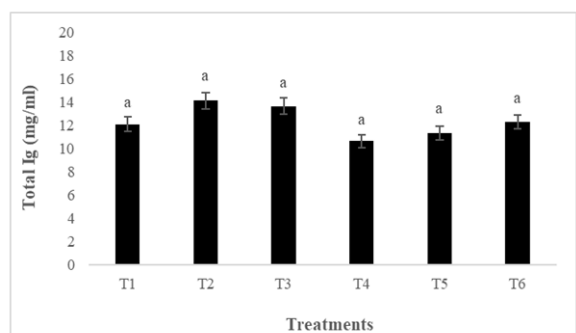


Figure 2. Total Ig secreted in common carp mucus fed on diets with (T2, T3, T5, T6) and without (T1, T4) *Chlorella vulgaris* dry powder for 4 weeks in the presence (T4, T5, T6) and absence (T1, T2, T3) of 12.5% LC<sub>50</sub> Imidacloprid. Treatments are the same as mentioned in Table 2

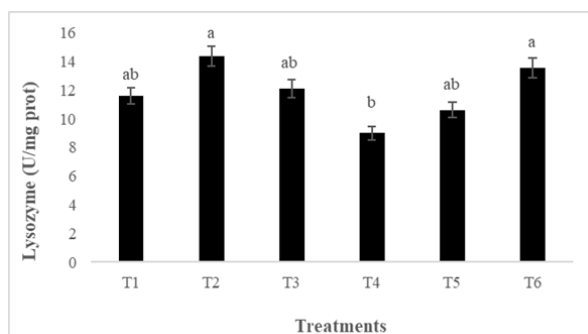


Figure 3. Lysozyme enzyme secreted in common carp mucus fed on diets with (T2, T3, T5, T6) and without (T1, T4) *Chlorella vulgaris* dry powder for 4 weeks in the presence (T4, T5, T6) and absence (T1, T2, T3) of 12.5% LC<sub>50</sub> Imidacloprid. Treatments are the same as mentioned in Table 2

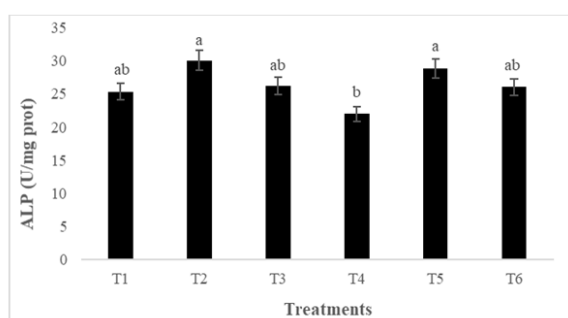


Figure 4. ALP (alkaline phosphatase) levels in common carp mucus fed on diets with (T2, T3, T5, T6) and without (T1, T4) *Chlorella vulgaris* dry powder for 4 weeks in the presence (T4, T5, T6) and absence (T1, T2, T3) of 12.5% LC<sub>50</sub> Imidacloprid. Treatments are the same as mentioned in Table 2

### Intestinal microbial population

The maximum values for TBC and LAB in Figures 6 and 7 were detected in T3 (diet with 10% *Chlorella vulgaris* dry powder and no LC<sub>50</sub> Imidacloprid pollution) and T2 (diet with 5% *Chlorella vulgaris* dry powder and no LC<sub>50</sub> Imidacloprid pollution) respectively; while the minimum value was observed in the fish fed with no *Chlorella vulgaris* powder and polluted by 12.5% LC<sub>50</sub> Imidacloprid (T4) for both TBC and LAB. The TBC and LAB levels significantly showed a decreasing tendency when 12.5% LC<sub>50</sub> Imidacloprid was used ( $P < 0.05$ ; Figures 6 and 7) and an increasingly significant value when *Chlorella* dietary is applied; yet, T5 and T6 groups are still less in TBC value compared to Control even when 5 and 10% of *Chlorella vulgaris* powder are added to the diet respectively.

