Scientific Papers. Series D. Animal Science. Vol. LXVII, No. 2, 2024
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

# KRILL OIL SUPPLEMENTATION AMELIORATES FRUCTOSE-INDUCED HYPERTRIGLYCERIDEMIA IN *Carassius auratus calico*

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***Abstract***

*Fructose is a highly lipogenic sugar and excessive fructose intake stimulates endogenous glucose production and lipid synthesis in the liver. Therefore, the present study aims to investigate the amelioration of liver impairment induced by high fructose dietary doses following dietary supplementation with krill oil. 45 exemplars of Carassius auratus calico weighing 150 grams, were randomly distributed into three experimental diets including V1 - Control, fish fed with a normal diet, V2 - fish fed with 1% fructose, V3 - fish fed with 2% fructose, respectively, at the end of 90 days: V1 - Control, fish fed with a normal diet, V2 - fish fed with 1% krill oil, V3 - fish fed with 2% krill oil. The blood tissues were collected to analyze hematological parameters (red blood cell counts (RBCc, x 106/µl), the hematocrit (PCV, %), hemoglobin concentrations (Hb, gl/dl,) HEM, VEM, CHEM and serum biochemistry parameters (GLU, TRIG, ALT and AST). In addition, the organosomatic indices (HIS, VSI) and proximate composition (water, lipid, ash, and protein content) were analyzed. No differences between the control and treated fish from the two experimental diets were observed in hematological parameters. After 90 days fructose-treated fish showed increased AST and ALT levels. Our results showed that daily krill oil supplementation in the Carassius auratus calico prevented fructose-induced hypertriglyceridemia.*

***Key words****: Aquaculture krill oil, hematological and serum biochemistry parameters, organosomatic indices.*

## INTRODUCTION

The Goldfish (*Carassius auratus calico*) belongs to the family *Cyprinidae* and is one of the most popular freshwater ornamental fish in the world, including in Romania. Being omnivorous, goldfish consume invertebrates and zooplankton from natural water bodies. However, goldfish are commonly fed high-quality granulated or pelleted feeds in artificial breeding systems (Hafeez-ur-Rehman et al., 2015).

Because more than half of the operational costs of ornamental species are associated with food costs, feed is an important factor that can affect fish welfare, growth, and profitability (Jamu & Ayinla, 2013; Fry et al., 2018). The goldfish, a modified variety of *Cyprinus carpio*, could be used as an ideal model for nutritional studies in larval and juvenile cyprinids. Carbohydrates such as glucose and fructose are the least expensive sources of energy for fish diets. Long-term consumption of diets high in carbohydrates leads to a significant accumulation of fat in the serum and liver. This is because carbohydrates can be converted into lipids through a process known as lipogenesis. The omnivorous fish have a greater ability to convert carbohydrates into lipids, as demonstrated in studies on gibel carp (*Carassius gibelio*) (Li et al., 2019).

Despite generally exhibiting higher plasma insulin levels than mammals (Moon, 2001), fish display lower insulin sensitivity and are classified as glucose intolerant. This characteristic makes fish more susceptible to hyperglycemia, which is linked to various metabolic diseases in fish (Prisingkorn et al., 2017; Goessling & Sadler, 2015). Herbivorous and omnivorous fish utilize carbohydrates more efficiently than carnivorous fish due to their more developed digestive system, enzyme activity of glucose metabolism, and complex hormone regulation (Legate et al., 2001). Hyperglycemia contributes to lipid deposition in fish, as Luo et al. (2020) reported.

The effect of high fructose levels on hepatopancreatic function in goldfish has not

been studied. In the case of diets with high levels of carbohydrates, functional ingredients are sought to reduce their negative effects. In this context, Krill can be a good candidate for several reasons. Antarctic krill (*Euphausia superba*) may represent the largest biomass of a single species worldwide, and it is a promising ingredient for use in fish feeds. Krill has a balanced amino acid profile, and krill oil contains the essential nutrient, choline, and an antioxidant, astaxanthin (Xie et al., 2019; Bengtson et al., 2014). In addition, Krill oil serves as a valuable alternative source of n-3 polyunsaturated fatty acids (PUFAs), containing a substantial content (30-65%) of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). (Hansen et al., 2011).

