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THESIS

THE INFLUENCE OF BIOACTIVE COMPOUNDS IN DIETARY SUPPLEMENTS ON THE METABOLIC PROCESSES OF AQUATIC ORGANISMS

Doctorand,
NĂSTAC (GRĂDINARIU) Lăcrămioara

Conducător științific,
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Doctorand

NĂSTAC (GRĂDINARIU) Lăcrămioara

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Introduction

In the context of the rapid development of the aquaculture industry, improving the immune status of fish and their effectiveness in combating and preventing diseases have become increasingly important aspects.

The growth of fish in intensive systems, such as recirculating aquaculture systems (RAS), promotes the multiplication and transmission of specific pathogens such as bacteria, viruses, fungi, and parasites, which can cause severe diseases and even mass mortality. At the same time, pharmaceutical pollutants such as antibiotics and chemicals used in disease treatment and prevention can enter the aquatic environment, negatively impacting fish and aquatic ecosystems.

In this context, dietary supplements are a promising solution, offering an effective way to enhance the immunity of fish and strengthen their ability of self-defence against the pathologies that occur, as well as against the harmful effects of pharmaceutical pollutants. By administering these supplements, the resistance of fish can also be strengthened to various pathogens and harmful chemicals, while promoting overall health and optimal fish development.

The doctoral thesis entitled "***The Influence of Bioactive Compounds in Dietary Supplements on the Metabolic Processes of Aquatic Organisms***", aimed to investigate the influence of dietary supplements on aquatic organisms in the context of improving the immune status and to assess the increase in the resistance of these organisms after the administration of pharmacological treatments, specific to parasitic diseases or after the exposure to certain pharmaceutical pollutants. Furthermore, the influence of dietary supplements on the growth performance and nutritional quality of the biological material (Common carp - *Cyprinus carpio* Linnaeus 1758) was evaluated. The nutrition provided to the fish was supplemented with specific food supplements.

This research aims to provide practical and applicable solutions with direct impact on aquaculture performance and efficiency. As a result, this will allow farmers to implement more effective strategies and prevention measures in order to maximize final yields in the context of promoting sustainable, healthy and environmentally responsible aquaculture.

In order to achieve the main purpose of this doctoral thesis, the work plan of the doctoral study program had the following scientific targets:

- Evaluation of the growth performance of carp brood and the nutritional value of their meat following the supplementation of their feed with Krill oil*;*
- Assessment of the technological comfort state of the cultured biomass (carp) after supplementing the carp brood's feed with Krill oil, through the analysis of hematological profile, blood biochemical analysis, and

the analysis of the level of oxidative stress induced by high-density fish farming;

- Evaluation of the hepatoprotective effect of Krill oil after the administration of an anthelmintic treatment with albendazole (ABZ);
- Evaluation of the growth performance of carp brood and the nutritional value of their meat following the supplementation of their feed with selected dietary supplements (silymarin and berberine);
- Assessment of the technological comfort state of the cultured biomass (carp) after supplementing the carp brood's feed with silymarin (SM) and berberine (BBR), through the analysis of hematological profile, blood biochemical analysis, and analysis of the level of oxidative stress induced by high-density fish farming;
- Evaluation of the hepatoprotective effect of the selected dietary supplements after conducting a "challenge test" with paracetamol.

The doctoral thesis is structured in two parts:

I. The DOCUMENTARY RESEARCH consists of 2 chapters, presenting recent literature information on physiological challenges of aquatic organisms in the context of therapeutic treatment use or exposure to various pharmaceutical pollutants, as well as nutritional strategies used in aquaculture in order to boost the immunity of aquatic organisms.

II. The EXPERIMENTAL RESEARCH includes the results of the experiments carried out during the doctoral internship and is structured in 4 chapters, as following:

Chapter 3, entitled "***Materials and Methods***", presents the research infrastructure, methodology, and equipment used for the experimental activities.

Chapter 4, entitled ***"Research Regarding the Influence of Krill Oil on Growth Performance in Carp Brood, Biochemical Composition and Health Status in Cyprinus Carpio"***, presents the results obtained after supplementing carp feed with krill oil. It examines the effects on growth performance, organosomatic indices, hematological profile, and oxidative stress markers. Additionally, the chapter presents the hepatoprotective effect of krill oil under the conditions of anthelmintic treatment.

Chapter 5, entitled "**Research on the Influence of Silymarin and Berberine on the Growth Performance of Carp Brood, Biochemical Composition of Meat and Technological Comfort State"**, presents the results obtained from the supplementation of carp feed with silymarin and berberine. The chapter focuses on the impact of these additives on growth performance, organosomatic indices, hematological profile, and oxidative stress markers. Additionally, the chapter discusses the hepatoprotective effect of the selected dietary additives under the conditions of a "challenge test" with paracetamol.

Chapter 6, entitled "***General Conclusions***", presents the main conclusions obtained from the experiments aimed at testing dietary supplements in carp brood's feed in order to improve growth performance and immune status.

The doctoral thesis comprises 122 pages, including 33 figures and 25 tables. The documentary research represents 33% of the thesis, while the experimental research represents 67%.

3. Materials and Techniques Used for Experimental Activities

3.1 Biological Material and Research Infrastructure

The biological material used in the experiments was represented by carp broods (*Cyprinus Carpio* Linnaeus, 1758), which was purchased from a local farm and transported to the Recirculating Systems Pilot Station, at Dunarea de Jos University of Galati. Prior to storing the biological material in the growth units of the recirculating system, a random sample of 7 specimens were randomly examined from a clinical and anatomopathological point of view. The purpose of the examination was to detect the presence of any infectious and parasitic agents which, due to their potential to trigger diseases, could have jeopardized the conduct of the experiments.

The experimental research was conducted at the Romanian Center for Modeling Recirculating Aquaculture Systems (MoRAS, http://moras.ugal.ro/), Dunărea de Jos University of Galați, Romania. Thus, the infrastructure consisted of the recirculating aquaculture system (RAS) pilot station as well as the Physiology and Nutrition laboratories within the MoRAS Center.

The recirculating system (RAS) was designed to provide appropriate treatment of the process water and, consequently, the well-being of the fish biomass. The flow scheme includes a well-thought-out sequence of components distributed on two levels (basement, ground floor). There are *main components* (1), such as: growth units (24 tanks of $1m^3$, 109 cm diameter), mechanical filter, biological filter, equipment for dissolved gas transfer (carbon dioxide degassing, oxygen injection, and contactors), UV disinfection systems, pumps, water quality monitoring and control equipment, as well as *auxiliary components* (2), such as automatic feeders, ozone generator, and an independent power generator (Appendix 1, Image 3.1).

Image 3.1. *The recirculating system used during the experiments (original image).*

At the end of each experiment, resistance tests were conducted. To ensure proper conditions for the tests, the fish were transferred to an experimental system consisting of 12 glass aquariums of 130 L each (Image 3.2).

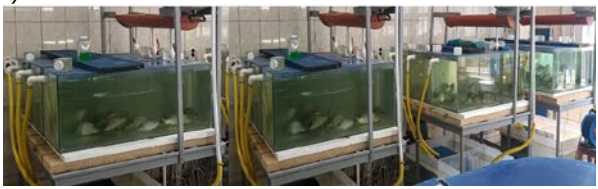

Image 3.2. *The recirculating system used for conducting the resistance tests (original photograph).*

3.2. Methodology for Calculating Growth Performance Indicators

At the end of the experimental periods, the biological material was weighed in order to calculate the following growth parameters:

- **Actual growth rate** [Sr],
- **Specific growth rate** [SGR]
- **Feed conversion ratio** [FCR]
- **Protein efficiency ratio** [PER]

3.3. Determination of Somatic Indices

At the end of each experimental period, six fish from each tank were sampled, individually weighed, and gutted. Internal organs (liver, viscera, heart, spleen) were weighed to calculate body indexes using the following formulas:

- **Hepato-somatic index** (HSI, %)
- **Visceral somatic index** (VSI, %)
- **Spleno-somatic index** (SSI, %)
- **Cardio-somatic index** (CSI, %)

3.4. Working Methods Used for Assessing the Haematological Profile

Blood samples were collected using the caudal puncture method, a widely used technique for investigating the health and physiology of aquatic organisms. Prior to blood sampling, the fish were anesthetized in a water bath containing 2-phenoxyethanol (0.7 mL/L) (Shualei et al., 2012).

The blood was collected using sterile syringes and transferred into Eppendorf tubes. The tubes containing the blood samples were kept on ice until they were transferred to the laboratory for further analysis. Each collected sample was divided into two Eppendorf tubes: one part was transferred into a sterile 2 ml Eppendorf tube with anticoagulant (Heparin) for hematological analysis, while the second part was transferred into an Eppendorf tube used for serum separation. The serum was obtained by centrifuging the blood at 3500 rpm for 10 minutes and was used for subsequent biochemical analyses.

The determination of hemoglobin concentration (*Hb, g/dL*) was performed using the cyanmethemoglobin method with Drabkin's reagent (DIALAB, Wiener Neudorf, Austria) to convert the blood hemoglobin into stable methemoglobin (Hesser, 1960). The absorbance of the samples was read at a wavelength of 540 nm compared to the Drabkin reagent (control) using the Specord 210 UV-Vis spectrophotometer. (Analytic Jena, Jena, Germany).

The determination of hematocrit (*Ht, %*) was performed using the microhematocrit method. For this purpose, 30 µL of blood was introduced into microhematocrit capillaries and then centrifuged at 12,000 rpm for 5 minutes using a HETTICH MIKRO 120 microcentrifuge (HettichZentrifugen, Tuttlingen, Germany). After centrifugation, the ratio between the formed elements and blood plasma was read on a nomogram.

The determination of red blood cells (*RBC × 106/mm^3*) was performed using Vulpian's dilution fluid, prepared in the laboratory from sodium citrate, potassium iodide, and metallic iodine (Sigma-Aldrich, St. Louis, MO, USA), the Neubauer counting chamber, and the Potain pipette for erythrocytes. The red blood cells were counted using the ZEISS-AXIO IMAGER microscope (Zeiss International, Thornwood, NY, USA) at a magnification of 10 oc. × 40 ob.

The determination of erythrocyte indices, represented by mean corpuscular volume (*MCV, µm^3*), mean corpuscular hemoglobin (MCH, pg), and mean corpuscular hemoglobin concentration (MCHC, g Hb/100 mL), was performed using the calculation formulas described by Ghergariu, 1985.

The mean corpuscular volume (*MCV*) represents the average volume of the erythrocyte.

3.5. Working Methods for Assessing the Biochemical Parameters of Blood Plasma

3.5.1. Determination of Serum Parameters

The determination of serum parameters was performed using the VetTest® IDEXX chemical analyzer (Image 3.6.), using compatible IDEXX VetTest kits (IDEXX Laboratories, Inc., Westbrook, ME, USA).

Thus, the following parameters were determined: albumin (ALB, g/dL), globulin (GLOB, g/dL), total serum protein (TP, g/dL), glucose (GLU, mg/dL), alanine aminotransferase (ALT, U/L), aspartate aminotransferase (AST, U/L), alkaline phosphatase (ALP, U/L), gamma-glutamyl transferase (GGT, U/L), direct bilirubin (BIL D, mg/dL), cholesterol (CHOL, mg/dL), high-density lipoprotein (HDL, mg/dL), low-density lipoprotein (LDL, mg/dL), and triglycerides (TG, mg/dL).

Image 3.6. *The VetTest® chemical analyzer and compatible kits for determining serum parameters (original image)*

3.5.2. Determination of Lysozyme Activity

Lysozyme (LZM, U/mL) is one of the numerous antimicrobial proteins associated with the first line of defense of innate immunity in fish. This enzyme (muramidase) breaks the β-1,4 glycosidic bond between N-acetylmuramic acid and N-acetylglucosamine in the cell wall (sacculus) of Gram-positive bacteria and, in association with complement components, can also affect Gram-negative bacteria (Paulsen et al., 2001). The enzymatic activity of the protein can be found in mucus, serum, eggs, spleen, liver, skin, mucus, gills, muscles, and can be influenced by various stressors. (Bowden 2008; Bulut et al., 2012).

The principle of the method. Lysozyme activity was determined from serum using the working protocol for enzymatic determination of lysozyme from Sigma (Sigma, EC 3.2.1.17).

Reagents and equipment required: I) potassium phosphate buffer solution; II) Micrococcus lysodeikticus substrate (Sigma, M3770); III) lysozyme enzymatic solution (Sigma, L6876); IV) pH meter WTW InoLab 7110, Xylem Analytics Germany Sales GmbH & Co. K, Germany; V) magnetic stirrer FALC, FALC Instruments, Italy; VI) UV-Vis spectrophotometer SPECORD 210 Analytikjena 250.

3.6. Analysis Methods for Determining Oxidative Stress Parameters

3.6.1. Determination of Malondialdehyde Concentration (MDA) (Ohkawa, 1979)

Malondialdehyde (MDA) represents the indicator of membrane lipid peroxidation and is often measured through the lipid peroxidation index. Similar to other higher vertebrates, lipid peroxidation in fish results from the oxidation of unsaturated fatty acids and serves as a crucial indicator of oxidative stress in cellular components (Ateş et al., 2016).