### Analysis of biochemical and enzymatic parameters of the serum

The values obtained for digestive enzymes, including amylase, lipase, and protease show an increase in the fish fed with *Chlorella vulgaris* supplement than the control group and T4 ( $P < 0.05$ ; Table 7). The maximum amounts for amylase, lipase, and protease were found in the treatment containing 5% *Chlorella vulgaris* powder and without LC<sub>50</sub> Imidacloprid pollution (T3). Adding *Chlorella vulgaris* powder can also increase three enzymes in fish within the polluted environment (T5 and T6) compared to T4 but the results are not significant for protease and lipase. Using 10% of dietary *Chlorella vulgaris* dry powder has been slightly more effective than 5% of this supplement (T5 versus T6) whilst it is not the same in non-polluted environments (T2 versus T3) (Table 7).

Table 8 shows the results for digestive hepatic enzymes. T4 does not show a significant increase in ALT, AST, and ALP while increasing the values for these parameters compared to control slightly. T2 shows a significant difference in all three parameters compared to the control group, indicating the positive effects of 5% dietary *Chlorella vulgaris* on the elimination of toxic compounds which causes less enzyme secretion. There is also no significant difference between different *Chlorella vulgaris* doses (T2/T3 and T5/T6).

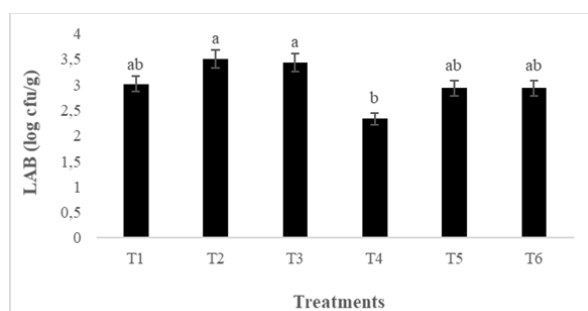


Figure 6. LAB (lactic acid bacteria) levels in common carp serum fed on diets with (T2, T3, T5, T6) and without (T1, T4) *Chlorella vulgaris* dry powder for 4 weeks in the presence (T4, T5, T6) and absence (T1, T2, T3) of 12.5% LC<sub>50</sub> Imidacloprid. Treatments are the same as mentioned in Table 2

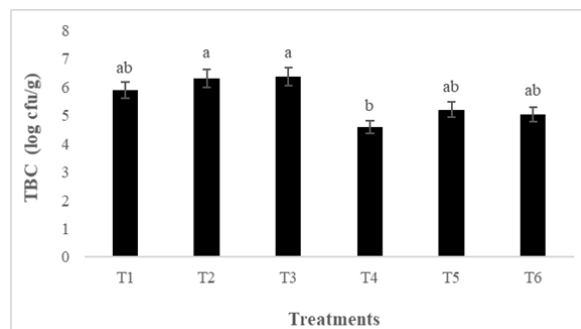


Figure 7. TBC (total bacterial count) levels in common carp fed on diets with (T2, T3, T5, T6)/without (T1, T4) *Chlorella vulgaris* dry powder for 4 weeks in the presence (T4, T5, T6) and absence (T1, T2, T3) of 12.5% LC<sub>50</sub> Imidacloprid. Data represented as mean  $\pm$  SE. Bars with different letters (a–c) in the parameter indicate statistically significant differences ( $P < 0.05$ ). Treatments are the same as mentioned in Table 2

Table 6. Serum antioxidant indices and MDA content of common carp fed on diets with (T2, T3, T5, T6)/without (T1, T4) *Chlorella vulgaris* dry powder for 4 weeks in the presence (T4, T5, T6) and absence (T1, T2, T3) of 12.5% LC<sub>50</sub> Imidacloprid