Within the aquaculture sector, krill has primarily been investigated as a protein source (Choi et al., 2020; Mørkøre et al., 2020; Olsen et al., 2006), and its utilization for pigmenting fish flesh with carotenoids has also been explored (Arai et al., 1987). However, some research specifically delves into the potential of krill oil (KO) as a functional ingredient to manage stress and promote health in intensively cultured fish (Li et al., 2021). The supplementation with 5 g/kg feed krill oil of the carp diet has been found to enhance growth performance, with no observed alterations in the proximate composition of the meat (Năstac et al., 2023). KO feeding also reduced oxidative damage in the liver, with a decrease in malondialdehyde content and an increase in total antioxidant capacity, thus improving the lysozyme activity and immune status of the carp fingerlings held under different stocking densities (Năstac et al., 2023).

Therefore, the present study aims to investigate the amelioration of liver impairment induced by high fructose dietary doses following dietary supplementation with krill oil.

## MATERIALS AND METHODS

### Experimental Design

*Phase I.* 90 Fingerlings of *Carassius auratus calico,* with an individual mean weight of 3.33 ± 0.09 g, were randomly selected to create three experimental variants: V1 - Control, fish fed with a normal diet, V2 - fish fed supplemented with 1% fructose, V3 - fish fed supplemented with 2% fructose, respectively.

*Phase II.* After 90 days of feeding, 12 from each experimental variant were selected, and the experimental variants were as follows: V1 - Control, fish fed with a normal diet, V2- fish fed supplemented with 1% krill oil, V3 - fish fed supplemented with 2% krill oil (Figure 1). This phase lasted for 40- days. During this trial, fish were fed two times a day at 08:00 a.m. and 4:00 p.m. at a rate of 5% of their body weight, with a commercial diet (37% protein, 12% fat, 4% fiber, 6% ash).

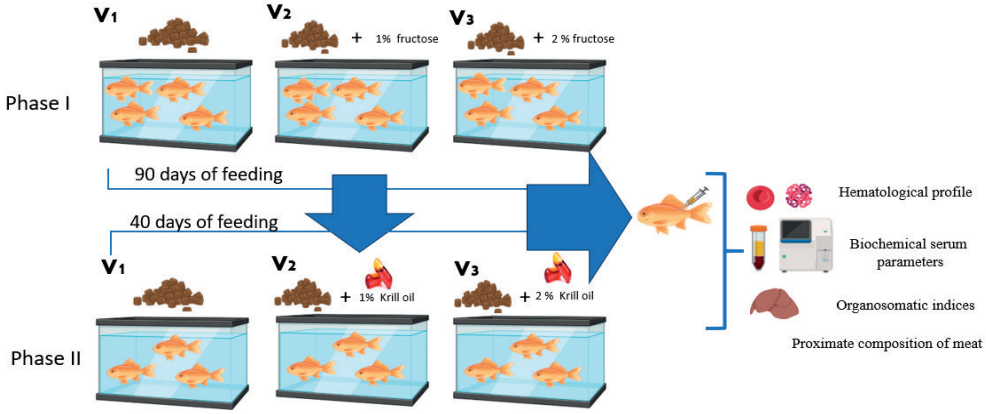


Figure 1. Experimental design (original - Created with BioRender.com)

The experimental activity took place at the Romanian Center for Modelling Recirculating Aquaculture Systems (www.moras.ugal.ro) from the "Dunărea de Jos" University of Galați, Romania. For the experimental activity, six aquariums were used, with a volume of 40 L each. During the experimental period, the water quality parameters such as dissolved oxygen,

temperature, and pH were measured daily with the YSI multi-parameter (YSI Pro1020). Weekly we quantify the concentration of nitrogen compounds with the help of the Spectroquant Nova 400 photometer compatible with Merck kits. The average and standard deviation (±SD) of water quality parameters monitored throughout the research were: water temperature was 23.40 ± 0.61°C, pH 7.4, DO was 6.6 ± 0.03 mg $L^{-1}$, nitrate was 4.9 ± 0.6 mg $L^{-1}$, nitrites 0.01±0.02 mg $L^{-1}$ and ammonia 0.05 ± 0.01 mg $L^{-1}$.

