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HEPATOPROTECTIVE EFFECT OF *Ulva lactuca* AND *Spirulina platensis* IN *Cyprinus carpio* EXPOSED TO IMAZALIL

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Abstract

The objective of the current investigation was to assess the hepatoprotective capabilities of two algal species, Ulva lactuca and Spirulina platensis, in carp (Cyprinus carpio) fingerlings subjected to imazalil exposure. Consequently, the experimental groups were simultaneously exposed to the fungicide (5mg/kg feed) and provided with feed containing either Ulva lactuca alone (IMZ-Uv variants – 5 g/kg ulva), Spirulina platensis alone (IMZ-Sp variants – 5 g/kg spirulina), or a combination of both algae (IMZ-Uv-Sp variants – 2.5 g/kg ulva and 2.5 g/kg spirulina). Two control variants were employed in the experiments: a negative control receiving normal feed and a positive control receiving feed with 5mg/kg imazalil. Each variant involved 15 fish per tank, and after the experiment, five fish from each tank were utilized for biological samples. The assessment of biochemical serum parameters and oxidative stress markers for each specimen revealed that imazalil induced alterations in biochemical parameters, resulting in physiological dysfunctions in carp. However, the administration of algae, in particular Spirulina platensis, demonstrated significant potential in mitigating the adverse effects of imazalil.

Key words*: carp, fish blood, imazalil, seaweed, spirulina.*

INTRODUCTION

Along with the increase in the number of the global population, the demand for food quantities also increased. To meet this high demand and to feed the growing urbanized population, both aquaculture and agriculture expanded cultivated areas and shifted technology towards intensive production. Thus, to obtain maximum productivity, intensive agriculture under the protection of crop protection measures, increased the amount of pesticides, thus causing contamination of surface waters (de Souza et al., 2020). Similarly, in the last decades, due to a lack of suitable sites, cage aquaculture has expanded into previously unexplored open-water culture zones in inland, coastal, and marine environments (FAO, 2020). Farming fish in cages offers numerous advantages such as low investment costs, high production, high stock survival, and easy harvesting. However, the fish biomass is continuously exposed to water quality and, in some cases, to different pollutants emerging in the aquatic environment through various pathways such as municipal sewage, industrial wastewater discharges, or agricultural runoff underground contamination

Imazalil (enilconazole) is one of the fungicides frequently used by farmers to combat and treat various pests that appear especially on fruits (citrus and bananas) but also on various seeds before planting (cereals and oilseeds) (Diedhiou et al., 2014; Kellerman et al., 2016). Thus, with its large-scale use, it increased significantly the risks of contamination of the aquatic environment (Chitescu et al., 2015) and therefore the potential to induce severe effects on fish populations (Şişman & Türkez, 2010) (Belenguer et al., 2014; Ccanccapa et al., 2016). With all the benefits that come from the use of pesticides, the risks associated with their use exceed the advantages because they produce a major imbalance in the life of aquatic ecosystems, and also produce harmful effects on