Principle of the method. In the TBA (thiobarbituric acid) test reaction, one molecule of MDA reacts with two molecules of TBA to produce a pink-colored pigment with a maximum absorption at 532-535 nm.

Reagents and equipment required: I) 10% TCA solution, II) 1% TBA solution, III) 1 mM stock solution of 1,1,3,3-tetraethoxypropane (TEP), IV) 1% sulfuric acid solution, 10 mL glass tubes, tips, and micropipettes, V) FALC magnetic stirrer, FALC Instruments, Italy; VI) SONICA® water bath, Soltec, Soluzione, Italy; VII) UV Vis spectrophotometer SPECORD 210 Analytikjena 250.

3.6.2. Determination of Total Antioxidant Capacity (TAC)

The determination of total antioxidant capacity (TAC) was performed using the method described by Re, 1999, and Van Den Berg, 1999. The method utilizes ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)), which measures the ability of antioxidants to convert the colored ABTS^0+ (2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonate)) radical cation into a colorless non-radical form.

Reagents and equipment needed: I) Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) (Sigma) - antioxidant standard; II) ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)) (Sigma); III) potassium persulfate K2S2O8 (Sigma); IV) phosphate-buffered saline (PBS) saline solution, pH 7.4.

3.7. Working Methods Used for Determining the Biochemical Composition of Fish Meat

After weighing the viscera, the skin and bones of the fish were removed, and biochemical determinations were performed on the muscular tissue. Analysis of the biochemical composition was carried out using standard AOAC (Association of Official Agricultural Chemists) methods from 1997.

The determination of dry matter was performed by drying the samples to a constant weight at 105±0.5 °C for 24 hours in a convection oven (Jeiotech, JeioTech Co., Inc, South Korea).

The lipid content (%) was analyzed using the Soxhlet extraction method with petroleum ether as the solvent (Gerhardt GmbH & Co. KG, Germany).

The ash content (%) was determined by calcining the muscle tissue samples at 525±25 ˚C until a constant weight was obtained. The Nabertherm type incinerator, Applied Scientific Instruments Co., Ltd., Thailand, was used for this purpose.

The crude protein content (%) was determined using the Dumas method, which involves burning the dried samples at 1100°C (Primacs SNC 100, Skalar Analytical B.V., Netherlands).

Total carbohydrates were calculated as follows: total carbohydrates (%) = 100 - (Moisture (%) + Crude Protein (%) + Lipids (%) + Ash (%)).

3.8. Statistical Data Analysis Techniques

Statistical analyses were performed using SPSS software for Windows, version 21.0 (SPSS Inc., Chicago, United States). The experimental data were analyzed using *t-tests* and ANOVA. Prior to the statistical analyses, both normality and variance homogeneity were confirmed using Shapiro-Wilk and Levene's tests. ANOVA analysis was followed by the Duncan post-hoc test when significant differences were detected. The *paired-sample t-test* was used to compare the average difference. The significance level was set at $p<0.05$ for all the analyses.

All the experiments were conducted with the permission of the Ethics Committee of "Dunărea de Jos" University of Galați. (RF2458/25.06.2021).

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4. Research on the Influence of Krill Oil Regarding Growth Performance in Carp Brood, Biochemical Composition of Meat and Technological Comfort State

4.1. Introduction

In intensive aquaculture systems, economic efficiency is influenced by unit production, which depends on the growth performance of fish and stocking density. High stocking densities represent a chronic stress factor that leads to physiological responses, jeopardizing homeostasis and fish health (Yarahmadi et. al., 2016). The main corticosteroid triggered by stress generated by high stocking densities is cortisol, which, at high concentrations in the blood, increases, inducing immunosuppressive and catabolic actions (Barton, 2002). Therefore, humoral immune parameters,

serum metabolites (osmolarity, albumin, and globulin values), hepatic parameters, and lipid metabolism are affected (Jia et. al., 2022).

Therefore, practicing excessively high stocking densities can lead to increased stress among fish biomass and, consequently, increased susceptibility to disease (Montero et al., 1999; Swain et al., 2022). In aquaculture, the impact of disease outbreaks can be fatal, resulting in significant economic losses due to reduced production efficiency caused by high mortality rates. In this context, determining the optimal stocking densities for each species is an important aspect to maximize final yields.

To counteract the negative effects induced by high stocking densities practiced in intensive production systems, current studies are focused on implementing new solutions to enhance the immune response of fish to various stress factors.

In this regard, the main approach is nutritional, primarily aiming at supplementing the feed with various antioxidant compounds, such as medicinal plants and their extracts (Sahin et al., 2014; Adineh et al., 2021), essential oils (De Freitas Souza et al., 2019; Shourbela et al., 2021), or other functional food additives (Hoseini et. al., 2021).

In parallel with researchers' efforts to develop alternative sources of proteins and lipids, there continues to be a significant interest in developing alternatives to traditional means of disease control. In aquaculture, diseases caused by parasites can result in major damages, leading to excessive mucus production on the skin and gills, hyperplasia, and gill necrosis (Tavares-Dias et al., 2021). Foodborne diseases caused by helminth parasites transmitted through the consumption of fish products pose a significant public health issue.

In the context of the aforementioned, this study aimed to evaluate the effects of supplementing the diet of carp brood with krill oil on growth performance, somatic indices, and biochemical composition. Additionally, the potential of krill oil to alleviate the stress induced by high stocking densities and its hepatoprotective action after anthelmintic treatment with ABZ were also assessed.

4.2. Experimental Design

The carp brood (*Cyprinus Carpio*) were purchased from a local farm. For a period of two weeks, the biological material was stored in a quarantine tank (Ewos tank, dimensions 1.4×1.4×0.6 m). During this period, the fish were fed a Skretting feed containing 43% crude protein, 12% lipids, 4% fiber, and 6% ash (Skretting, Vignetto, Italy).

After the acclimatization period, a total of 300 fish (with an average individual weight of 113.58 ± 11.09 g) were distributed into 12 tanks with a water volume of 500 L, forming four experimental groups with triplicates each (Figure 4.1).:

- LD – low stocking density of 3,5 kg/m^3;
- LD-KO – low density with KO supplementation feed of 3,5 kg/m^3;

- HD – high stocking density of 8 kg/m^3;
- HD-KO- high density with KO supplementation of 8 kg/m^3.

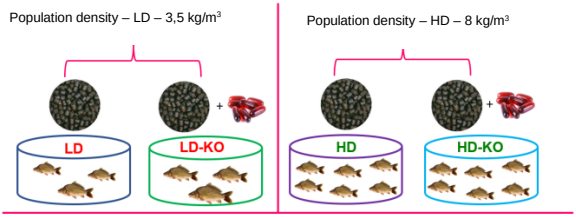

Figure 4.1. *Experimental Design Schema*

For the LD-KO and HD-KO experimental groups, the feed was supplemented with 5 g/kg of KO (containing 500 mg of pure krill oil, 200 mg of phospholipids, 90 mg of Omega-3 fatty acids (EPA/DHA), and 400 µg of astaxanthin). To incorporate the oil, the feed was ground and mixed with the specified oil, and then transformed into pellets (2 mm in diameter). After drying, the pellets were transferred to plastic bags and stored in the refrigerator until feeding. The fish were fed the mentioned diets for a period of 60 days. The feeding was done manually to avoid food competition, and the feeding intensity was of 2% of the body biomass.

The photoperiod was set to 16 hours of light and 8 hours of darkness. The biomass in each growth unit was weighed weekly to adjust the amount of feed.

Throughout the experimental research, water quality parameters were monitored daily. Water temperature, pH, and dissolved oxygen were automatically measured using the Endress+Hauser monitoring system (Endress+Hauser AG, Switzerland) through probes placed in each growth tank, while nitrogen compound concentrations were quantified weekly using a Skalar SAN++ analyzer (SkalarAnalytical, Netherlands).

Thus, the statistical analysis of the main physico-chemical parameters of the water did not show significant differences ($p>0.05$) among the described experimental variations, falling within the optimal range recommended by the specialized literature for carp growth (Goran et al., 2016). The recorded average values were as follows: temperature 22.4 ± 2.04 ˚C; pH = 7.25 ± 1.24; dissolved oxygen 7.56 ± 1.23 mg L-1; ammonia 0.16 ± 0.19 mg L-1; nitrates < 0.20 mg L-1; and nitrites < 0.010 mg L-1.

After 60 days of experimentation, 7 fish from each experimental group were randomly selected for albendazole (ABZ) treatment. Subsequently, the fish were transferred to an experimental system consisting of 12 glass aquariums of 130 L each. For the ABZ treatment, a 10% ABZ solution (100 mg/ml oral suspension) (Dopharma, Romania) was used. The dose was administered orally (5 mg ABZ/kg body weight) using a syringe attached to a tube. The ABZ treatment was administered for 7 consecutive days.

4.3. Results and Discussions

4.3.1. Evaluation of Growth Performance in Carp Brood

At the beginning of the experiment, the average individual body weight for the four experimental groups was 119.27±10.22 g for the LD group, 117.47±11.02 g for the LD-KO group, 112.97±12.42 g for the HD group, and 114.14±12.56 g for the HD-KO group. No statistical differences were observed among the four experimental groups (ANOVA, $p>0.05$) (Table 4.1).

At the end of the experimental period, the survival rate of carp was 100% in all the groups, indicating that the stocking densities used in our experiment were optimal for carp growth.

Table 4.1. Technological Indicators of Growth Performance in Carp Brood after 60 days of experimentation

Growth parameters	***Experimental variants***			
	LD	***LD-KO***	***HD***	***HD-KO***
Initial biomass (g)	1789±10,41^{a}	1762±9,96^{a}	3954±12,15^{b}	3995±10,42^{b}
Initial biomass (kg/m^3)	3,58±0,52^{a}	3,52±0,36^{a}	7,91±0,22^{b}	7,99±0,31^{b}
Initial fish number	15	15	35	35
Initial average mass (g)	119,27±10,22^{a}	117,47±11,02^{a}	112,97±12,42^{a}	114,14±12,56^{a}
Survival rate (%)	100	100	100	100
Final biomass (g)	3865±30,16^{a}	3969±29,23^{a}	7742±41,17^{b}	7965±37,65^{c}
Final biomass (kg/m^3)	7,73±0,09^{a}	7,94±0,06^{a}	15,48±0,08^{b}	15,93±0,05^{b}
Initial average mass (g)	257,67±28,12^{a}	264,60±26,70^{a}	221,2±33,64^{b}	227,57±34,62^{b}
Growth increment of biomass (g)	2076±32,26^{a}	2207±28,16^{b}	3788±36,12^{c}	3970±41,12^{d}
Individual growth increment (g)	138,40±12,16^{a}	147,13±15,42^{a}	108,23±10,56^{b}	113,43±11,21^{b}
SGR (% day^{-1})	1,28±0,05^{a}	1,35±0,05^{a}	1,12±0,10^{a}	1,15±0,09^{a}
FCR (g/g)	1,06±0,02^{a}	0,95±0,01^{a}	1,32±0,03^{c}	1,21±0,04^{b}
PER (g)	2,19±0,07^{b}	2,44±0,04^{b}	1,76±0.11^{a}	1,93±0,08^{a}

The values represent the average ± S.E. (n = 3). Different letters within a row indicate significant differences (ANOVA, $p < 0.05$) between experimental groups. SGR - Specific growth rate; FCR - Feed conversion ratio; PER - Protein efficiency ratio.

After 60 days of feeding, the final average fish weight and individual growth rate were significantly higher ($p < 0.05$) for the lower stocking density groups, with better values observed in the LD-KO groups. Conversely, the final fish weight in the higher stocking density groups was significantly lower ($p < 0.05$). At the end of the experiment, the stocking

density was 7.73 ± 0.09 kg/m^3 in LD and 7.94 ± 0.06 kg/m^3 in LD-KO, and 7.91 ± 0.22 kg/m^3 in HD and 7.99 ± 0.31 kg/m^3 in HD-KO..

The feed conversion ratio (FCR) ranged from 0.95 to 1.32 g/g, with significantly better values ($p < 0.05$) observed in the groups with lower stocking densities. Additionally, the specific growth rate was significantly better ($p < 0.05$) for the fish in the experimental groups with lower densities and fed with KO (0.95 g/g) compared to the fish stocked at higher densities. Moreover, the addition of KO to the carp diet improved the values of protein efficiency ratio (PER), with better values observed in the fish from the lower stocking density group.

In this study, both stocking density and the addition of KO to the feed significantly influenced nutrient utilization efficiency as well as growth parameters. Growth performance was superior in the LD-KO group, followed by the HD-KO group, with KO significantly improving the obtained technological indicators.

Our results are similar to those reported by other authors (Yoshitomi et al., 2006; Hansen et al., 2011; Kousoulaki et al., 2013), who have stated that supplementing or partially replacing fish oil with krill meal stimulates appetite, improves visceral weight indices, and consequently leads to superior growth performance in rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*). However, there is limited information available in the specialized literature regarding the supplementation of feed with KO.