*Hematological and biochemical parameters.* After 90 days of fructose administration (phase I) and after 40 days of feeding with a supplemented diet with krill oil (Phase II) blood samples were collected. Before blood collection fish were gently caught and placed in phenoxyethanol (0.7 mL/L) until deep anesthesia. Blood samples were obtained from five fish in each aquarium through caudal venous puncture, utilizing lithium heparin as an anticoagulant. Subsequent blood analysis employed routine methods commonly applied in fish hematology (Svobodova et al., 1991)

The red blood cells (RBC × $10^6$/µL) were counted using glass blood diluting pipette Vulpian diluting solution and a Neubauer hemocytometer. Hemoglobin concentration (Hb, g/dL) was determined utilizing the cyanmethemoglobin method with Drabkin's reagent. Hematocrit (PCV, %) was assessed through the microhematocrit method, involving the centrifugation of blood at 12,000 rpm for 5 minutes. 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 the values of PCV, Hb, and RBC, following the methodology outlined by Blaxhall & Daisley in 1973.

The VetTest® Chemistry Analyzer and IDEXX VetTest kits (IDEXX Laboratories, Inc., Westbrook, ME, USA) were employed for the biochemical analysis. Plasma required for the determination of biochemical parameters such as glucose (GLU, mg/dL), alanine aminotransferase (ALT, U/L), aspartate aminotransferase (AST, U/L), and triglycerides (TG, mg/dL), was obtained by centrifuging blood at 3500 rpm for 10 minutes.

*Organosomatic Indices.* After the end of phase I and II, 6 fish from each experimental variant were sacrificed and the weight of the liver, gonads, and viscera were assessed, for the calculation of hepatosomatic and viscerosomatic indices:

- Hepatosomatic index (HSI, %) = [liver weight (g)/body weight (g)] × 100;
- Viscerosomatic index (VSI, %) = [viscera weight (g)/body weight (g)] × 100;

*Proximate Composition Analysis.*

The proximate composition analyses were conducted by the established procedures outlined by the Association of Official Analytical Chemists (AOAC, 1997). In this context, 6 fish from each experimental variant were used for the determination of water, lipid, ash, and protein content. To determine dry matter, samples were subjected to drying until a constant weight was achieved at 105°C for 24 hours in a convection oven (Jeiotech, Jeio Tech Co., Inc, Korea). After moisture content determination, the dry samples were finely ground for the analysis of lipids, protein, and ash. Lipid content (%) was assessed through the Soxhlet extraction method, utilizing petroleum ether as the solvent (Gerhardt GmbH & Co. KG, Germany). The ash content (%) was determined by employing a muffle furnace (Nabertherm, Applied Scientific Instruments Co., Ltd. Thailand) at 525±25°C for 8 hours. The crude protein content (%) was calculated by converting the nitrogen content, quantified by Dumas's method through the combustion of dry samples at 1100°C (Primacs SNC 100, Skalar Analytical B.V., The Netherlands), using the common conversion factor of N×6.25.

The fish experiments were conducted with ethical approval obtained from the Ethics Committee of the "Dunărea de Jos" University of Galați, Romania.

*Statistical analysis* of the data was performed using SPSS software (version 26.). Data are expressed as mean ± SD. One-way ANOVA was employed to assess statistical differences between the experimental variants. The Duncan multiple range test further evaluated significant

differences among the groups. The chosen level of significance was set at $p < 0.05$.

## RESULTS AND DISCUSIONS

At the end of the two experimental stages were analyzed the fish hematological parameters such as hemoglobin (Hb), red blood cell count (RBC), hematocrit (PCV), and the erythrocytes indices (MCV, MCH, MCHC) (Table 1).

Table 1. Hematological parameters of goldfish

| | Hematological parameters | V1 | V2 | V3 |
|---|---|---|---|---|
| Phase I | Hb (g/dL) | 9.77± 0.88[a] | 9.33± 0.75[a] | 9.51± 1.03[a] |
| | PVC (%) | 32.04± 1.63[a] | 31.67± 3.04[a] | 32.42± 5.11[a] |
| | RBCc ($\times 10^6/\mu L$) | 1.49± 0.18[a] | 1.52± 0.26[a] | 1.51± 0.17[a] |
| | MCV (fL) | 215.03± 12.44[a] | 208.35± 19.08[a] | 214.70± 21.52[a] |
| | MCH (pg) | 65.57± 9.21[a] | 61.38± 12.15[a] | 62.98± 20.31 |
| | MCHC (g/dL) | 30.49± 5.97[a] | 29.46± 8.65[a] | 29,33± 6.02 |
| Phase II | Hb (g/dL) | 10.21± 1.86[a] | 9.26± 1.10[a] | 9.47± 1.21[a] |
| | PVC (%) | 31.79± 2.41[a] | 30.19± 5.35[a] | 32.8± 5.66[a] |
| | RBCc ($\times 10^6/\mu L$) | 1.56± 0.19[a] | 1.50± 0.34[a] | 1.66± 0.09[a] |
| | MCV (fL) | 192.01± 16.41[a] | 208.51± 78.98[a] | 197.37± 33.91[a] |
| | MCH (pg) | 61.67± 11.49[a] | 64.40± 18.14[a] | 57.11± 8.78[a] |
| | MCHC (g/dL) | 32.26± 6.68[a] | 33.49± 12.34[a] | 29.42± 5.08[a] |