human health causing numerous health problems (Kumar Goswami et al., 2018; Fisher et al., 2022).
The studies available so far provide results whose evaluation correlates with the fact that exposure to imazalil of different fish species can produce changes in the locomotor system as well as in the development of zebrafish larvae, they can also cause dysbiosis on the composition of the intestinal microbiota, as well as imbalances on hepatic metabolism in adult zebrafish (Jin et al., 2016; Jin et al., 2017; Huang et al., 2022).
To avoid the risks associated with this excessive dependence on fungicides, new strategies, and alternatives are being sought to encourage ecological practices that mitigate their impact on water and soil quality. Researchers' attention in recent years has been directed to seaweeds, which due to their properties in the treatment of wastewater become promising sources of bioremediation technology (Karthik et al., 2020; Areco et al., 2021). At the same time, it is essential to explore strategies aimed at mitigating the risk of aquatic animal exposure to contaminants or discovering nutritional approaches to enhance the health of aquatic animals and attain higher-quality aquatic feed within the aquaculture industry. From this perspective, including algae in fish diet proved to have the potential for both directions: to effectively replace fish meal and fish oil (Shah et al., 2018) and to ameliorate the negative effects of contaminants in the fish (Abdel-Latif et al., 2022).
Spirulina platensis is a blue-green, microalga that can survive in unfavourable environments (with a high content of heavy metals, organic pollutants, and extreme environmental conditions) also due to its chemical composition rich in vitamins, minerals, essential fatty acids, amino acids, this pigment possesses antioxidant and immunomodulatory qualities, improving the growth and physiological response to stress and diseases of several species of fish (Bortolini et al., 2022; Youssef et al., 2023).
Ulva lactuca is a green macroalga that belongs to the phylum Chlorophyta whose diversification has shown an increasing trend in the last decades, both due to the global distribution and the composition and properties of the bioactive compounds identified in the composition of this macroalga (Øverland et al., 2018; Khora & Navya, 2020). The studies carried out so far, have indicated a high potential for integration into the fish diet, with beneficial effects on growth performance, oxidative stress, and immunity, in terms of improving intestinal microbiota and antimicrobial activity, but at the same time, these valuable compounds can be exploited in sectors such as the pharmaceutical industry, food nutraceuticals, agricultural or biorefineries (Kidgell et al., 2019; Thépot et al., 2021; Negreanu-Pirjol et al., 2022; Vijayaram et al., 2022)
Although those algal species were extensively explored for their potential to improve fish health there are no current studies investigating their role in mitigating the imazalil toxicity in fish. Therefore, the main aim of the present study was to assess the protective potential of the two algal species, *Ulva lactuca* and *Spirulina platensis*, in carp (*Cyprinus carpio*) fingerlings subjected to imazalil exposure. For this purpose, some hematological, oxidative stress, and plasma biochemical markers were considered.

MATERIALS AND METHODS

Experimental design and fish feeding

This experiment was conducted at the Aquaculture Pilot Station of the Faculty of Food Science and Engineering, "Dunărea de Jos" University of Galați, Romania. The fish (*Cyprinus carpio*) were obtained from the Research and Development Institute for Aquatic Ecology, Fishing and Aquaculture, Galați, Romania. All fish were acclimatized for 2 weeks during which were fed with a commercial diet (40% protein, 10% fat, 1.5% fiber). After the acclimatization period, 140 individuals, with an initial body weight of 65.4 ± 0.61 g, were randomly distributed into five experimental groups, as follows: Control (commercial diet), IMZ (commercial diet and 5 mg/kg feed), IMZ+Sp. (IMZ 5 mg/kg feed and *Spirulina platensis* 5 g/kg feed), IMZ+Uv (IMZ 5 mg/kg feed and *Ulva lactuca* 5 g dw/kg feed), and IMZ+Uv+Sp. (IMZ 5mg/kg feed, *Spirulina platensis* 2.5 g/kg feed and *Ulva lactuca* 2.5 g/kg feed). The green macroalgae *Ulva lactuca* were purchased from Algas Atlanticas Algamar S.L., the collection area being the Bay of Biscay

Galicia, Spain. The algal dry biomass was crushed with the help of a mill and later incorporated into the feed. *Spirulina platensis* was purchased from the company Cerasus, China. Imazalil was purchased from Sigma-Aldrich Production GmbH, Switzerland.

The experiment took place over a period of 27 days and was conducted in glass aquariums, with a capacity of 140 L each. Each aquarium has an independent water filtration system. Fish were fed two times per day (09:00, and 17:00) with a daily ration of 2.6% of fish body weight (BW). The survival was surveyed daily.

Hematological analysis and blood biochemistry. At the end of the experimental period, 2 mL of blood was taken by caudal venous puncture, from 7 fish randomly chosen from each experimental unit. For the biochemical analysis, plasma was obtained by centrifuging the blood at 3500 rpm for 10 minutes.

The hematological profile was determined using the routine methodology of fish hematology. Red blood cell count (RBC × 106/µL) was determined by utilizing a Potain pipette and Vulpian diluting solution. The calculation of RBC (10^6/µL) involved the number of cells counted, the number of squares in which they were counted, square volume, and blood dilution (Svobodova et al., 2012). For hematocrit determination (Ht, %), the microhematocrit method was employed following blood centrifugation at 12,000 rpm for 5 minutes. Hemoglobin concentration (Hb, g/dL) was measured using the cyanmethemoglobin method with Drabkin's reagent (DIALAB, Wiener Neudorf, Austria), and the absorbance was read at a wavelength of 540 nm (Hesser, 1960) using a Specord 210 UV–Vis spectrophotometer (Analytic Jena, Jena, Germany).