Some authors have reported that supplementing shrimp feed with KO leads to improved body weight and final production (Castro et al., 2017). Additionally, Nunes et al., 2020 reported that the use of KO in the diet of shrimp larvae reduces the production cycle duration and mortality, even under stressful conditions. Xiuling et al., 2019 conducted a study on golden pompano shrimp (Trachinotus ovatus Linnaeus, 1758) using different lipid sources (FO-fish oil, KO-krill oil, SO-soybean oil, and CO-corn oil), and the results showed improved growth performance when a 1:1 combination of FO and KO, as well as KO-CO, were used in their feed. The authors also concluded that the use of KO in the diet of golden pompano larvae may regulate certain physiological indicators.

The present study has demonstrated that higher stocking density negatively influences nutrient retention efficiency and, consequently, growth performance. Several authors have reported that growth performance is negatively affected when stocking density exceeds certain "optimal densities". Hayat et al., 2018, reported a decrease in growth performance for common carp Majalaya at stocking densities of 125 fish/m3. Enache et al., 2011, reported superior growth performance for carp (average individual weight of 65 g/fish) stocked at lower densities (32 kg/m3) compared to carp stocked at densities of 64 kg/m3. The lower growth performance observed at higher stocking densities is correlated with the reduced space available for each fish, increased food competition, and additional energy requirements induced by stress (Montero et al., 1999; Costas et al., 2008).

4.3.2. Evaluation of Somatic Indices and Biochemical Composition of Meat

Table 4.2 presents the values of somatic indices after 60 days of experimentation. In our experiment, ANOVA statistical analysis did not reveal significant differences ($p>0.05$) in terms of CSI values, while HSI, VSI, and SSI values showed significant differences ($p<0.05$) among the four experimental variants.

Table 4.2. ***Somatic index values after 60 experimental days***

Body indices	LD	LD-KO	HD	HD-KO	p
HSI	2,94±1,03^{a}	3,27±0,54^{b}	2,62±0,23^{a}	2,7±0,4^{a}	0,04
VSI (%)	11,46±1,94^{b}	10,38±0,69^{a}	10,87±1,53^{a}	11,67±0,92^{b}	0,04
SSI (%)	0,34±0,14^{a}	0,35±0,07^{a}	0,51±0,09^{b}	0,40±0,12^{a}	0,03
CSI (%)	0,21±0,38^{a}	0,11±0,07^{a}	0,16±0,03^{a}	0,20±0,02^{a}	0,85

HSI - hepatosomatic index; VSI - viscerosomatic index; SSI - splenosomatic index; CSI - cardiosomatic index. The values represent the average ± S.E. Different letters in the same row indicate significant differences (ANOVA, p<0.05) between the experimental variants.

The hepatosomatic index is an indirect measure that reflects the levels of glycogen and carbohydrates and can be used to indicate the nutritional status of fish. On the other hand, the splenosomatic index reflects both the immune status and hematopoietic capacity of fish (Tavares-Dias, 2000).

The HSI showed a significantly higher value ($p<0.05$) in the LD-KO variant (3.27±0.54%), while a significant reduction ($p<0.05$) was observed in the HD variant (2.62±0.23%). The reduction in HSI at high stocking densities has also been reported by Leatherland and Cho, 1985 in rainbow trout. The authors explain this phenomenon by the accumulation of lower total hepatic reserves of lipids and hepatic glycogen.

The VSI values were significantly lower ($p<0.05$) in the LD-KO and HD groups, with no significant differences ($p>0.05$) observed between the LD and HD-KO groups. Regarding the SSI values, a significant increase ($p<0.05$) was recorded for the fish in the HD group.

The spleen is considered to be the main organ in fish where erythrocytes, neutrophils, and granulocytes mature, and it plays a vital role in their immune response, being responsible for the high production of melano-macrophages (Kumaran et al., 2010). In our experiment, the SSI index showed significantly higher values ($p<0.05$) in the HD group. Storing fish at high densities can lead to an increase in spleen volume, allowing the organism to maintain its organic functions in balance.

The figures 4.1 to 4.4 present the values of the biochemical composition of the flesh after the 60-day experimental period. The statistical analysis ANOVA did not show significant differences ($p>0.05$) among the four experimental groups. However, slightly better values were obtained for the fish fed with KO. Similar results were reported by Choi et al., 2020, after including Krill meal in the diet of Alaska pollock (*Gadus Chalcogrammus*, Pallas,1811).

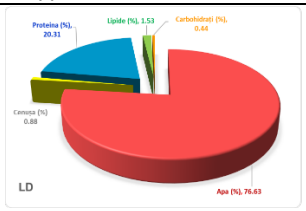

Figure 4.1. *Biochemical composition of meat after 60 days of experimental period in the LD variant*

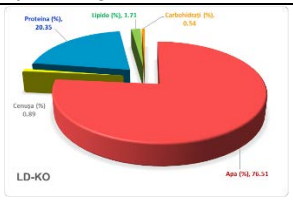

Figure 4.2. *Biochemical composition of meat after 60 days of experimental period in the LD-KO variant*

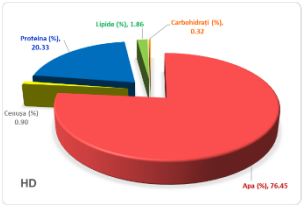

Figure 4.3. *Biochemical composition of meat after 60 days of experimental period in the HD variant*

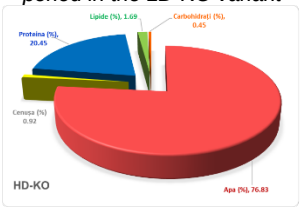

Figure 4.4. *Biochemical composition of meat after 60 days of experimental period in the HD-Ko variant*

4.3.3. Evaluation of Haematological Parameters

After 60 days of the experimental period, as well as after the treatment with ABZ (after 7 days of exposure), hematological parameters were determined: hemoglobin (Hb), red blood cell count (RBC), hematocrit (Ht), as well as erythrocyte constants (MCV, MCH, and MCHC) (Table 4.3).

At the end of the feeding experiment, although the statistical analysis did not show significant differences ($p>0.05$), the hematological parameters showed a slight decrease in red blood cell count (RBC) in the higher stocking density group, and a non-significant increase in the groups fed krill oil diet. Furthermore, both the chosen stocking densities and the supplementation of the diet with krill oil did not have a significant effect ($p>0.05$) on Hb, Ht, VEM, HEM, and CHEM. However, after the ABZ treatment, Hb values showed a significant decrease ($p<0.05$) in the HD and HD-KO groups, while Ht, VEM, and HEM values decreased only in the HD groups.

Overall, high stocking densities can compromise the health of the fish, causing alterations in hematological and metabolic profiles.

Table 4.3. *Hematological parameter values recorded after 60 experimental days and after the application of the ABZ test*

Parameter	After 60 days of experimentation			
	LD	LD-KO	HD	HD-KO
RBC ($x10^6$ /mm^3)	1.24±0.14^{a*}	1.31±0.16 ^{a*}	1.14±0.14 ^{a*}	1.28± 0.05 ^{a*}
Hb(g/dL)	8.42±0.63 ^{a*}	8.83±0.69 ^{a*}	9.71±0.51 ^{a*}	9.34 ±0.27 ^{a*}

Ht(%)	28.22±2.10 ^{a*}	29,13±1.31 ^{a*}	31.59±1.80 ^{a*}	27.93 ±2.65 ^{a*}
VEM (μm^3)	238.59±36.97 ^{a*}	233.77±34.33 ^{a*}	286.63±12.3 ^{a*}	220.36 ±14.88 ^{a*}
HEM (pg)	71.71±11.45 ^{a*}	70.68±10.97 ^{a*}	88.01±10.31 ^{a*}	73.46 ±4.46 ^{a*}
CHEM (g/dL)	30.02±2.05 ^{a*}	30.21±1.21 ^{a*}	30.93±2.02 ^{a*}	34.42 ±3.44 ^{a*}
Parameter	**After the test using ABZ (7 days)**			
	LD	**LD-KO**	**HD**	**HD-KO**
RBC (x10^6 /mm^3)	1.40±0.12 ^{a*}	1.67±0.15 ^{a*}	1.36±0.21 ^{a*}	1.41±0.11 ^{a*}
Hb (g/dL)	8.44±0.31^{c*}	9.48±0.39 ^{d*}	7.09±0.73 ^{a**}	7.28±0.72 ^{b**}
Ht (%)	31.30± 2.17^{c*}	31.80±1.77^{c*}	23.00±2.62 ^{a**}	28.87±2.02 ^{b*}
VEM (μm^3)	231.23±12.30 ^{a*}	196.57±9.74 ^{a*}	194.52±16.87 ^{a**}	213.77±11.01^{a*}
HEM (pg)	62.40±6.37 ^{a*}	58.68±6.07 ^{a*}	56.48±9.06^{a**}	53.01±7.38 ^{a*}
CHEM (g/dL)	27.33±1.57 ^{a*}	30.35±2.52 ^{a*}	30.55±5.63 ^{a*}	25.56±2.71^{a**}

*The values represent the average ± S.E. (n = 3). Values with different letters in the same row indicate significant differences (ANOVA, p<0.05) between experimental groups. Values with different symbols */** in the same row indicate significant differences after the ABZ treatment (paired t-test, p<0.05). RBC - red blood cell count (erythrocytes), Hb - hemoglobin, Ht - hematocrit, VEM - mean corpuscular volume, HEM - mean corpuscular hemoglobin, CHEM - mean corpuscular hemoglobin concentration.*

The results of our experiment showed that the hematological parameters were not influenced by fish stocking density or the supplementation of feed with KO. It is well-known that the hematological profile of fish is influenced by external stressful factors, as it represents the link between the surrounding environment and the internal environment. The reduction in hemoglobin concentration in the HD groups after exposure to ABZ could be the cumulative inhibitory effect of both stress factors, density and ABZ, on the enzymatic system responsible for hemoglobin synthesis (Ahmed et al., 2020).

The determination of erythrocyte indices (VEM, HEM, CHEM) is of particular importance for evaluating anemia conditions in fish and is widely used to determine the size, content, and density of Hb in red blood cells (Ambili et al., 2013). In our study, after 60 days of feeding, the values of VEM, HEM, and CHEM did not show significant changes ($p>0.05$) among the four experimental groups, supporting the hypothesis that higher population density did not induce stress or pathological conditions. However, after the ABZ treatment, a significant decrease was observed in the values of Ht, VEM, and CHEM in the HD groups ($p<0.05$), and a significant decrease in CHEM values in the HD-KO groups due to a decrease in Hb levels.

Our results are similar to those reported by other authors. The reduction in these hematological indices is attributed to the effect of ABZ on the hematopoietic system, which can decrease cell size or disrupt blood components (Nwani et al., 2015). According to Islam et al., 2015, this could hinder the oxygen-carrying capacity of the blood, leading to hypoxia. Additionally, the decrease in RBC, Hb, VEM, Ht, and CHEM values could

be due to the oxidative stress resulting from the metabolic interaction of ABZ in fish.

In our study, the values of hematological parameters showed a range within the optimal range for this species at this age (Baghizadeh et al., 2015). However, fish in the high-density groups (HD-KO) that received KO-supplemented feed exhibited less alteration in the hematological profile.

4.3.4. Evaluation of Serum Parameters

Biochemical measurements of blood reflect the health status of fish. Therefore, at the end of the experiment, as well as after the ABZ treatment, the metabolic profile of the fish was determined: albumin (ALB), globulin (GLOB), the albumin/globulin ratio (A/G), total serum proteins (TP), glucose (GLU), alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma-glutamyltransferase (GGT), direct bilirubin (BIL D), total bilirubin (BIL T), high-density lipoproteins (HDL), low-density lipoproteins (LDL), cholesterol (CHOL), triglycerides (TG), and total lipids (TL) (Table 4.4).

Prior to the ABZ treatment, fish stocked at a higher density and fed a simple diet showed a significant increase ($p<0.05$) in GLU, AST, ALT, and GGT values compared to the groups fed with KO. KO did not induce significant changes ($p>0.05$) in ALP, BIL D, BIL T, TL, and lipid fraction values, except for HDL, which had higher values in the LD-KO and HD-KO variants.

After the ABZ treatment, ALB, GLOB, and TP values increased in all experimental variants, indicating a significant interaction ($p<0.05$) between density and diet for these parameters. Additionally, GLU values significantly increased after ABZ treatment, with the highest value observed in the HD variant (97.60±3.80 mg/dl) and the lowest value in the LD-KO variant (75.20±3.11 mg/dl), followed by HD-KO (85.60±4.73 mg/dl) and LD (86.80±4.95 mg/dl). Furthermore, ABZ treatment led to an increase in ALT and AST values in all experimental variants, with significantly higher average values ($p<0.05$) observed in the HD groups.

HDL values increased after the ABZ treatment in all experimental variants, but without significant differences ($p>0.05$) between the experimental groups. At the end of the 60-day experiment, LDL values were not influenced ($p>0.05$) by the administered diet or population density, but they significantly increased ($p<0.05$) after the ABZ treatment, with the highest average value observed in the HD groups (95.20 ± 2.52 mg/L).

After 60 days of the experimental period, the values of CHOL, TL, and TG were not significantly influenced ($p>0.05$) by the stocking density or the supplementation of the diet with KO. However, after the ABZ treatment, a significant decrease ($p<0.05$) in TG and TL values and a significant increase ($p<0.05$) in CHOL values were observed in all experimental variants. Therefore, after the ABZ treatment, the highest average values of CHOL and TG, and the lowest mean value of TL, were recorded in the HD groups.