Data are expressed as the Mean ± SD. Different letters indicate significant differences between experimental variants (ANOVA, $p < 0.05$).

Regarding the influence of different concentrations of fructose, we noticed that there are no statistically significant differences ($p > 0.05$) in the main hematological parameters (Hb, PVC, RBC). At the end of the feeding krill oil experiment, although no significant differences ($p > 0.05$) were detected, the data showed a slight increase in values of RBC in the V3 variant. Haemoglobin content (Hb), hematocrit (PVC), mean corpuscular volume (MCV), mean corpuscular hemoglobin content (MCH), and mean corpuscular hemoglobin concentration (MCHC) were not significant ($p > 0.05$) affected by dietary krill oil. At the end of the trial and after the krill oil supplementation, the metabolic profile of the fish was analyzed, plasma biochemical parameters values (mean ± SD) (AST, ALT - aspartate and alanine aminotransferase, TRIG - triglycerides, and GLU - glucose) are presented in Table 2.

Table 2. Serum metabolic profile parameters of goldfish

| Exp. design | Biochem Param. | V1 | V2 | V3 |
|---|---|---|---|---|
| Phase I | AST (U/L) | 61.33± 2.34[a] | 59.50± 3.65[a] | 71.67± 5.07[b] |
| | ALT (U/L) | 417.00± 14.08[b] | 341.50± 16.05[a] | 532.67± 17.64[c] |
| | GLU (mg/dL) | 74.67± 3.49[a] | 77.00± 4.06[a] | 78.33± 4.21[a] |
| | TRIG (mg/dL) | 158.00± 5.22[a] | 184.50± 4.88[b] | 205.00± 7.21[b] |
| Phase II | AST (U/L) | 56.00± 3.16[b] | 49.00± 3,31[a*] | 46.00± 2.40[a*] |
| | ALT (U/L) | 398.00± 15.44[b] | 315.00± 21.17[a*] | 423.50± 17.23[b*] |
| | GLU (mg/dL) | 80.50± 2.78[a] | 81.00± 2.44[a] | 81.50± 3.07[a] |
| | TRIG (mg/dL) | 155.5± 7.02[a] | 180.5± 8.87[b] | 174.00± 8.05[b*] |

Values are Mean±S.D., Values with different superscripts in a column differ significantly (ANOVA, $p < 0.05$). Values with different symbols * in a row differ significantly after krill oil ($p < 0.05$).

Before the Krill oil supplementation, fish fed with 1% and 2% fructose registered significant changes in some biochemical parameters compared with groups fed with a normal diet. Fructose 1% did not induce significant changes in AST, ALT, and GLU ($p > 0.05$), except TRIG, which had higher values, bat AST, ALT, and TRIG significant increase in fish feed with fructose 2% ($p < 0.05$). After Krill oil supplementation, significant decreases were observed in the ALT, AST, and TRIG values from V3 ($p < 0.05$), while in version V2 the changes in biochemical parameters were statistically insignificant ($p > 0.05$).The effects of different concentrations of dietary krill oil on the chemical composition of the whole body of *Carassius auratus* var. *calico* are shown in Figures 2, 3, and 4. No significant differences were shown in whole-body moisture, protein, lipid, and ash among the dietary treatments ($p>0.05$).