Hematological indices, including mean corpuscular volume (MCV, fL), mean corpuscular hemoglobin (MCH, pg), and mean corpuscular hemoglobin concentration (MCHC, g/dL), were calculated based on PCV, Hb, and RBC values (Ghergariu et al., 1985; Svoboda, 2001).

The plasma **biochemical parameters** determinations were made with the help of VetTest® Chemistry Analyser, using IDEXX VetTest kits for albumin (ALB, g/dL), Calcium (Ca, mg/dL), Total protein (TP, g/dL), Glucose (GLU, mg/dL) and Globulins (GLOB, g/dL).

Oxidative stress. Lipid peroxidation was quantified by malondialdehyde (MDA, nmol/mL) concentrations. The MDA was assessed using the method outlined by Ohkawa et.al 1979. The optical density of the samples was measured at 532 nm, and the results were expressed as nmol of MDA per mL of plasma or per gram of liver homogenate (nmol/g liver).

Lysozyme activity (LZM, U/mL) was determined through a turbidimetric assay, following the Enzymatic Activity of Lysozyme Protocol (Sigma, EC 3.2.1.17, Sigma-Aldrich, St. Louis, MO, USA). Total antioxidant capacity (TAC, mMol Trolox) was measured spectrophotometrically at an optical density of 734 nm, utilizing the ABTS - (2,2-azinobis 3-ethylbenzothiazoline-6-sulphonic acid) method as described by Re et al. (1999).

Statistical analysis. Statistical analyses were conducted utilizing the SPSS statistical software for Windows, Version 16.0, by SPSS Inc., Chicago, United States. Hematological and serum parameters were presented as means ± S.E. of the replicates. A one-way ANOVA analysis was performed on the data. If significant differences were detected, a Ducan's post hoc test was employed. The significance level for all analyses was established at $p < 0.05$.

RESULTS AND DISCUSSIONS

Growth performance

At the outset of the experiment, there was no statistically significant variation ($p>0.05$) in the initial body mass among the six experimental groups of carp specimens. However, by the end of the trial, the mean weight of the carp exhibited significant differences ($p<0.05$) among the five experimental groups. Thus, IMZ did not affect significantly the growth performance. Notably, the IMZ-Sp experimental variants demonstrated the highest final average weight (FW) and specific growth rate (SGR) at 87.40±11.65 g and 1.04±0.09%, respectively (Table 1).

Table 1. Effect of different treatments on specific growth rate and feed conversion efficiency of *Cyprinus carpio*

Parameters	Experimental groups				
	Control	**IMZ**	**IMZ-Sp**	**IMZ-Uv**	**IMZ-Sp-Uv**
Initial average weight IW (g)	66.14±10.15[a]	66.92±9.02[a]	65.78±5.9[a]	64.64±7.2[a]	64.92±6.76[a]
Final average weight FW (g)	83.14±18.61[a]	84.50±8.22[a]	87.40±11.65[b]	84.93±10.16[a]	83.14±07.65[a]
Specific growth rate SGR (%)	0.85±0.11[a]	0.86±.0.07[a]	1.04±0.09[c]	1.01±0.12[c]	0.92±0.03[b]
Feed conversion ratio FCR	2.04±0.19[c]	1.98±0.12[c]	1.64±0.15[a]	1.71±0.03[a]	1.91±0.02[b]

Different letters reflect significant differences between groups

Hematological parameters

At the end of the experiment, fish hematological parameters were determined, and the results are presented in Table 2. Thus, for the fish from IMZ groups, statistical analysis revealed significant ($p < 0.05$) lower values for Ht, Hb, and RBC and higher values for MCV and MCH compared to the control. Nevertheless, the highest values of Ht and MCV were recorded in the IMZ-Uv-Sp groups while the lowest MCV and MCH were observed for IMZ-Sp groups, followed by imazalil and respectively imazalil and ulva, while the highest value of MCH was for the diet with imazalil with ulva, followed by the diet with imazalil and the one with imazalil ulva and spirulina. The higher values for MCV and MCH were observed for the diet with imazalil and spirulina. Regarding the MCHC value, the highest level was for the treatment with IMZ-Uv, followed by IMZ-Sp while the lowest value was observed for IMZ-Uv-Sp treatment.