In our study, after 60 days of feeding, fish raised under high stocking density (HD) exhibited the lowest values of TP and GLOB. Although the groups that received the KO-supplemented diet showed higher serum concentrations of TP and GLOB compared to the groups fed a normal diet and kept at the same density, significant differences ($p<0.05$) were observed only in the high-density groups. ALB was not affected by density or feeding regimen ($p>0.05$).

The alteration of biochemical parameters under high stocking density conditions has been reported by other authors as well (Kpundeh et al., 2013; Abdel-Tawwab et al., 2014; Mahmoud et al., 2021). Naderi et al. (2017) reported a significant decrease in globulin and albumin values, without changes in serum protein levels, in rainbow trout (*Oncorhynchus mykiss*) raised at a high stocking density (80 kg/m3).

Table 4.4. *Serum parameter values at the end of the feeding experiment and after the ABZ-test application*

Parameter	**Experimental variants**				**Interaction**
	LD	**LD-KO**	**HD**	**HD-KO**	**Feed × Density**
After 60 days of feeding					
ALB (g/dl)	1.09± 0.03^{a*}	1.00 ± 0.05^{a*}	1.13 ± 0.10^{a*}	1.03 ± 0.07^{a*}	p=0.084
GLOB(g/dl)	2.76 ± 0.25^{c*}	2.96 ± 0.10^{c*}	2.33 ± 0.06^{a*}	2.56 ± 0.15^{b*}	p=0.034
A/G	0.39 ± 0.03^{b*}	0.33 ± 0.02^{a*}	0.48 ± 0.03^{c*}	0.40± 0.01^{b*}	p=0.020
TP (g/dl)	3.83 ± 0.18^{b*}	3.96 ± 0.25^{b*}	3.36 ± 0.05^{a*}	3.59 ± 0.10^{b*}	p=0.235
GLU (mg/dl)	48.33 ± 3.79^{a*}	43.67 ± 2.42^{a*}	68.67 ± 3.21^{c*}	51.50 ± 5.03^{b*}	p=0.967
ALT (U/L)	81.33 ± 3.59^{a}	54.33 ± 3.46^{a*}	110.00 ± 4.94^{c*}	96.00 ± 3.58^{b*}	p=0.560
AST (U/L)	120.67 ± 8.36^{a*}	106.67 ± 5.16^{a*}	189.67 ± 4.98^{c*}	150.33 ± 6.53^{b*}	p=0.872
ALP (U/l)	129.20 ± 4.44^{a*}	103.60 ± 4.50^{a*}	135.33 ± 4.31^{a*}	113.50 ± 6.79^{a*}	p=0.403
GGT (U/L)	0.50 ± 0.71^{a*}	0.50 ± 0.71^{a*}	3.00 ± 0.83^{c*}	1.33 ± 1.15^{b*}	p=0.457
BIL D(mg/dl)	0.20 ± 0.07^{a*}	0.22 ± 0.08^{a*}	0.20 ± 0.05^{a*}	0.18 ± 0.04^{a*}	p=0.213
BIL T (mg/dl)	0.29 ± 0.10^{a*}	0.25 ± 0.11^{a*}	0.32 ± 0.09^{a*}	0.27 ± 0.06^{a*}	p=0.187
HDL (mg/dl)	79.73 ± 6.85 ^{a*}	90.07 ± 6.38 ^{b*}	74.63 ± 5.03 ^{a*}	87.73 ± 8.24 ^{b*}	p=0.578
LDL (mg/L)	27.67 ± 6.00^{a*}	26.33 ± 2.65 ^{a*}	25.67 ± 4.85^{a*}	27.67 ± 12.22^{a*}	p=0.858
CHOL (mg/dl)	211.67 ± 15.57 ^{a*}	204.67 ± 8.50^{a*}	203.50 ± 5.69^{a*}	181.33 ± 8.02^{a*}	p=0.961
TG (mg/dl)	449.33 ± 9.29 ^{a*}	435.00 ± 3.51^{a*}	440.00 ± 4.43^{a*}	421.33 ± 27.74^{a*}	p=0.642
TL (mg/dl)	1026.33 ± 3.01^{a*}	983.00 ± 4.64^{a*}	999.00 ± 9.81^{a*}	931.33 ± 43.25^{a*}	p=0.734
After the test using ABZ (7 days)					

ALB (g/dl)	1.28 ± 0.06^{b**}	1.18 ± 0.06^{a*}	1.42 ± 0.05^{c**}	1.21 ± 0.03^{a**}	p=0.044
GLOB (g/dl)	3.02 ± 0.04^{b**}	3.22 ± 0.22^{c**}	2.75 ± 0.18^{a**}	3.11 ± 0.13^{b**}	p=0.026
A/G	0.40± 0.02^{a*}	0.36 ± 0.03^{a*}	0.51 ± 0.01^{b**}	0.38 ± 0.01^{a*}	P=0.312
TP (g/dl)	4.30 ± 0.15^{b**}	4.40 ± 0.22^{c**}	4.17 ± 0.32^{a**}	4.32 ± 0.15^{b**}	p=0.031
GLU (mg/dl)	86.80 ± 4.95^{b**}	75.20 ± 3.11^{a**}	97.60 ± 3.80^{c**}	85.60 ± 4.73^{b**}	p=0.751
ALT (U/L)	92.40 ± 2.40^{b**}	65.00 ± 2.47^{a**}	148.50 ± 1.44^{d**}	122.00 ± 2.99^{c**}	p=0.011
AST (U/L)	233.50 ± 10.32^{b**}	197.75 ± 6.79^{a**}	367.25 ± 7.25^{d**}	281.00 ± 7.06^{c**}	p=0.012
ALP (U/l)	53.20 ± 0.73^{a*}	69.60 ± 4.33^{a**}	74.80 ± 2.19^{a*}	75.53 ± 3.95^{a**}	p=0.494
GGT (U/L)	2.40 ± 2.30^{a**}	2.50 ± 1.29^{a**}	4.00 ± 1.00^{b**}	2.40 ± 1.14^{a**}	p=0.148
BIL D(mg/dl)	0.11 ± 0.04^{a**}	0.11 ± 0.05^{a**}	0.13 ± 0.04 ^{a**}	0.12 ± 0.03^{a**}	p=0.830
BIL T (mg/dl)	0.19 ± 0.06^{a**}	0.19 ± 0.09^{a**}	0.21 ± 0.06^{b**}	0.24 ± 0.05^{c**}	p=0.860
HDL (mg/dl)	75.40 ± 3.15 ^{a**}	82.10 ± 6.50 ^{a**}	70.16 ± 7.45 ^{a**}	80.00 ± 6.08 ^{a**}	p=0.571
LDL (mg/L)	89.00 ± 3.76^{a**}	81.60 ± 5.66^{a**}	118.40 ± 9.07^{c**}	95.20 ± 2.52^{b**}	p=0.459
CHOL (mg/dl)	228.40 ± 9.58^{a*}	214.80 ± 7.29^{a*}	247.60 ± 6.89^{b**}	229.00 ± 4.16^{a**}	p=0.771
TG (mg/dl)	263.80 ± 8.94^{a**}	266.60 ± 7.04^{a**}	334.40 ± 6.89^{b**}	285.80 ± 9.05^{a**}	p=0.252
TL (mg/dl)	899.00 ± 10.23^{a**}	959.40 ± 7.87^{b**}	871.60 ± 2.46^{a**}	903.00 ± 5.23^{a**}	p=0.268

*The values represent the average ± S.E., n = 3. Different letters in a row indicate significant differences (ANOVA, p<0.05). Different symbols */** in a column indicate significant differences after treatment (T dependent, p<0.05).*

Globulin is a significant diagnostic biomarker in fish. Therefore, the increase in serum globulin and the decrease in the albumin/globulin (A/G) ratio represent important indicators of nonspecific immunity and protective mechanisms in fish (Opiyo et al., 2019). In this study, after 60 days of feeding, the A/G ratio was influenced by both the diet and density factors, as well as their interaction (p<0.05). A higher A/G ratio was observed in the HD groups, while lower values were observed in the groups that received KO-supplemented feed.

After the ABZ treatment, the levels of total serum proteins and globulin increased in all experimental groups (p<0.05). Total proteins and globulin showed significantly higher values in the groups fed the KO diet and in the groups kept at lower densities. However, based on our results, it can be concluded that fish fed a normal diet, kept under density stress conditions, and treated with ABZ exhibited a more impaired nonspecific immune response compared to fish kept under the same conditions but fed with KO.

Glucose is also considered to be a good indicator of stress in fish (acute or chronic) and is often used as a biomarker of welfare (Martínez-Porchas et al., 2009). The results of this study highlighted a significant increase (p<0.05) in serum glucose concentration in carp in both HD groups, even in those fed with KO. After the ABZ treatment, serum glucose

concentration increased in all experimental groups, with the highest value observed in the HD group fed a normal diet (97.60±3.80 mg/dl), suggesting a stronger physiological response to the stress induced by high population density and ABZ toxicity.

Serum CHOL and TG provide important information about the stress status of fish as they are linked to energy and lipid metabolism (Wu et al., 2018). It is recognized that in fish kept under high population density conditions, energy reserves are affected in order to cope with the increased energy demand, either through consumption or reallocation of these reserves.

In our experiment, after 60 days of feeding, CHOL levels were not significantly affected by population density ($p>0.05$) or supplementation of the diet with KO ($p>0.05$). However, lower values were observed in the higher population density and KO-fed groups.

Population density or KO supplementation did not induce significant differences in LDL concentration ($p>0.05$), while HDL showed higher values in fish fed the KO diet. However, treatment with ABZ significantly increased LDL levels in all experimental groups ($p<0.05$), with the highest value observed in HD and the lowest in LDK, suggesting that KO reduced the negative impact of ABZ on lipids. HDL significantly decreased ($p<0.05$) after the ABZ treatment, but without significant differences ($p>0.05$) between the experimental groups.

Chronic stress also affects liver function, as evaluated through biomarkers such as ALT, AST, ALP, and GGT. Normally, these enzymes have a constant variation, unless environmental factors or certain pathological challenges are encountered. In teleost fish, ALT and AST enzymes are the main aminotransferases involved in amino acid metabolism in the liver (Coz-Rakovac et al., 2008). In the case of the current study's carp subjected to density stress, AST and ALT levels, measured after 60 days of feeding, significantly increased ($p<0.05$) in the higher density groups, while ALP showed no significant changes ($p>0.05$) due to diet supplementation or population density.

Our results are consistent with those reported by other authors. Onxayvieng et al. (2021) and Adineh et al. (2021) reported increased values for AST, ALT, and ALP in species such as gibel carp (*Carassius gibelio*) or grass carp (*Ctenopharyngodon idella*) reared at high densities. However, some authors did not report significant changes in serum ALT, AST, and ALP values for American bass (*Micropterus salmoides*) (Wang et al., 2019). Supplementation of the diet with KO led to significantly lower AST values ($p<0.05$) in the high-density groups, as well as significant changes ($p<0.05$) in ALT in both tested densities. Regarding ALP values, they were not affected by density or feeding regime ($p>0.05$).

After the treatment with ABZ, the concentration of hepatic enzymes ALT and AST increased in all experimental groups, with the highest values observed in the HD groups. However, it is worth noting the significantly lower values ($p<0.05$) recorded for both density groups that received KO-

supplemented feed. For these parameters, a significant interaction (diet × density) was observed ($p<0.05$).

The values of alkaline phosphatase (ALP) measured in the ABZ-exposed carp showed a significant decrease ($p<0.05$) for the groups that received a normal diet, regardless of density. In the current study, the decrease in ALP activity in fish after exposure to ABZ may result from the disruption of the membrane transport system, as ALP is an enzyme directly involved in membrane transport activities (Bernet et al., 2001).

The values of direct bilirubin, among other biomarkers, provide information regarding liver function or the nutritional status of fish. Total bilirubin concentrations in fish serum are considered to be almost negligible compared to mammals, due to the lower activity of biliverdin reductase (Fang and Bada, 1982). In the present study, total bilirubin (BIL T) was not affected by population density or supplementation of the diet with KO, but it was significantly reduced ($p<0.05$) after treatment with ABZ, with higher values observed in the HD groups.

The activity of GGT was enhanced by high density and ABZ treatment, indicating, together with AST and ALT, liver dysfunction or injury. The use of KO in the carp diet significantly reduced ($p<0.05$) the level of GGT in the serum of fish kept at high densities..

4.3.5. Evaluation of Oxidative Stress, Antioxidant Capacity and Lysozyme Activity

In Table 4.5, oxidative stress parameters and lysozyme activity are presented after the 60-day experimental period and after the application of the ABZ test.

After the 60-day experimental period, plasma and liver MDA concentrations were significantly increased ($p<0.05$) in the groups of fish fed a normal diet and kept at high density. Significantly higher concentrations ($p<0.05$) of MDA were recorded after the application of the ABZ test in all experimental groups. However, the lowest concentration was observed in the LD-KO groups, while the highest concentration was found in the HD variant.