| Parameters    | T1                  | T2                  | T3                  | T4                  | T5                  | T6                   |
|---------------|---------------------|---------------------|---------------------|---------------------|---------------------|----------------------|
| CAT (U/ml)    | 112.00 $\pm$ 6.92 a | 116.33 $\pm$ 5.48 a | 114.00 $\pm$ 6.35 a | 98.00 $\pm$ 4.61 a  | 113.66 $\pm$ 4.05 a | 111.16 $\pm$ 5.05 a  |
| SOD (U/ml)    | 29.16 $\pm$ 1.30 bc | 35.33 $\pm$ 1.16 a  | 34.66 $\pm$ 1.20 ab | 25.33 $\pm$ 1.33 c  | 31.63 $\pm$ 1.21 ab | 30.66 $\pm$ 1.50 abc |
| MDA (nmol/ml) | 46.06 $\pm$ 1.50 ab | 38.13 $\pm$ 2.42 b  | 39.30 $\pm$ 2.11 b  | 47.76 $\pm$ 1.58 a  | 42.06 $\pm$ 1.18 ab | 45.33 $\pm$ 1.45 ab  |
| GPx (U/ml)    | 171.33 $\pm$ 6.11 a | 177.33 $\pm$ 6.06 a | 174.50 $\pm$ 5.34 a | 140.56 $\pm$ 3.62 b | 173.33 $\pm$ 6.69 a | 172.33 $\pm$ 5.81 a  |

CAT: catalase; SOD: superoxide dismutase; MDA: malondialdehyde; GPx: glutathione peroxidase. Data represented as mean  $\pm$  SE. Treatments are the same as mentioned in Table 2. Means with different letters (a–d) in the same row indicate significant differences ( $P < 0.05$ ).

Table 7. Digestive enzymes of serum of common carp fed on diets with (T2,T3,T5,T6)/without (T1,T4) *Chlorella vulgaris* dry powder for 4 weeks in presence (T4,T5,T6) and absence (T1,T2,T3) of 12.5% LC<sub>50</sub> Imidacloprid

| Parameters              | T1                  | T2                 | T3                  | T4                | T5                  | T6                  |
|-------------------------|---------------------|--------------------|---------------------|-------------------|---------------------|---------------------|
| Amylase (U/mg protein)  | 11.83 $\pm$ 0.72 ab | 13.66 $\pm$ 0.92 a | 12.60 $\pm$ 0.87 ab | 9.50 $\pm$ 1.07 b | 12.20 $\pm$ 0.81 ab | 11.56 $\pm$ 0.65 ab |
| Protease (U/mg protein) | 7.37 $\pm$ 0.49 ab  | 7.89 $\pm$ 0.59 a  | 7.55 $\pm$ 0.53 ab  | 5.28 $\pm$ 0.59 b | 7.06 $\pm$ 0.46 ab  | 7.33 $\pm$ 0.33 ab  |
| Lipase (U/mg protein)   | 1.53 $\pm$ 0.12 a   | 1.77 $\pm$ 0.09 a  | 1.71 $\pm$ 0.06 a   | 1.34 $\pm$ 0.07 a | 1.46 $\pm$ 0.09 a   | 1.49 $\pm$ 0.11 a   |

Data represented as mean  $\pm$  SE. Treatments are the same as mentioned in Table 2. Means with different letters (a–d) in the same row indicate significant differences ( $P < 0.05$ ).

Table 8. Hepatic enzymes activity of common carp fed on diets with (T2, T3, T5, T6)/without (T1, T4) *Chlorella vulgaris* dry powder for 4 weeks in presence (T4, T5, T6) and absence (T1, T2, T3) of 12.5% LC<sub>50</sub> Imidacloprid

| Parameters | T1                    | T2                  | T3                  | T4                  | T5                   | T6                   |
|------------|-----------------------|---------------------|---------------------|---------------------|----------------------|----------------------|
| ALT (U/L)  | 35.53 $\pm$ 1.58 ab   | 33.06 $\pm$ 1.09 b  | 32.33 $\pm$ 1.20 b  | 39.60 $\pm$ 0.97 a  | 34.16 $\pm$ 1.16 ab  | 32.93 $\pm$ 1.09 b   |
| AST (U/L)  | 76.40 $\pm$ 1.93 ab   | 64.83 $\pm$ 2.16 c  | 69.16 $\pm$ 1.48 bc | 84.43 $\pm$ 2.76 a  | 79.20 $\pm$ 1.33 a   | 80.40 $\pm$ 1.51 a   |
| ALP (U/L)  | 104.00 $\pm$ 3.21 ab  | 93.50 $\pm$ 2.29 b  | 92.16 $\pm$ 2.24 b  | 115.90 $\pm$ 3.05 a | 104.83 $\pm$ 2.31 ab | 102.50 $\pm$ 3.17 b  |
| LDH (U/L)  | 480.00 $\pm$ 11.54 ab | 450.00 $\pm$ 8.66 b | 440.16 $\pm$ 9.67 b | 495.00 $\pm$ 7.63 a | 477.16 $\pm$ 7.94 ab | 478.66 $\pm$ 7.79 ab |