Table 2. Hematological parameters of *Cyprinus carpio* in different experimental groups

Group experimental	Ht (%)	Hb (g dL^{-1})	RBC (10^6/μL)	MCV ($μm^3$)	MCH (pg)	MCHC (g dL^{-1})
Control	41.44±3.76[b]	9.67±0.38 [b]	1.87[b]	223.33 [b]	52.13 [a]	23.51[b]
IMZ	39.73±4.63[a]	9.26±2.07[a]	1.72[a]	236.95[bc]	55.49 [b]	23.64[b]
IMZ-Uv	42.68±7.00[b]	10.91±2.67[b]	1.95[b]	225.53 [b]	57.93 [b]	25.52[c]
IMZ-Sp	41.51±6.37[b]	9.96±0.91[c]	2.12[c]	195.75 [a]	47.55 [a]	24.33[bc]
IMZ-Uv-Sp	47.32±4.23[c]	10.22±0.81[b]	1.92[b]	249.80 [d]	54.12[bc]	21.64[a]

Different letters reflect significant differences between groups

Serum Metabolic Profile

At the end of the experiment, several parameters of the metabolic profile of the fish were analyzed, namely glucose (GLU), total proteins (TP), albumin (ALB), calcium (Ca), and globulins (GLOB), represented in Table 3. The highest value of the total protein was in the IMZ-Uv variant (4.26±0.47 mg/dl), while the lowest value was recorded in the control groups (3.08±0.06 mg/dl). The levels of the protein fractions of albumin and globulin varied between the different experimental treatments so that significantly higher values of globulin and albumin were recorded for the IMZ groups (3.20±0.57 mg/dl, respectively 1.12±0.14 mg/dl) comparing with control where TP and ALB had the lowest level (3.08±0.06 mg/dl respectively 0.74±0.07 mg/dl). Regarding the blood glucose concentration, it varied significantly between all treatments, the lowest value being in the treatment IMZ-Sp (133.90±8.14 mg/dl) and the highest for the IMZ treatment (172.80±11.12 mg/dl). The calcium level showed a high value in the case of the IMZ treatment (11.60±0.28 mg/dl), while the lowest value was indicated by the control group (8.62±1.20 mg/dl).

Table 3. Effect of different treatments on serum biochemical parameters for *Cyprinus carpio*

Parameters	Experimental groups				
	Control	**IMZ**	**IMZ-Sp**	**IMZ-Uv**	**IMZ-Sp-Uv**
GLU (mg/ dL)	163.66±9.50[a]	172.80±11.12[b]	133.90±8.14[a]	162.20±44.23[a]	157.67±19.01[a]
TP (g/dl)	3.08±0.06[a]	4.20±0.64[c]	3.82±0.38[b]	4.26±0.47[b]	4.00±0.50[b]
ALB(g/dl)	0.74±0.07[a]	1.12±0.14[b]	0.98±0.07[b]	1.10±0.07[b]	1.00±0.07[b]
GLOB(g/dl)	2.4±0.0 [a]	3.20±0.57 [c]	2.65±0.35 [b]	3.08±0.07 [b]	3.00±0.35 [b]
Ca(mg/dl)	8.62±1.20[a]	11.60±0.28[b]	10.68±1.84[ab]	11.20±1.63[b]	10.60±0.64[ab]

Different letters reflect significant differences between groups

Oxidative Stress Parameters

Biomarkers of oxidative stress showed some changes compared to the control group. Thus, for groups exposed to IMZ both plasma and gut MDA increased significantly ($p<0.05$) compared with the control variant. Although the plasma and gut MDA values were higher in all fish groups exposed to imazalil regardless of the diet, the increase was not significant for groups fed with a diet containing spirulina.

Total antioxidant capacity dropped in all experimental groups but significant differences were found only for IMZ, IMZ-Uv, and IMZ-Sp-Uv groups. Also, the lysozyme (LYZ), which represents an indicator of the immune function evaluated in this study was affected by IMZ. However, the least affected group was represented by IMZ-Sp-Uv (Table 4).