In addition, TAC values were significantly influenced (interaction $p<0.05$) by stocking density and diet supplementation. Before the ABZ treatment, TAC significantly increased ($p<0.05$) in the LD-KO group of fish. After the ABZ challenge, significant decreases in TAC values were observed, with the lowest values recorded in the HD group.

Table 4.5. *The values of malondialdehyde, antioxidant capacity, and lysozyme at the end of the feeding experiment and after the ABZ test*

Parameter	After 60 days of feeding			
	LD	LD-KO	HD	HD-KO
MDA (nmol/mL plasma)	2.10 ± 0.09 ^{b*}	1.67 ± 0.13^{a*}	2.67 ±0.16 ^{c*}	2.00 ± 0.15 ^{b*}
MDA (nmol/g liver)	8.12 ± 0.17 ^{b*}	5.49 ± 0.10 ^{a*}	8.47 ±0.21 ^{c*}	8.12 ±0.22 ^{b*}

TAC (mMTrolox equivalent)	21.78 ± 0.25 ^{b*}	24.64 ±0.29 ^{c*}	19.31 ±0.26 ^{a*}	21.48 ±0.31 ^{b*}
LYZ (U/mL)	7.70 ± 0.29 ^{b*}	9.10 ± 0.12 ^{c*}	7.33±0.14 ^{a*}	9.33 ±0.11 ^{d*}
Parameter	**After the test using ABZ**			
MDA (nmol/mL)	2.44 ±0.11 ^{b**}	1.88 ±0.17 ^{a**}	2.98 ±0.15 ^{c**}	2.38 ±0.13 ^{b**}
MDA (nmol/g liver)	10.77 ±0.19 ^{b**}	8.80 ±0.14 ^{a**}	11.84 ±0.18 ^{b**}	8.61 ±0.19 ^{a**}
TAC (mMTrolox equivalent)	18.97 ±0.22 ^{a**}	18.95 ±0.24 ^{a**}	14.37 ±0.21 ^{b**}	17.21 ±0.23 ^{a**}
LYZ (U/mL)	9.18 ±0.18 ^{b**}	9.73 ±0.16 ^{c**}	8.54 ±0.11 ^{a**}	9.10 ±0.14 ^{b**}

*The values represent the average ± S.E. (n=3). Different letters in the same row indicate significant differences (ANOVA, p<0.05) between experimental groups. Different symbols */** in the same row indicate significant differences after the ABZ treatment (paired t-test, p<0.05). MDA - lipid peroxidation index; TAC - total antioxidant capacity; LYZ - lysozyme activity*.

The lysozyme activity in the blood serum of Cyprinus carpio brood was significantly higher (p<0.05) after 60 days in the groups fed diets supplemented with HO. After the ABZ test, lysozyme activity increased significantly (p<0.05) in all groups except LD-KO, with the lowest values recorded in the HD groups.

As a result of metabolic processes at the cellular level, reactive oxygen species (ROS) are produced and eliminated through complex physiological mechanisms designed to maintain a continuous dynamic balance. When ROS are in excess, free radicals cause lipid peroxidation. The main component of lipid peroxides, malondialdehyde (MDA), affects cellular structure and functions and is characterized by its biotoxicity (Ming et al., 2015).

In the present study, both serum and hepatic concentrations of MDA were affected by the feeding regimen as well as the stocking density, with significantly higher values observed in the groups fed a single diet and those reared under high-density conditions. Therefore, the highest values were observed in the HD groups, followed by the HDKO, LD, and LDKO groups.

After the application of the ABZ test, MDA values increased for all experimental groups, although they maintained the same pattern: the lowest levels were observed in the groups that received KO-supplemented diet, and the highest levels were observed in the HD groups fed a normal diet.

The total antioxidant capacity (TAC) was significantly reduced (p<0.05) after the application of the ABZ test in all four experimental groups. Similar results were reported by Nwani et al., 2016 for African catfish exposed to ABZ. They observed higher levels of lipid peroxidation and, correspondingly, greater inhibition of antioxidant activities, indicating a higher risk of damage.

However, in the present study, the lower reduction in TAC in the groups supplemented with KO may indicate that the tissues avoid depletion of total antioxidant reserves in order to neutralize oxidative stress. Thus, the high content of n-3 PUFA and astaxanthin in KO may be responsible for

activating protective mechanisms and/or exerting antioxidant action, limiting the utilization of internal TAC reserves.

Lyzozyme (LZM) is a commonly accepted biomarker for assessing the immune status of fish following exposure to various stimuli, as it plays a significant role in the immune defense system (Saurabh et al., 2008). The decrease in plasma LZM concentration has been correlated with immune system impairment in different fish species reared at high population densities (Costas et al., 2013; Liu et al., 2019). Similarly, in our experiment, the level of LZM decreased in the HD groups fed with non-supplemented feed, indicating that high population density had a negative effect on immune status. However, in the groups of fish fed with KO, LZM activity was improved, and no significant differences were detected between the tested population densities for these variants. After the ABZ test, the concentration of LZM increased in all experimental groups, with the highest value observed in the LD-KO group and the lowest in the HD group. This suggests that the combination of anti-inflammatory and antioxidant agents found in KO stimulated the production of immune-related molecules such as lysozyme and enhanced the organism's ability to strengthen the defense system when exposed to stressful factors.

4.4. Conclusions and Recommendations

In general, in intensive growth systems such as recirculating aquaculture systems, fish biomass is subjected to stress. However, chronic exposure to stress leads to the deterioration of health status, resulting in reduced feed efficiency, weakened immunity, and ultimately, poor growth performance.

The use of dietary supplements in fish feed for aquaculture has been intensively studied in recent years. In this context, the present study was conducted in order to investigate the potential of KO as a functional ingredient for intensive aquaculture. As a result of the current experiment, several conclusions can be drawn, as presented below:

- administering krill oil in the feed of carp brood led to improved growth performance, both at stocking densities of 3.5 kg/m^3 and higher densities of 8 kg/m^3;
- somatic indices showed higher values in the experimental groups where krill oil was administered, indicating a superior maintenance condition compared to the groups whose feed was not supplemented.

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5. Research on the Influence of Silymarin and Berberine on the Growth Performance of Carp Brood, Biochemical Composition of Meat and Technological Comfort State

5. 1. Introduction

The liver, as the main organ responsible for metabolizing endogenous and exogenous compounds, plays a crucial role in the functioning of the organism. It is also one of the first organs to be affected by stress factors or toxic substances. The presence of stressful conditions or exposure to substances such as metals, biotoxins, and persistent organic pollutants can cause liver damage in fish. This is because the liver is responsible for the detoxification process and has the ability to accumulate various contaminants or associated metabolites. Therefore, maintaining liver function within normal limits is essential for the health and optimal growth of fish.

Plants and their extracts have been used for various purposes since ancient times. In aquaculture, they have started to be used as additives in fish feed to improve growth, stimulate immunity, and enhance disease resistance.

Milk thistle fruits (*Silybum marianum*) contain approximately 2% of a mixture called silymarin, which is composed of flavonolignans including silybin, silydianin, silicristin, and isosilybin A and B. Silymarin has strong antioxidant activity (Kvasnička et al., 2003) and has been found to possess anti-inflammatory, immunomodulatory, and liver-regenerating properties (Abenavoli et al., 2018). Silimarina (SM) possesses antioxidant and anti-inflammatory properties and has been reported to reduce cholesterol and blood lipids. It is used in human medicine for the treatment of biliary disorders and other gastrointestinal-related diseases. The antioxidant properties of SM have been shown to prevent metabolic disorders in liver cells by inhibiting lipid peroxidation, which has a positive effect on preventing altered metabolism of lipoproteins (Halim et al., 1997; Schonfeld et al., 1997). Additionally, SM has been found to reduce liver enzymes in the blood.

However, the mechanism of action of SM in fish is still unknown. Studies have shown that administration of SM in fish feed reduces oxidative stress, histopathological changes, and genotoxicity caused by exposure of common carp to a sublethal level of deltamethrin (an insecticide) (Jindal et al., 2019). Additionally, Al-Shawi et al. (2021) reported that dietary supplementation of fish feed with SM may modulate oxidative stress-induced injuries and hepatotoxicity in common carp exposed to sublethal cadmium chloride toxicity. In a recent study, El-Houseiny et al. (2022) found that supplementation of the diet of African catfish (*Clarias gariepinus*) with dried *S. marianum* plants (seeds, leaves, and stems) for 60 days resulted in reduced histopathological lesions and alleviated the immunosuppressive effects of fluoride toxicity.

Berberine (BBR) is a bioactive compound belonging to a class of compounds called alkaloids. It is isolated from several plants, found in the rhizome, roots, and stems of medicinal plants such as *Berberis aristata*, *Coptis chinensis Franch*, and *Berberis vulgaris L*. (Wang et al., 2022). According to Xu et al. (2021), the content of BBR in these plants ranges from 0.05 mg/g to 96.10 mg/g, with *Berberis* being the richest natural source of BBR.

In recent years, several studies have found promising results of BBR in the clinical treatment of fatty liver, obesity, hypertension, and type 2 diabetes (Ilyas et al., 2002; Shinjyo et al., 2020).

In aquaculture, BBR has been recently used as a functional additive in feed for the prevention and treatment of bacterial diseases (Xu et al., 2017). It has also been reported to improve immune response and antioxidant capacity, as well as to reduce lipid accumulation in the liver, total cholesterol (TC), and triglycerides (TG) (Chen et al., 2016; Xu et al., 2017).

In the context of the above-mentioned, the aim of this experiment was to evaluate the effects of administering SM and BBR on technological indicators and the biochemical composition of carp meat. Additionally, the influence of these dietary supplements on the technological comfort of carp and the hepatoprotective effect after a paracetamol "challenge test" was also assessed.

5.2. Experimental Design

The carp brood (*Cyprinus carpio*) were obtained from a local farm. After a two-week acclimation period, a total of 180 fish (with an average individual weight of 118.4±11.09 g) were randomly distributed into the recirculating system to create six experimental groups in triplicates (Figure 5.1).

Throughout the experimental period, the fish were fed with Skretting feed, which contained 40% crude protein (CP), 10% lipids, 1.5% fiber, and 6.8% ash (Skretting, Vignetto, Italy). The feed was supplemented with silimarin (SM) and berberine (BBR), resulting in the following experimental groups;

- The M variant – control variant, in which the fish were fed with feed containing 40% crude protein (CP).
- The SM variant - Feed with 40% CP, supplemented with 1 g of silimarin/kg of feed;
- The BBR100 variant - Feed with 40% CP, supplemented with 100 mg of berberine/kg of feed;
- The BBR200 variant - Feed with 40% CP, supplemented with 200 mg of berberine/kg of feed;
- The SM+BBR100 variant - Feed with 40% CP, supplemented with 1 g of silimarin/kg of feed + 100 mg of berberine/kg of feed;
- The SM+BBR200 variant - Feed with 40% CP, supplemented with 1 g of silimarin/kg of feed + 200 mg of berberine/kg of feed.

The feeding intensity was set at 1.5% of the biomass per day (BW/day), and the biomass of each growth unit was weighed after 36 days of the experiment to adjust the amount of feed.

The photoperiod was set to 16 hours of light and 8 hours of darkness. Throughout the experimental period, water quality parameters were monitored daily. Water temperature, pH, and dissolved oxygen were automatically measured using the Endress+Hauser monitoring system (Endress+Hauser AG, Switzerland) with probes placed in each rearing tank. Nitrogen compound concentrations were quantified weekly using a Skalar SAN++ analyzer (Skalar Analytical, Netherlands).

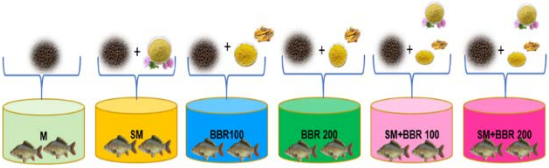

***Figure 5.1**. Experimental Design Diagram*

Therefore, monitoring water quality in a recirculating aquaculture system is essential to ensure an optimal environment for fish growth and health. Inadequate water quality can negatively impact fish physiology and growth, leading to pathological reactions in internal organs and potentially increasing mortality rates. Throughout the experiment, daily monitoring of water quality parameters was conducted, and the average values fell within the optimal range for carp growth: temperature 21.8 ± 3.14 ˚C; pH = 7.41 ± 1.04; dissolved oxygen 7.29 ± 1.13 mg/L^{-1}; ammonia 0.09 ± 0.05 mg/L^{-1}; nitrates < 0.16 mg/L^{-1}; and nitrites < 0.09 mg/L^{-1}.

After 64 days of experimentation, 7 fish from each experimental group were randomly selected to undergo a "challenge" test with paracetamol. Paracetamol, a widely used medication for pain relief and fever reduction, can enter the aquatic environment through various pathways, including direct excretion of the active substance by humans or the disposal of medication waste. It is also known that paracetamol, an antipyretic, has long been associated with hepatic toxicity when administered at doses exceeding therapeutic levels. Therefore, the purpose of the paracetamol "challenge" test was to evaluate whether the administered supplements influenced the level of hepatic toxicity induced by paracetamol.