ALT: alanine aminotransferase; AST: aspartate aminotransferase; ALP: alkaline phosphatase; LDH: lactate dehydrogenase. Data represented as mean  $\pm$  SE. Treatments are the same as mentioned in Table 2. Means with different letters (a–d) in the same row indicate significant differences ( $P < 0.05$ ).

## Discussion

Growth performance comes under stress response. Fish growth, feed intake, and immunity parameters are proved to be adversely affected by Imidacloprid toxicity; showing the lowest performance at T4; and were improved significantly by addition of *Chlorella vulgaris* dry

powder to BD. However, in some cases, no significant differences were observed between *Chlorella vulgaris* dry powder treatments while in many immunity indices; the highest indices were observed in the fish fed with a diet containing the 5% *Chlorella vulgaris* dry powder level. The primary stress response is cortisol which is elevated in fish exposed to poison and high temperature

(Kumar et al., 2021). Imidacloprid can target several sites on a hypothalamus-pituitary-internal axis which might be the reason for increased cortisol secretion in this study. Our results were similar to those previously reported in other studies, where the outcomes showed an increase in growth rates and immune responses of fish as referred to below.

Mohammadiazarm et al. (2021) studied the effect of spirulina meal (*Spirulina platensis*) as a feed additive on the growth and physiological response of Oscar fish (*Astronotus ocellatus*). The result showed that the spirulina dieted fish had statistically better growth performance, feeding parameters, total protease activity, total protein and lipid contents than the control fish. The spirulina-dieted fish showed higher total protein and albumin levels, but lower triglycerides, cholesterol levels, and liver enzyme activities than the control. Also, serum complements (C3, C4), lysozyme activity and total carotenoid contents in fish skin significantly increased in the spirulina added treatments compared to control.

Similar results were also found in earlier studies that reported fish grew better on algae-enriched (*Spirulina platensis*) diets (Reitan, 1997; Stoneham et al., 2018; Kandathil Radhakrishnan et al., 2020; Reverter et al., 2021). It was reported that Caspian brown trout (*Salmo trutta caspius*) fed with 52.8 g/kg SP in formulated feed, showed significantly higher weight gain than those fed a fishmeal-based diet (Stoneham et al., 2018). Also, similar results were found in parrot fish (*Oplegnathus fasciatus*) (Kim et al., 2013) and rainbow trout (*Oncorhynchus mykiss*) (Teimouri et al., 2013) fed diets including spirulina dry powder.

Improved digestive enzyme activities in the intestine of fish were reported by dietary *Spirulina* sp. (Teimouri et al., 2013; Kandathil Radhakrishnan et al., 2020). A similar result was observed in studies on gilthead sea bream (*Sparus aurata*) fed with plant ingredients (Santigosa et al., 2011; Silva et al., 2010).

In the present study, the positive effect of *Chlorella vulgaris* on the growth efficiency may be related to its inducing effects on digestive enzymes (Bilen et al., 2019, 2020; Rashidian et al., 2020 a, b), as digestive enzymes (amylase, protease, and lipase) were significantly increased in *Chlorella vulgaris* supplemented fish compared to the control group. However, the change patterns for enzymes varied between *Chlorella vulgaris* treatments, in the presence and absence of 12.5% LC<sub>50</sub> Imidacloprid which may be due to different secretion thresholds in response to insecticide pollution. For example, maximum amylase, protease, and lipase were observed in 5% dietary *Chlorella vulgaris* with no pollution adjusted, while a minimum of all 3 enzymes was secreted when there was 12.5% LC<sub>50</sub> Imidacloprid and no dietary *Chlorella vulgaris* supplemented.