Table 4. Effect of different treatments on oxidative stress markers for *Cyprinus carpio*

Parameters	Experimental groups				
	Control	**IMZ**	**IMZ-Sp**	**IMZ-Uv**	**IMZ-Sp-Uv**
MDA (nmol/ml)	5.49 ± 1.17^{a}	7.28 ± 0.29^{b}	5.54 ± 0.64^{a}	6.66 ± 1.06^{ab}	5.67 ± 0.39^{a}
MDA gut (nmol/g)	7.45 ± 0.43^{a}	8.51 ± 0.37^{c}	7.58 ± 0.29^{a}	8.30 ± 0.41^{b}	7.53 ± 0.27^{a}
TAC (mm Trolox)	20.42 ± 2.94^{c}	15.17 ± 0.85^{a}	20.32 ± 2.71^{c}	19.04 ± 1.08^{b}	17.81 ± 2.59^{b}
Lysozyme (U/mg solid)	223.79 ± 0.78^{b}	204.88 ± 2.86^{a}	207.52 ± 0.94^{a}	206.64 ± 1.58^{a}	212.96 ± 6.51^{b}

Different letters reflect significant differences between groups.

Water pollution has become a real problem and quite difficult to control due to the various harmful substances (insecticides, pesticides, heavy metals, petroleum waste) that are frequently released into the aquatic environment (Khoshnood, 2017; Shah & Parveen, 2020). Part of the polluting substances are the fungicides that are used in agricultural practices, which once in the water can produce unwanted effects on the health of aquatic organisms and implicitly can also affect the health of people through their consumption (Amenyogbe et al., 2021)**.** One of the disturbing factors that involve changes in the morpho-physiological state of the fish is the stress caused by water pollution (Sinha et al., 2022). The hematological and serum/plasma biochemical parameters represent a valuable tool in toxicological research and environmental monitoring due to their high sensitivity in the presence of contaminants, thus producing physiological and pathological changes (Bhatnagar et al., 2017; Kumar Maurya et al., 2019; Ahmed et al., 2020)**.**

The present study aims to evaluate the potential protective effect of the two algae *Ulva lactuca* and *Spirulina platensis* for the *Cyprinus carpio* fish when exposed to imazalil.

The results of our study indicate that the fish exposed to imazalil (5 mg/kg body weight) through the feed registered a decreased number of RBC, Ht, and Hb. Similar results were reported by other authors who found low values of Ht, Hb and RBC in fish exposed to different types of pesticides for species such as *Oreochromis mossambicus* (Ghayyur et al., 2019), *Oreochromis niloticus* (Dawood et al., 2020), *Cyprinus carpio* (Chatterjee et al., 2021), *Cirrhinus mrigala*, (Ghayyur et al., 2021) and *Mystus keletius* (Barathinivas et al., 2022). This suggests that anemia may have been triggered by toxic stress, leading to the breakdown of red blood cells by reactive oxygen species (ROS) generated in reaction to exposure to the fungicide (Bloom & Brandt, 2008).

Similar reports, emphasizing the negative impact of different pesticides (herbicides, insecticides, and fungicides) on oxygen transportation with consequences on fish metabolic rate, are available (Bojarski & Witeska, 2020; Lutnicka et al., 2016).

For the experimental groups, where the diet was supplemented with algae, a slight increase of RBC, Ht, and Hb was observed. However, the significant increase of RBC was associated with IMZ-Sp groups while significantly higher Ht was observed in IMZ-Uv-Sp groups. Therefore, spirulina had a protective role in reducing cytotoxic effects induced by imazalil. Similar studies (Sharma et al., 2005) showed that spirulina could alleviate also the toxicity stress of azo dye-methyl red on the red blood cells (RBCs) of guppies (*Poecilia reticulata*). Moreover, the groups fed with Ulva algae showed the highest level of hemoglobin, which indicates the potential of *Ulva lactuca* to improve the immune response. This ability is supported by the content of various secondary metabolites such as carotenoids, polyphenols, and flavonoids that improve health and increase fish resistance (Abu Hafsa et al., 2021; Pratiwi & Pratiwy, 2022).

An important role in the description of the state of anemia is also played by the blood parameters MCV, MCH, and MCHC, which represent important indicators in determining the size, density, and hemoglobin concentration of red blood cells (Witeska et al., 2023). In our study, significant variations were observed between the different experimental groups, compared to the control group. The elevated MCV and MCH values observed in IMZ-Uv variants may suggest a macrocytic anemia, while the low value of MCV and MCH observed in IMZ-Sp indicates a microcytic anemia, all of which indicate a defense reaction against exposure to pesticides.