Thus, immediately after the feeding experiment, a group of fish were transferred to an experimental system consisting of 12 glass tanks of 130 L each. The dose of paracetamol was administered orally (500 mg paracetamol/body weight) using a syringe attached to a tube. The administration of paracetamol was done in a single dose.

5.3. Results and Discussions

5.3.1. Evaluation of Growth Performance in Carp Brood

The primary goal of aquaculture is to achieve rapid growth, and as a result, a low feed conversion ratio (FCR). Therefore, a primary objective of our experiment was to evaluate how the dietary supplements used in carp feed influenced growth performance indicators (Table 5.1.). At the start of the experiment, the initial average body weight of the carp specimens did not show statistically significant differences ($p>0.05$) among the six experimental groups.

After 36 days, a significant increase ($p<0.05$) in body weight is observed in the SM and M variants, while the other experimental variants show statistically insignificant differences in body weight ($p>0.05$) (Figure 5.2).

At the end of the experiment (after 64 days), the body weight of the carp shows significant differences ($p<0.05$) among the six experimental groups. The highest average body weight is recorded in the experimental variants where silimarin (SM) was administered (195.60±10.12 g), as well as in the variant where the feed was supplemented with BBR at a concentration of 200 mg/kg feed (198.70±18.16 g). On the other hand, the experimental variants BBR100, SM+BBR100, and SM+BBR200 have the lowest average final body weight (Figure 5.2; Table 5.1.)..

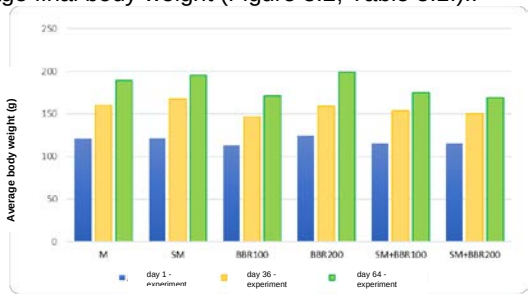

Figure 5.2. *The evolution of the average body weight of carp throughout the experimental period*

Table 5.1. Technological indicators of performance *in carp brood after 64-day experimental period*

Growth parameters	M	SM	BBR100	BBR200	SM+BBR 100	SM+BBR 200
Initial fish number	10	10	10	10	10	10
Initial biomass (g)	1207±8,56^{a}	1216±7,23^{a}	1131±9,76^{a}	1245±9,76^{a}	1151±5,23^{a}	1154±3,12^{a}
Initial average mass (g)	120,70±10,25^{a}	121,60±9,12^{a}	113,10±7,16^{a}	124,50±5,2^{a}	115,10±6,24^{a}	115,40±4,65^{a}
Survival	100	100	100	100	100	100

rate (%)						
Final biomass (g)	1891,50±16,65^{b}	1956±19,65^{a}	1714±18,50^{c}	1987±20,16^{a}	1750±16,24^{c}	1694±14,29^{c}
Final average mass	189,15±18,61^{b}	195,60±10,12^{a}	171,40±19,65^{c}	198,70±18,16^{a}	175,03±17,46^{c}	169,47±10,12^{c}
Growth increment of biomass (g)	684,50±6,65^{b}	740±4,95^{a}	583±9,67^{c}	742±10,10^{a}	599,33±9,96^{c}	540,67±8,49^{c}
FCR, (g/g)	1,70±0,04^{b}	1,47±0,08^{a}	1,86±0,05^{b}	1,46±0,03^{a}	1,83±0,02^{b}	2,10±0,05^{c}
SGR (% zi^{-1})	0,70±0,02^{a}	0,74±,01^{b}	0,65±0,04^{c}	0,73±0,02^{b}	0,65±0,03^{c}	0,60±0,03^{c}

Note: The values represent the average of triplicates ± S.D. Different letters within a row indicate significant differences (ANOVA, $p < 0.05$) between experimental variants. FCR - Feed Conversion Ratio; SGR - Specific Growth Rate.

At the end of the experimental period, it was observed that the survival rate of the carp in all experimental variants reached 100%. This confirms that both the environmental conditions and the feeding practices were maintained within optimal limits.

Regarding the values of the Feed Conversion Ratio (FCR) calculated for the six experimental variants, statistical comparison revealed significant differences ($p<0.05$). The best FCR was recorded in the experimental variants where silymarin was administered (FCR-1.47±0.01 g/g), as well as in the BBR200 variant (FCR-1.46±0.03 g/g), indicating a more efficient utilization of nutrients. The SM+BBR200 variant showed a poorer nutrient utilization with an FCR of 2.10±0.05 g/g. Achieving superior technological indicators in the SM and BBR200 variants can be attributed to the hepatoprotective and immunostimulatory properties of these plants. However, the effect on growth is closely correlated with the administered dose. Similar studies conducted by other researchers have provided conclusive results in this regard.

For example, in a study conducted by Wang et al., 2019, the addition of SM to the diet of turbot (*Scophthalmus maximus*, L) at different concentrations (100, 200, and 400 mg/kg feed) was investigated. The results showed superior growth performance at the concentration of 100 mg/kg feed. The authors observed that the administration of SM at higher concentrations led to immunomodulatory effects and activation of inflammatory processes in the body, which reduced nutrient retention efficiency.

Contrary to the previously mentioned findings, Shahin et al., 2023, obtained superior growth performance in the case of *Dicentrarchus labrax* larvae when using much higher concentrations of SM. By using concentrations of 0,200, 400, and 600 mg SM kg-1 feed, the authors achieved a significant increase in the growth performance of the sea bass larvae directly proportional to the increase in the amount of SM in the diet.

Regarding the effect of BBR on growth performance, studies have demonstrated that its administration in the diet can have beneficial effects on the growth performance of fish. Thus, VanDoan et al., 2020, state that the digestion and absorption of nutrients in the digestive tract of Nile tilapia brood (*Oreochromis niloticus*) were improved after the administration of BBR in the diet for eight weeks. Additionally, the authors achieved a significant increase in the specific growth rate, growth performance, and fish body weight, with the highest levels observed in the case of supplementation with 1 g BBR/kg feed compared to 3, 6, and 9 g BBR/kg feed..

In a study conducted by Yi et al., 2012, the addition of BBR to the diet of *C. aurtaus gibelio* at a level of 4 mg/kg feed did not result in superior growth outcomes for the fish. The differences reported by these studies may be due to the different doses incorporated into the feed or to the different metabolism of each species.

Most studies have focused on evaluating the effect of BBR when administered in diets with a high lipid content. In intensive aquaculture, diets rich in lipids or carbohydrates are commonly used due to their advantages in protein conservation and fish growth stimulation. However, the administration of these diets raises concerns regarding excessive fat accumulation in the liver, which represents an unnecessary energy loss, considering that an energy nutrient is stored in the liver or visceral mass.

Thus, studies emphasize the potential of BBR for use in aquaculture as a feed additive to treat hepatic dysfunction caused by diets high in lipids and carbohydrates, due to its proven effects in treating metabolic disorders such as fatty liver and hyperlipidemia, suggesting its important role in glucose and lipid metabolism (Xu et al., 2021). Furthermore, supplementation with berberine has also been shown to improve growth performance, intestinal health, antioxidant capacity, and immune status of fish in multiple studies (Doan et al., 2020).

After 64 days, the specific growth rate (SGR) showed significantly better values in the SM and M variants, followed by the M variant. A lower SGR was recorded in the SM+BBR200, BBR100, and SM+BBR100 variants.

Considering the significant variation in the results reported in the literature regarding the optimal doses at which these supplements (SM and BBR) could be incorporated into the feed, it is necessary to establish them for each fish species as well as for each stage of development.

Based on the information presented in the literature, there is currently no available information regarding the supplementation of fish feed with SM and BBR. In our study, it was observed that adding 1 g of SM/kg feed, in combination with 100 mg and 200 mg of BBR/kg feed, did not lead to an improvement in fish growth performance, final average weight, or biomass growth rate. These experimental variants recorded the lowest values in terms of growth parameters.

Therefore, the relationship between growth performance and the supplementation of feed with SM and BBR can vary depending on the fish species, feeding method, diet used, as well as the dosage and duration of administration. Some authors have reported that prolonged use of BBR in fish feed may reduce its beneficial effects (Shan et al., 2013). This aspect has been reported in mammals, suggesting that the intestinal expression of P-glycoprotein becomes overwhelmed by a high dose or long-term administration of BBR, inhibiting the absorption of berberine by the intestine (Shan et al., 2013). However, this mechanism has not been reported in fish and should be further studied.

5.3.2. Evaluation of Somatic Indices and Biochemical Composition of Meat

Table 5.2 presents the values of somatic indices. Thus, after the 64 experimental days, the statistical analysis using ANOVA did not reveal significant differences ($p>0.05$) among the experimental variants for the analyzed somatic indices.

Table 5.2.*Somatic indices at the end of the feeding experiment*

Body Index	M	SM	BBR100	BBR20	SM+BBR100	SM+BBR200
HSI	2,37±0,18 a	1,91±0,24 a	1,92±0,38 a	1,69±0,68 a	1,65±0,21 a	2,09 ±0,74[a]
IGS (%)	6,82±2,36 a	3,03±2,35 a	4,32±1,43 a	4,96±0,09 a	5,40±1,80 a	7,62±5,68 a
SSI (%)	0,27±0,07 a	0,28±0,06 a	0,18±0,01 a	0,33±0,19 a	0,24±0.06 a	0,23±0,08 a

Note: The values represent the average ±SD of triplicates. Values with different letters indicate significant differences (ANOVA, $p<0.05$) among the experimental variants.

However, a slight decrease in the HSI index can be observed in the variants where the feed was supplemented with the selected dietary supplements, with the highest HSI recorded in the control variant. A lower HSI indicates a reduction in fat accumulation or toxic substances in the liver, suggesting that SM and BBR had a beneficial effect on the liver health of the fish. It is known that fatty liver can lead to metabolic pathologies in mammals, ranging from steatosis to hepatocellular injury, fibrosis, or liver failure (Dai et al., 2015). On the other hand, fish are more susceptible to fat accumulation in the liver since the liver is the main storage site for lipids. Additionally, deficiencies in growth and a high mortality rate caused by fatty liver have been observed in aquaculture, resulting in significant economic consequences.

After 64 days of feeding, the statistical analysis did not show significant differences ($p>0.05$) in terms of water, protein, and ash content. However, the lipid content showed significantly lower values ($p<0.05$) in the variants where the dietary supplements were administered in the fish feed.

Table 5.3. *The biochemical composition of carp meat after 64 days of experimentation*

	M	SM	BBR100	BBR200	SM+BBR 100	SM+BBR 200
Water (%)	78,44±1,26[a]	78,52±0,64[a]	79,30±0,22[a]	79,04±1,32[a]	79,09±1,32[a]	79,54±0,26[a]
Protein (%)	17,06±0,73[a]	18,63±0,17[a]	17,54±0,47[a]	17,94±0,53[a]	17,69±0,61[a]	17,42±0.56[a]
Lipids (%)	2,44±0,88[a]	1,44±0,46[b]	1,39±0,08[b]	1,32±0,22[b]	1,68±0,28[a]	1,31±0,13[b]
Ash (%)	1,12±0,07[a]	1,29±0,09[a]	1,26±0,05[a]	1,07±0,05[a]	1,72±0,65[a]	1,20±0,24[a]

Note: The values represent the average ± SD of triplicates. Values with different letters indicate significant differences (ANOVA, $p < 0.05$) between experimental variants.

Although the protein values did not show significant differences between the experimental variants, a slight increase in the protein percentage can be observed in the variants where the dietary additives were used in the fish feed. These results suggest that dietary additives can be effectively used to improve the quality of fish meat and meet the nutritional requirements of consumers who aim to reduce lipid intake and seek a healthier source of protein.

5.3.3. Evaluation of Haematological Parameters

After the 64-day experimental period and the administration of a single dose of paracetamol, hematological parameters were measured, including hemoglobin (Hb), red blood cell count (RBC), hematocrit (Ht), as well as erythrocyte indices (MCV, MCH, and MCHC).

At the end of the feeding experiment, statistical analysis revealed significant differences ($p<0.05$) regarding the values of Ht and RBC, while the values of Hb, MCV, MCH, and MCHC did not show significant differences ($p>0.05$) among the six experimental variants.

After the paracetamol test, the hematological parameters did not show significant differences ($p>0.05$) among the six experimental variants.

The hematocrit percentage reflects the proportion of red blood cells in relation to leukocytes and plasma. After the 64-day feeding period, the hematocrit levels exhibited significantly different values ($p<0.05$) among the experimental variants (Figure 5.3). Therefore, the SM variant had the lowest Ht values (36.62±0.31%), followed by the M variant (41.15±3.35%), while the BBR100 (42.14±3.80%), BBR200 (44.36±1.11%), SM+BBR100 (43.98±3.77%), and SM+BBR200 (44.11±3.63%) variants showed similar values without significant differences ($p>0.05$).