Other specific enzymes like the liver enzyme activities are considered critical diagnostic indices, as they relate to the general nutritional status and the liver function of target animals (Ahmad et al., 2021). Thus, the re-

sults of the present study suggest an improvement in fish health due to reduced liver enzyme activities (ALT, AST, and ALP) and the unchanged albumin–globulin ratio in fish fed by dietary *Chlorella* sp. for 56 days. These results are in agreement with the results reported by Ansarifard et al. (2018).

On the other hand, data on bacterial counts also confirmed that the intestine microflora number increased after a drastic reduction affected by the poison. Antagonistic activity of *Chlorella vulgaris* against Imidacloprid has been proved as the TBC and LAB value raised in T2 and T3 compared to control and in T5–T6 compared to T4. Additionally, esterase secretion is strongly and positively impressed by the dietary *Chlorella vulgaris* which can be explained by a positive increase of TBC and LAB in the intestine due to *Chlorella vulgaris* supplementation in the fish diet. Esterase is widely expressed by bacteria in the intestine. For the longest time esterase has been used as a marker system of somatic embryogenesis and organogenesis of bacteria and thus, reflecting the bacterial activity (Wheeler et al., 2010; Liu et al., 2020; Belinskaia et al., 2021).

Similarly, Kahyani et al. (2021) revealed that feeding with the *Weissella confusa* supplemented diet with host-derived potential probiotics significantly enhanced the lactic acid bacteria levels. Total bacterial count was also significantly increased and the growth performance of supplemented treatments remarkably improved compared to the control group ( $P < 0.05$ ). These results confirmed the beneficial effects of *W. confusa* as a host-derived potential probiotic in rainbow trout.

To our best knowledge, in fish, total protein, albumin, and globulin are known as important components of the innate immune system (Rao et al., 2006). Albumin is needed for the transportation of metal, hormones, vitamins, drugs, bilirubin, and fat metabolites and regulates the free available hormones and is mainly secreted from the liver in fish to fulfill the high energy demand during stress through protein synthesis (Abdel-Tawwab and Ahmad, 2009; Kumar et al., 2021). In the present investigation, the albumin was less than globulin, however rapid utilization of albumin to meet the immediate energy demand could also increase its rate of synthesis in the liver. Further, it was also revealed that fish with low globulin is more susceptible to water pollution with 12.5% LC<sub>50</sub> Imidacloprid due to poor immune resistance. Abdel-Tawwab and Ahmad (2009) reported that fish fed a diet including 5.0–10 g/kg spirulina dry powder had higher protein, albumin, and globulins than the control which was similar to that investigated in this study.

In the present study, the blood cortisol and LDH levels significantly decreased in the *Chlorella vulgaris* supplemented fish compared to the control group before and after pollution. Results are clearer in non-polluted diet fed fish which may be due to the ameliorating effects of the *Chlorella vulgaris* dry powder on fish stress. In fish, elevated cortisol levels are the most prominent physiological response to stressors like insecticides. Cortisol

is an endocrine hormone secreted from interreginal cells of the fish kidney, which is regulated by corticotropin-releasing hormone (CRH). It can be used as an indicator of fish health due to its role in metabolism, immunity, and osmoregulation. Cortisol mobilizes free fatty acids, glucose, and amino acid to meet the immediate energy demands of the animal, however, excess mobilization of these metabolites by cortisol reduced the body and muscle mass due to increased energy expenditures (Vijayan et al., 2010; Ellis et al., 2012). In agreement with our results, Paray et al. (2020) indicated that oak leaf extract partially mitigates stress responses in common carp by reducing cortisol and LDH levels.

This study suggests that the dietary *Chlorella vulgaris* inclusion on fish could regulate the activation of the innate immune system, and this increase of lysozyme activity levels can be attributed to lipopolysaccharides and carotenoids present in *Chlorella* sp. that play an important role in stimulating the immune status (Kamoshida et al., 2020). In addition, the complement system (ACH<sub>50</sub>), another innate defensive factor constituted by some serum proteins, also showed increases in all treatments with inclusion of *Chlorella vulgaris* in common carp. In fish, increase in complement system levels is usually related to a higher energy demand under stress conditions (Hoseini and Tarkhani, 2013; Giri et al., 2020; Zargar et al., 2020), but the exact mechanisms are unknown.