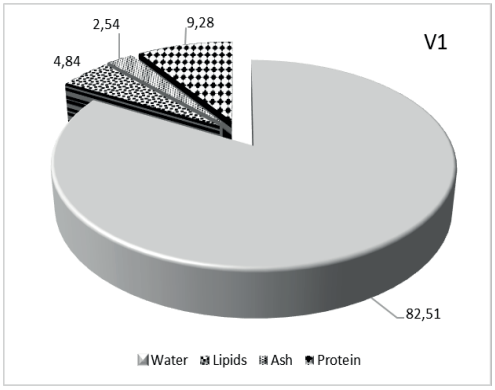


Figure 2. The biochemical composition of fish at the end of phase II

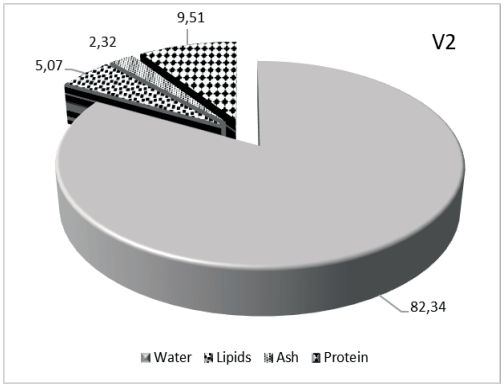


Figure 3. The biochemical composition of fish at the end of phase II

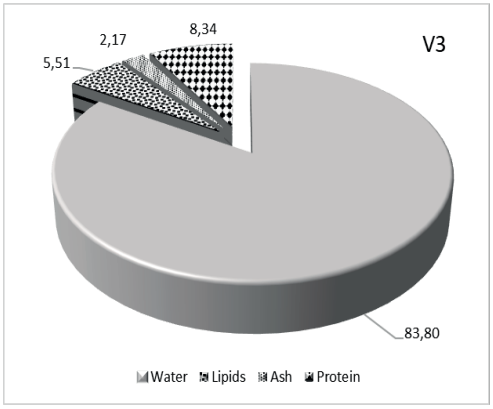


Figure 4. The biochemical composition of fish at the end of phase II

Table 3 presents the values of organosomatic indices at the end of the two experimental stages. In the case of our experiment, ANOVA statistical analysis revealed no significant differences (P>0.05) for the values of HIS and VSI indices.

Table 3. The viscerosomatic and hepatosomatic index of goldfish

| Period | Experimental variants | HSI | VSI |
|---|---|---|---|
| Phase I | V1 Control | 3.33± 0.07[a] | 9.66± 1.68 |
| | V2 1% fructose | 3.97± 0.03 | 10.37± 0. 85[a] |
| | V3 2% fructose | 4.20± 0.07 | 10.99± 1.13 |
| Phase II | V1 Control | 3.80± 1.13[a] | 16.72± 3.81[a] |
| | V2 1% Krill | 3.67± 1.49 | 13.09± 5.23[b] |
| | V3 2% krill | 3.97± 1.06 | 13.29± 5.10[b] |

Data are expressed as the mean ± SD. Different letters indicate significant differences between experimental variants (ANOVA, $p < 0.05$).

Carbohydrates are an important ingredient in fish diet, but the dosage is critical. Fructose is known as a pro-inflammation metabolite in mammals (Jones et al., 2021). The excessive intake of fructose is harmful to health, especially for organisms with chronic diseases (Velickovic et al., 2019). High fructose (10.5 g/kg) significantly increases uric acid and proinflammatory cytokines in serum (Wang et al., 2020). On the other hand, some results indicated the beneficial effect of fructose as a metabolite in promoting *C. carassius* survival and also report that fructose mainly enhances the expression of lysozyme and complement, which enables the bacteria to be reduced more efficiently (Cao et al., 2022).

Several studies examining the influence of diet on various hematological parameters have determined that alterations in these parameters occur in response to changes in dietary composition. This underscores a correlation between food intake and hematological variations in the investigated fish species. (Abdulrahman et al., 2019; Satheeshkumar et al., 2012; Hoseinifar et al., 2011; Abdel et al., 2006).

The findings from our current study indicate that a diet containing fructose had no discernible impact on hematological parameters. Similarly, after the experiment, supplementation with krill oil also demonstrated minimal effects, with only a slight increase observed in the number of erythrocytes. The slight increase in erythrocyte count following dietary supplementation with krill oil may be due to krill's iron intake (John et al.,

1983), which is known to be an essential component of hemoglobin. Krill also contains vitamins and minerals (B12, B9) (Xie et al., 2019) essential for the formation of red blood cells.

Analyzing blood parameters provides insights into the physiological condition of fish and can be indicative of various health-related factors. The interest in such studies has increased in recent years due to the importance of understanding the baseline values of blood parameters and how they can be influenced by a variety of factors (Ahmed et al., 2020). Nutrition plays a crucial role in the overall health of fish, and the composition of their diet can affect blood parameters (Esmaeili, 2021; Kenari et al., 2011). For example, the quality and quantity of nutrients in the fish's diet can influence parameters like hematocrit, hemoglobin levels, and various metabolic indicators (Azaza et al., 2020).