These similar effects were also reported in the comparative study of two species of fish, *Clarias gariepinus* and *Oreochromis niloticus* exposed to several types of pesticides on several exposure models, where it was shown that continuous exposure to pesticides is as dangerous as short-term exposure to an increased level of pesticides (Kanu et al., 2023).

To assess the metabolic profile and nutritional well-being of the fish, it is highly beneficial to determine the total content of plasma proteins, including albumins and globulins. This evaluation aids in assessing the health status of the fish and the effectiveness of both specific and non-specific defense mechanisms against harmful agents. Consequently, levels of total protein (TP), albumin (ALB), and globulin (GLOB) increased across all experimental groups compared to the control.

However, it should be mentioned that for all experimental groups where the feed was supplemented with algae, these parameters were significantly higher ($p<0.05$) than the negative control, IMZ group. This suggests that both varieties of algae possess the ability to mitigate the negative impacts of IMZ and restore the concentration of serum proteins

Glucose is also a biochemical indicator that can be used to evaluate the degree of normality of the general physiological state, which can be easily modified under the influence of external or internal factors, having an important role in establishing the level of stress in fish. (Ray & Sinha, 2014).

The data of the current study indicated that the level of serum glucose in carp exposed to imazalil significantly increased ($p<0.05$) compared with the control. In fish exposed to IMZ but fed with algae, the glucose decreased to the level found in control excepting the IMZ-Sp groups which had the lowest glucose level. The reduced level of glucose could highlight the role of the two algae in suppressing the state of stress. Moreover, spirulina has a glucose-lowering effect demonstrated also in other studies. Thus, this effect was emphasized in studies conducted to evaluate the potential of spirulina to mitigate the effect of chlorpyrifos in *Clarias gariepinus* (Mokhbatly et al., 2020) or the effect of diazinon in *Oreochromis niloticus* (Abdelkhalek et al., 2017).

It is well known that both *Ulva lactuca* and *Spirulina platensis* are good candidates for oxidative stress alleviation. Thus, *U. lactuca* contains bioactive compounds such as ulvan or phlorotannins (Holdt & Kraan, 2011) while *S. platensis* contains vitamins, minerals, carotenoids, polysaccharides, and γ-linolenic acid, compounds with a great role in antioxidant defense (Adel et al., 2016).

In our investigation, both types of algae demonstrated the ability to reduce the MDA levels (in plasma and gut tissue) amplified by IMZ. Nevertheless, *S. platensis* proved to have a more pronounced MDA lowering effect compared with *U. lactuca*, since the lowest values were observed in the IMZ-Sp group and, respectively, IMZ-Sp-Uv group. The same pattern was observed also for total antioxidant capacity which was less affected by IMZ in spirulina-fed fish, IMZ-Sp variant.

The primary effect of immunostimulant agents is to enhance the function of phagocytic cells and boost their activity in animals (Sakai, 1999). Previous experiments demonstrated that polysaccharides derived from seaweed exert a beneficial impact on cultured fish by modifying the activity of certain components of the innate immune system (Akbary & Aminikhoei, 2018). In this study, it was observed that by the end of the 4 weeks, fish-fed diets containing Ulva and Spirulina exhibited higher levels of lysozyme and phagocytic activity compared to those fed only Ulva or only spirulina diets.

The adverse impact of imazalil on growth performance parameters was not detected. This could be attributed to the relatively short experimental period during which the tertiary stress level may not have had adequate time to install.

CONCLUSIONS

In our study, imazalil did not influence the growth of *C. carpio*, but it did alter the health condition as indicated by selected hemato-biochemical markers. The inclusion of algae in the carp's diet had a positive impact on enhancing antioxidant levels and bolstering fish immunity. *Spirulina platensis* exhibited superior outcomes concerning the plasma biochemical profile, while both *S. platensis* and *U. lactuca* contributed to improving oxidative stress biomarkers. The potential mutualistic relationship between *Ulva lactuca* and *Spirulina platensis* was highlighted. However, further research is required to substantiate this relationship.

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