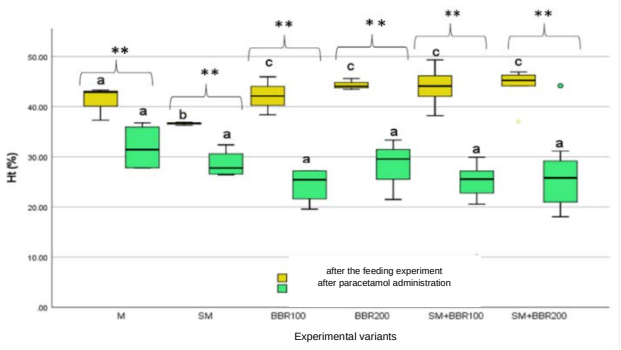

Figure 5.3. *Hematocrit variation – box plot graph (the median, minimum, maximum values and quartiles)*
*Values with different letters indicate significant differences (ANOVA, p<0.05) between experimental groups. Values with * indicate non-significant differences (t-dependent, p>0.05) after the paracetamol test. Values with ** indicate significant differences after the paracetamol test (t-dependent, p<0.05).*

After the paracetamol test, the Ht values did not show significant differences (p>0.05) among the six experimental variants.

Statistical comparison (t-test) of Ht values after the feeding experiment and after the paracetamol test revealed significant differences in all experimental variants. Although the hematocrit values showed a significant decrease after the overdose of paracetamol, the obtained values fall within the recommended optimal range according to the literature (Table 5.4).

Table 5.4. *Reference values for haematological parameters in Cyprinuscarpio*

Body index (g)	Ht (%)	Hb (g/dL)	RBC ($\times 10^6$/mm^3)	VEM (μm^3)	HEM (pg)	CHEM (g/dL)	Reference
138,3±28,7	29±3	8,63±0,76	1,64±0,14	180,30±15,3	52,9±4,7	29±2,0	Sudova et. al., 2009
200	31,8±5,5	6,94±1,6	1,81±0,2	178,2±31,7	40,2±6,5	21,6±3,3	Tripathi et. al., 2004
61,2±7,3	22-39	3,76-8,76	0,90-2,02	133,7-248,4	36,9-57,8	15-32	Mikula et. al., 2008
138±28,7	26±3	6,84±0,9	1,05±0,03	248,1±36	65,2±10	26,3±0,13	Velisek et. al., 2010
297,4±55,6	29,2±2,7	7,43±0,7	1,63±0,13	179,5±13,5	45,8±4,2	25,5±0,78	Velisek et. al.,

							2012
41±0,2	42,2±0,5	11,24±0,41	1,80±0,02	234,5±2,0	62,5±1,6	26,67±0,7	Gholami-Seyedkolaei et. al., 2013
67,5±9,1	30,9±3,5	7,18±0,13	1,40±0,09	217,2±24,2	51,9±8,2	24,1±0,31	Yonar, 2013
43,3±7,7	34,2±5,7	7,66±0,7	1,49±0,12	230±37,9	51±7,5	23±3,79	Yonar et. al., 2014
61,9±2,4	42,8±4	9,4±0,8	1,37±0,19	317,9±60,9	-	22,2±3,3	Kuhlwein et. al., 2014
80±5	24±1	10,2±0,8	1,47±0,2	176,6±13,5	78,8±10	44,6±0,4	Bojarski et. al., 2015

Regarding the hemoglobin values, the statistical analysis did not show significant differences (p>0.05) after the 64 days of feeding. However, the Hb values were slightly lower in the SM variant (Figure 5.4). After the administration of a single dose of paracetamol, the hemoglobin values did not exhibit significant differences (p>0.05).

The statistical comparison of hemoglobin concentration values after the 64 days of experimentation with those obtained after the administration of a single dose of paracetamol did not reveal significant differences (p>0.05). However, a slight increase in Hb can be observed in all experimental variants.

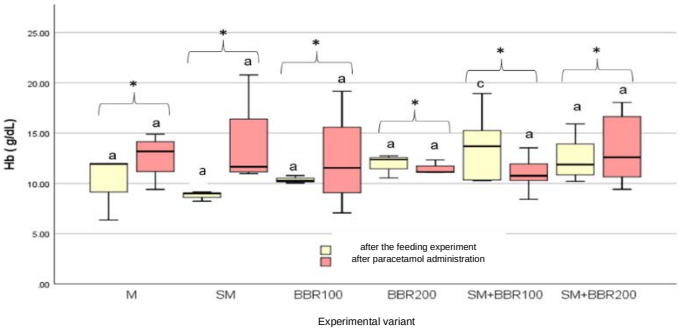

Figure 5.4. *Variation of hemoglobin – box plot graph (median, minimum values, maximum values, and quartiles)*

Values with different letters indicate significant differences (ANOVA, p<0.05) between experimental variants. Values with * indicate non-significant differences (t-dependent, p>0.05) after the paracetamol test. Values with ** indicate significant differences after the paracetamol test (t-dependent, p<0.05).

In general, stressful situations can lead to an increase in hemoglobin levels in fish blood. This can be an adaptation of the organism to enhance oxygen transport capacity during demanding periods.

After the feeding period, the number of erythrocytes significantly increased in the SM+BBR200 variant (1.91±0.20 ×106 mm^3) (Figure 5.5.). However, after the administration of the paracetamol dose, erythrocyte values did not show significant differences ($p>0.05$) among the six experimental variants.

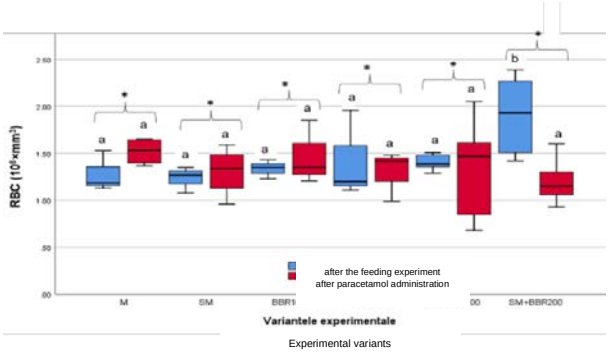

Figure 5.5. *Variation of red blood cell count – box plot graph (median, minimum and maximum values, quartiles)*

Values with different letters indicate significant differences (ANOVA, $p<0.05$) between experimental variants. Values with * indicate non-significant differences (t-dependent, $p>0.05$) after the paracetamol test. Values with ** indicate significant differences after the paracetamol test (t-dependent, $p<0.05$).

Comparing the values of erythrocyte count after the 64-day experimental period with those obtained after the administration of the single dose of paracetamol, the T-test did not reveal significant differences ($p>0.05$).

Regarding the RBC variables, VEM, HEM, and CHEM, the statistical analysis did not indicate significant differences ($p>0.05$) after the feeding experiment or after the administration of the paracetamol dose (Figures 5.6; 5.7; 5.8). However, in terms of the statistical comparison of erythrocyte constant values after the feeding experiment with the values after the administration of the paracetamol dose, the T-test revealed significant differences ($p<0.05$) only in the case of CHEM in the BBR200 variant.

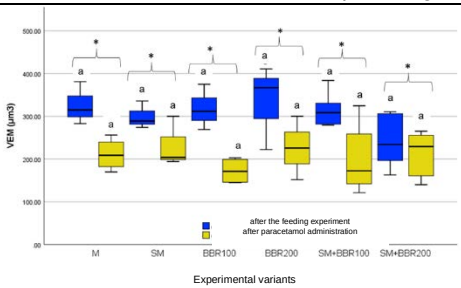

Figure 5.6. *Variation of VEM – box plot graph (median, minimum and maximum values, and quartiles)*

Values with different letters indicate significant differences (ANOVA, p<0.05) between experimental variants. Values with * indicate non-significant differences (t-dependent, p>0.05) after the paracetamol test. Values with ** indicate significant differences after the paracetamol test (t-dependent, p<0.05)

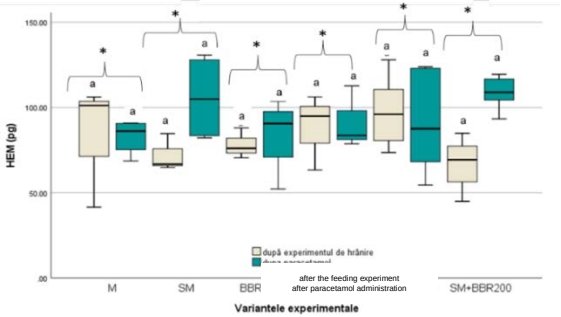

Figure 5.7. *HEM variati* *edian, minimum and maximum values, and quartiles)*

Values with different letters indicate significant differences (ANOVA, p<0.05) between experimental variants. Values with * indicate non-significant differences (t-dependent, p>0.05) after the paracetamol test. Values with ** indicate significant differences after the paracetamol test (t-dependent, p<0.05).

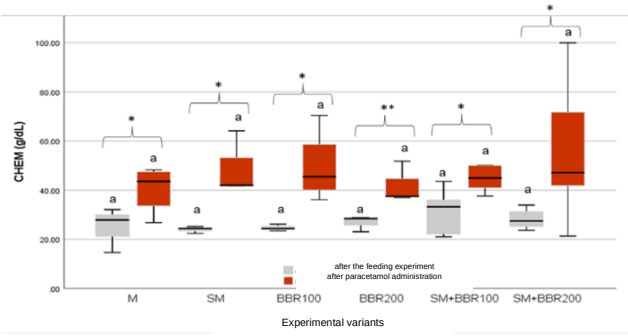

Figure 5.8. *CHEM variation- box plot graph (median, minimum and maximum values, and quartiles)*
Values with different letters indicate significant differences (ANOVA, p<0.05) between experimental variants. Values with * indicate non-significant differences (t-dependent, p>0.05) after the paracetamol test. Values with ** indicate significant differences after the paracetamol test (t-dependent, p<0.05).

In our study, the analysis of hematological parameters did not show major changes after the administration of selected dietary supplements, except for Ht and RBC. Ht exhibited a significant decrease in the SM variant, while the number of erythrocytes showed a significant increase in the SM+BBR200 variant.

Our results are similar to those reported by other authors. For instance, Lukanov et al., 2018, did not observe significant changes in the number of erythrocytes, Ht, or Hb concentration after administering SM (0.5% and 1%) in the diet of Japanese quails. Ahmadi et al., 2012, observed a significant increase in the number of erythrocytes, hematocrit, and hemoglobin concentration after administering SM (0.0, 0.1, 0.4, and 0.8 g/kg of feed) in the diet of rainbow trout, indicating the influence of SM on hematopoietic viscera such as the spleen and kidney, which play an important role in haemopoiesis.

Regarding the influence of paracetamol on hematological parameters, a significant decrease (p>0.05) in hematocrit was observed in all experimental variants. Additionally, a decrease in CHEM was observed in the BBR200 experimental variant. These findings do not suggest the occurrence of cellular lesions.

5.3.4. Evaluation of Serum Parameters

One of the main objectives of this study was to evaluate the potential of phytobiotic supplements such as SM and BBR, as well as nutraceutical formulas (a mixture of BBR and SM), on the health status of carp, and assess their hepatoprotective potential after conducting a "challenge" test

where hepatotoxicity was induced by an overdose of paracetamol. At the end of the long-term experiment, in which feeds supplemented with the mentioned compounds were administered, a series of biochemical parameters relevant for assessing the general health status were quantified. The same parameters were also quantified after the acute toxicity test.

Table 5.5 presents the values of serum parameters at the end of the feeding experiment and after the paracetamol test. When administered in overdose, paracetamol causes hepatotoxicity in both humans and laboratory animals, which is easily identified by the elevated levels of hepatic enzymes alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (Kumar et al., 2004; Yen et al., 2007). It has been demonstrated that an increase in ALT and AST values reflects liver damage, while an elevation in alkaline phosphatase (ALP) levels can indicate both kidney and liver injuries (Bhattacharya et al., 2005).

In general, for the evaluation of liver injuries caused by paracetamol, TGO (AST) and TGP (ALT) are commonly used as marker enzymes. TGO is an enzyme found in the mitochondria and cytoplasm of all cells, while TGP is a cytoplasmic enzyme specific to hepatocytes, and its elevation in the blood indicates liver damage. Therefore, TGP (ALT) is considered a more specific marker for the liver and is thus a more suitable parameter for detecting liver injuries and assessing their extent. Increased levels of TGO, TGP, and ALP (alkaline phosphatase) can indicate cellular leakage and loss of functional integrity of hepatocyte membranes. Elevated serum levels of ALP also indicate an increase in biliary pressure.

In the feeding experiment, after 64 days of administration of SM and BBR doses, no significant differences ($p>0.05$) were observed between the tested variants regarding the levels of TGO and TGP enzymes in the blood. These values fell within the range of normal values for the studied species (Nicula et al., 2010). However, slightly lower TGP values were observed in the experimental variants where BBR was administered at concentrations of 100 and 200 mg/kg feed (8.00±1.82 U/L and 8.12±0.63 U/L for BBR100, respectively, BBR200 variants).

After the administration of a single dose of paracetamol, the level of serum TGP enzymes increased for all tested experimental variants, but a significant increase ($p<0.05$, paired T-test) was detected only for the control variant (M), which recorded an average TGP value of 42.50±8.58 U/L, approximately 400% higher than the initial value. The same trend of increased serum values after exposure to paracetamol, especially in the control variant, was also observed for TGO. Thus, for the M batches, the average TGO value recorded was 364.25±78.88 U/L, a significantly higher value compared to the pre-exposure level ($p<0.05$, paired T-test) and compared to the values recorded for the experimental batches, which ranged from 218.00±44.21 U/L to 304.50±32.27 U/L. The differences

between these values were not statistically significant ($p<0.05$, ANOVA test).