Alterations in the composition of blood serum components can be utilized to detect specific functional disorders in the organs and assess the overall health status of fish (Li et al., 2023). In our study, we evaluated the variation of some biochemical parameters (AST, ALT, GLU, TRIG) to highlight the beneficial role of krill oil in the case of administration of a high-carbohydrate diet. After ingesting a high-carbohydrate diet (1 % and 2% fructose), there was no apparent effect on plasma glucose levels, indicating that goldfish are capable of regulating glycemia effectively. Other researchers have found comparable results: stable plasma glucose levels may result from the liver and muscle's increased lipogenesis potential and the absence of changes in gluconeogenesis (Li et al., 2019).

In omnivorous fish, as in other animals, AST (Aspartate Aminotransferase) and ALT (Alanine Aminotransferase) values can serve as indicators of liver health (Coz-Rakovac et al., 2008). These enzymes are predominantly found in the liver, and elevated levels may suggest liver dysfunction or damage (Ali et al., 2017).

The notable increase in ALT and AST levels in the V3 - fish feed with 2% fructose compared to the V1 variant, may indicate potential stress in the hepatopancreas of goldfish under the present study. However, lower ALT and AST values observed in V2 groups (fed with 1% fructose) may be associated with the anti-inflammatory effect of low fructose concentrations reported also for *C. carassius* by other authors (Cao et al., 2022). Therefore, in our study, 2% of fructose imposed a higher pressure on the hepatopancrease activity.

After 40 days of krill oil 2% supplementation there was an observed improvement in hepatopancrease function, as evidenced by a significant decrease in liver enzyme values (AST, ALT).

Fructose is known to stimulate lipogenesis, a process where the liver converts excess carbohydrates into triglycerides (Shikata et al., 1994); this can result in increased production and release of triglycerides into the bloodstream, an aspect also observed in our study at the end of stage I of growth. For goldfish, after the feeding trial, at the end of stage II, a krill oil 2% supplemented diet induced a significant decrease in TRIG concentration, suggesting that KO alleviated the negative impact of fructose on the metabolism of lipoproteins.

Gaining insights into specific quantitative aspects of fish is essential for delving into fundamental biological concepts, such as the examination of viscerosomatic and hepato-somatic indices. This is due to the significance of measuring and analyzing these indices in evaluating the nutritional value of administrated food (Ighwela et al., 2014). The hepatosomatic index serves as an indirect metric for assessing the nutritional status of fish by measuring glycogen and carbohydrate levels (Tavares-Dias et al., 2000).

Although, at the end of Phase I, no significant differences were observed between hepato-somatic and viscerosomatic indices, a slight increase of HSI (hepatosomatic index) and VSI (viscerosomatic index) can be noticed in the V3 variant (2% fructose). Typically, the increase in HSI observed alongside the rise in dietary carbohydrate levels could be associated with an increase in glycogen accumulation within the liver of fish fed to high-carbohydrate diets (Moreira et al., 2008; Li et al., 2015). The same trend of non-significant increase is observed at the end of Phase II for the HSI, while the VSI exhibited a significantly higher value in the variant where the fish diet was not supplemented with krill oil. Krill oil, rich in omega-3 fatty acids and antioxidants, may decrease viscerosomatic indices in fish through its anti-inflammatory effects (Ku et al., 2023),

regulation of lipid metabolism, antioxidant protection, and maintenance of cellular membrane integrity (Sun et al. 2017).

## CONCLUSIONS

The present study was conducted to investigate the amelioration of hepatopancreas impairment induced by high fructose dietary doses following dietary supplementation with krill oil for *Carassius auratus* reared in different strategy-feeding. Our results showed that daily krill oil supplementation in the *C. auratus* prevented fructose-induced hypertriglyceridemia. Therefore, monitoring the blood biochemical parameters can help in optimizing feeding practices to ensure the welfare and productivity of the fish. Research in this area is ongoing, and studies aim to optimize the incorporation of krill oil into aquafeeds, taking into account economic feasibility and environmental impact.

## ACKNOWLEDGMENTS

The equipment used in this study belongs to the infrastructure of *Research Center MoRAS,* "Dunărea de Jos" University of Galați (www.moras.ugal.ro)

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