As a result of above-mentioned mechanisms, the use of *Chlorella vulgaris* dry powder in the fish diet significantly improved the immune components of blood and mucus compared to the control diet. Although 12.5% LC<sub>50</sub> Imidacloprid also shows a negative reducing effect, yet, implementation of *Chlorella vulgaris* has improved fish immunity even after being polluted. Regarding serum, lysozyme is an essential enzyme of the non-specific immune system that lyses certain Gram-positive bacteria and even some Gram-negative bacteria (Kamoshida et al., 2020). In mucus, the highest values belonged to 5% supplemented fish. However, some variations were observed depending on the *Chlorella vulgaris* concentration.

Michelin et al. (2021) showed that aflatoxin can reduce immune parameters of fish such as lysozyme, ACH<sub>50</sub>, Ig, MPO, NBT, C3, and C4 while Fadl et al. (2021) reported an increase in the same parameters after adding *Dunaliella* algae to the diet. Likewise in this study, lysozyme activity was increased in the blood of common carp, under the dietary *Chlorella vulgaris* while the highest immune responses were observed in the fish fed by diets containing 5% *Chlorella vulgaris* except for Ig and peroxidase which are higher in 10% of *Chlorella vulgaris* dry powder fed fish. Also, gilthead sea bream and rainbow trout fed diets with other microalgae showed a significant increase in C3 and C4 levels in multiple earlier studies (Wheeler et al., 2010; Ellis et al., 2012; Zargar et al., 2020).

The antioxidant defense systems include a series of antioxidative enzymes that is susceptible to attack by reactive oxidative stress (ROS) (Tudi et al., 2021). Anti-

oxidant defense systems including antioxidant enzymes represent protection against oxidative damage in tissue (Adeshina et al., 2019; Khafaga et al., 2020). It is known that antioxidant enzymes in fish are usually affected by nutritional factors. Superoxide dismutase (SOD) production and catalase (CAT) activity are widely used as non-specific immune indices in fish as well as their activities are key indicators of the antioxidant capability of cells (Shiau et al., 2015; Nayak et al., 2020; Chen et al., 2021).

In our research, Imidacloprid interacts with the cellular antioxidant system and induces oxidative stress resulting in a higher inflammation rate and finally accumulating free radicals in the cell. Serum antioxidant indices show maximum values in treatment T2 with 5% *Chlorella* powder and no insecticide pollution. Although there is no significant change in data obtained for CAT and GPx, SOD changes significantly. MDA also is reduced using *Chlorella* powder in the diet before and after the pollution of fish living environment with 12.5% LC<sub>50</sub> Imidacloprid but changes are not significant. Our findings are in line but a bit different from the results of Abd El-Hameed et al. (2021) where research demonstrated that dietary supplementation with a 14.30 g activated charcoal/kg diet resulted in the lowest blood glucose and serum MDA levels of Nile tilapia.

## Conclusion

In conclusion, the findings of the present study showed that dietary supplementation of *Chlorella vulgaris* dry powder enhanced the growth, immunity, and resistance of common carp to Imidacloprid infection. In this regard, the optimum results were obtained primarily on fish supplemented with the 5% *Chlorella vulgaris* and no pollution with the insecticide which means dietary *Chlorella vulgaris* can improve the health and growth of the fish in every pure and poisonous environment. 5% *Chlorella vulgaris* successfully reduced the negative effects of 12.5% LC<sub>50</sub> Imidacloprid to bring the values somewhere around the values of the control group; yet, not strong enough to eliminate the negative effects. However, more investigations need to be applied to find the optimum dosage of supplementation to gain maximum resistance to Imidacloprid infection. It is also proposed to investigate *Chlorella vulgaris* extracts on fish health and immune system.

## Compliance with ethical standards

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

## Declaration of competing interest

The authors have no conflict of interest to declare for the publication of the present work.

## Author statement

All people who meet authorship criteria are mentioned as authors, and all authors certify that they have

taken part sufficiently in the work to take public responsibility for the content, including participation in the concept, design, analysis, writing, or revision of the manuscript. In addition, every author certifies that this material or similar material has not been and will not be submitted to or published in any other publication before its appearance in the Annals of Animals Science.

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