The results obtained from the acute toxicity test, conducted by administering a single dose of paracetamol (500mg/kg body weight), highlight the fact that both SM, BBR, and the tested nutraceutical combinations (SM+BBR100 and SM+BBR200) have a hepatoprotective effect, as neither TGP nor TGO values showed a significant increase after exposure.

The alkaline phosphatase (ALP) levels measured in carp exposed to paracetamol showed a significant increase ($p < 0.05$, paired T-test) in the control group, while the experimental groups exhibited a non-significant statistical increase ($p > 0.05$). At the end of the induced toxicity test, the lowest ALP values were observed in the BBR200 (154.00 ± 77.6 U/L), BBR100 (173.33 ± 49.80 U/L), and SM+BBR200 (179.25 ± 78.54 U/L) groups. This indicates that berberine had a more pronounced contribution as a hepatoprotective agent compared to silymarin, at least in terms of ALP. In the BBR200 group, the increase in ALP after exposure to paracetamol was only 7%.

Oral administration of SM in other species such as *Oncorhynchus mykiss* has also been found to stabilize the cell membrane structure and regulate the activity levels of AST, ALT, and ALP when using concentrations of up to 400 mg/kg body weight (Banaee et al., 2011). In a study conducted on *Cyprinus carpio*, it is shown that silymarin protects hepatocytes against tissue damage induced by cadmium chloride only at concentrations of 2400 mg/kg diet (Al-Shawi et al., 2021).

Throughout the history of medicine, the fruits of plants such as *Berberis vulgaris* have also been used for their beneficial effects on liver function and cardiovascular system by reducing triglycerides, cholesterol, low-density lipoproteins, and blood pressure, thanks to their unique bioactive compounds, such as berberine (Ardestani et al., 2013) (Zarei et al., 2015).

In our study, the levels of serum triglycerides (TG) in fish fed with feed enriched with BBR, SM, and BBR+SM showed a significant decrease ($p<0.05$, ANOVA test) compared to the control group. Although no statistically significant differences were observed between the experimental variants that were supplemented with either SM or BBR at different concentrations, it can be observed that the TG values were lower in the SM+BBR100 (343.5±53.78 mg/dL) and SM+BBR200 (342.6±48.71 mg/dL) variants, indicating a synergistic effect of SM and BBR.

Similar results have been reported for the sturgeon species *Acipenser baeri* (Ramezan et al., 2021), where a diet supplemented with BBR at concentrations of 150, 300, 600, and 750 mg/kg showed a negative correlation with serum TG levels in fish. BBR has been reported as an effective agent in reducing serum lipids by decreasing TG synthesis through the stimulation of AMP-activated protein kinase (AMPK) activity, which plays a key role in cellular energy homeostasis (Brusq et al., 2006).

Table 5.5. *The values (Average±S.D) of serum parameters at the end of the feeding experiment and after the paracetamol test*

Parameter	After the feeding experiment					
	M	**SM**	**BBR100**	**BBR200**	**SM+BBR100**	**SM+BBR200**
TG (mg/dL)	458±81,93^{c}	370,33±76,44^{b}	367,00±67,39^{b}	347,29±45,74^{b}	343,5±53,78^{a}	342,6+±48,71^{a}
CHOL (mg/dL)	268,33±33,07^{c}	234,00±16,7^{b}	196,75±9,81^{a}	225,00±22,99^{b}	225,66±12,5^{b}	214,5±20,69^{a}
LDL (mg/dL)	115,10+4,56^{d}	98,22±11,22^{c}	53,67±7,55^{b}	46,67±9,34^{a}	64.34±8,56^{b}	54,76±6,55^{b}
HDL(mg/dL)	44,71±6,32 ^{a}	42,73±8,12^{a}	77,50±9,10^{b}	89,88±8,45^{c}	75,43±6,77^{b}	82,12±7,45^{b}
TGP/ ALT (U/L)	10,25±1,25^{a}	10,00±3,00^{a}	8,00±1,82^{a}	8,12±0,63^{a}	9,33±4,03^{a}	8,33±1,96^{a}
TGO/ AST (U/L)	174±19,71^{a}	142,67±49,03^{a}	134,79±8,86^{a}	188,67±37,11^{a}	199,5±55,47^{a}	147,8±22,81^{a}
PHOSPHATASE/ ALP (U/L)	186±48,46^{a}	158,33±27,28^{a}	144,67±20,71^{a}	147±54,72^{a}	185,66±68,19^{a}	155,25±77,10^{a}
GGT (U/L)	1,33±0,47^{a}	1,23±0,75^{a}	0,92±0,09^{a}	1,00±0,44^{a}	1,38±0,33^{a}	1,45±0,32^{a}
BILD (mg/dL)	0,18±0,05^{a}	0,13±0,03^{a}	0,15±0,01^{a}	0,18±0,04^{a}	0,12±0,03^{a}	0,15±0,02^{a}
BILT(mg/dL)	0,31±0,06^{b}	0,20±0,02^{a}	0,21±0,06^{a}	0,23±0,09^{a}	0,19±0,02^{a}	0,21±0,01^{a}
Parameter	**After paracetamol**					
	M	**SM**	**BBR100**	**BBR200**	**SM+BBR100**	**SM+BBR200**
TG (mg/dL)	141,00±29,81^{a**}	189,25±38,59^{b*}	186,75±41,55^{b*}	181,75±38,81^{b**}	182,25±28,50^{b**}	163,25±27,73^{b**}
CHOL (mg/dL)	187,25±32,01^{a**}	173,25±14,10^{a**}	206,50±27,19^{a*}	168,25±24,40^{a**}	156,00±25,17^{a**}	170,50±18,77^{a*}
LDL (mg/dL)	95,10+4,56^{d**}	78,22±11,22^{c**}	44,67±7,55^{b**}	39.61±7,24^{a**}	44.98±7,65^{b**}	39,16±8,34^{b**}
HDL(mg/dL)	34,71±6,32 ^{a**}	35,73±8,12^{a**}	68,20±9,10^{b*}	82,33±4,95^{c*}	70,43±7,37^{b*}	77,12±7,45^{b*}
TGP/ ALT (U/L)	42,50±8,58^{b**}	25,50±8,54^{a*}	26,00±2,58^{a*}	29,75±12,39^{a*}	25,30±0,57^{a*}	29,75±4,71^{a*}
TGO/ AST (U/L)	364,25±78,88^{b**}	218,00±44,21^{a*}	295,25±88,68^{a*}	304,50±32,27^{a*}	242,00±24,12^{a*}	273,20±79,95^{a*}
PHOSPHATASE/ ALP (U/L)	294,50±15,02^{c**}	183,67±64,01^{b*}	173,33±49,80^{a*}	154,00±77,6^{a*}	199,5±23,01^{b*}	179,25±78,54^{b*}
GGT (U/L)	2,90±0,85^{c**}	1,55±0,42^{b*}	1,27±0,25^{a*}	1,15±0,20^{a*}	1,50±0,57^{b*}	2,00±1,55^{b*}
BILD (mg/dL)	0,26±0,04^{c**}	0,16±0,03^{a*}	0,22±0,01^{b**}	0,24±0,05^{b*}	0,15±0,04^{a*}	0,20±0,01^{b*}
BILT(mg/dL)	0,46±0,06^{c**}	0,27±0,04^{a*}	0,31±0,04^{a**}	0,35±0,10^{a*}	0,25±0,10^{a*}	0,34±0,09^{b*}

*Note: Values with different letters indicate significant differences (ANOVA, p<0.05) between experimental variants. Values with * indicate non-significant differences (t-dependent, p>0.05) after the paracetamol test. Values with ** indicate significant differences after the paracetamol test (t-dependent, p<0.05).*

Regarding total cholesterol (CHOL), after the feeding experiment, the average serum values were significantly lower in the experimental variants where feed enriched with food additives was administered compared to the control variant. It is noteworthy, however, that the lowest values were recorded in the groups where BBR was administered, either as a single supplement (in the BBR100 variant, the average concentration was 196.75±9.81 mg/dL) or in a nutraceutical combination with SM (in the SM+BBR100 variant, the average concentration was 214.5±20.69 mg/dL). Additionally, the cholesterol fraction values indicate that BBR positively influenced the HDL/LDL ratio (Table 5.4). Thus, the BBR200 variant recorded the lowest LDL concentration (46.67±9.34 mg/dL), followed by the BBR100, SM+BBR200, and SM+BBR200 variants; the highest value was recorded for the control variant (115.10±4.56 mg/dL), which was significantly higher compared to the avergare values recorded for the other experimental variants. The HDL fraction recorded the lowest values in the control and SM variants (44.71±6.32 mg/dL and 42.73±8.12 mg/dL, respectively).

High doses of paracetamol rapidly deplete hepatic levels of glycogen, triglycerides, and cholesterol, indicating impairment of carbohydrate and lipid metabolism. The loss of glycogen or lipids can occur as a direct effect of intoxication or may occur secondarily due to the alteration of the overall body condition caused by hunger, stress, or concurrent illness (Wolf and Wolfe, 2005).

It has also been demonstrated that both subtoxic and toxic doses of paracetamol reduce genes involved in energy-consuming biochemical pathways, including gluconeogenesis (glucose-6-phosphatase), fatty acid synthesis (sterol C4-methyl oxidase), and cholesterol synthesis (3-hydroxy-3-methylglutaryl-coenzyme A synthase 1), and regulate genes involved in energy-producing biochemical pathways, such as glycolysis/gluconeogenesis (6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 1) (Yuxia and Richard, 2010).

In the case of fish fed with feed supplemented with SM and BBR (SM and BBR100 experimental variants), the triglyceride levels decreased non-significantly after exposure to paracetamol, suggesting the regulatory action of these supplements.

Cholesterol has also been used in numerous previous research studies as a diagnostic tool for the biological monitoring of farmed fish (Clifton et al., 2010). Consequently, elevated energy metabolites such as total cholesterol (CHOL), HDL, and LDL may indicate lipid or lipoprotein metabolic disorders or liver dysfunction. The results obtained after the toxic challenge test revealed that the levels of total cholesterol and cholesterol fractions decreased for all experimental variants. However, it can be easily noted that from a lipid metabolism perspective, the control variant was the most exposed to the negative effects of paracetamol-induced hepatotoxicity, while the variants showed non-significant decreases in CHOL levels. Additionally, in the case of the BBR100 variant, the least depreciation of CHOL values was observed, while for the BBR200 and HDL and LDL variants, a similar trend was observed.

GGT (γ-glutamyltransferase), also known as gamma-glutamyltranspeptidase, is an enzyme primarily present in the liver, but also in other tissues such as the kidneys, pancreas, and spleen. The main function of GGT is to transfer γ-glutamyl groups from peptides and amino acids to other molecules, such as

aminotransferases, and to hydrolyze γ-glutamylcysteinylglycine (a precursor of glutathione). GGT is often used as an enzymatic marker for assessing liver function. Elevated GGT levels in the blood can indicate various liver disorders, particularly impairment of the biliary pathways.

Bilirubina is a yellow-orange pigment that results from the breakdown of hemoglobin in old red blood cells. It is primarily produced in the liver and is an important component of bile, which plays a crucial role in fat digestion. There are two main forms of bilirubin: total bilirubin and direct bilirubin (conjugated bilirubin). Total bilirubin represents the sum of direct bilirubin and indirect bilirubin (unconjugated bilirubin). Indirect bilirubin is the form of bilirubin that is initially formed in the process of hemoglobin degradation. It is insoluble in water and needs to be transported to the liver for processing and elimination. In the liver, indirect bilirubin is conjugated with glucuronic acid to form direct bilirubin.

In this study, after the feeding experiment, no significant differences were observed in the average values of GGT and BILD among the tested experimental groups ($p>0.05$, ANOVA test). However, BILT showed a significantly higher average value compared to the experimental groups ($p<0.05$, ANOVA test). Furthermore, after exposure to paracetamol, the levels of GGT, BILD, and BILT significantly increased ($p<0.05$, paired t-test) in the control group. In the experimental groups where phytobiotic compounds and the BBR+SM nutraceutical combination were tested, no significant increases were observed in the mentioned parameters, except for the BBR100 group, where BILD and BILT showed significant increases. It can also be noted that the lowest values of BILD and BILT were recorded in the groups where SM was administered as a single compound (0.16±0.03 mg/dL, respectively, 0.27±0.04 mg/dL), and in the groups where the combination of silymarin and berberine was administered in a dose of 100 mg/kg of feed.

The improved effects of BBR and SM were evidenced by their modulation of gamma-glutamyltransferase activity and bilirubin levels. Bilirubin is considered to be one of the most powerful endogenous antioxidants, and its serum concentrations are predominantly affected by hepatic bilirubin activity. Thus, bilirubin plays a role in cell protection when exposed to the negative effects of toxic compounds (Tomaro & Batlle, 2002). The high levels of bilirubin in the control group indirectly reflect the failure of liver function due to paracetamol-induced hepatotoxicity. Additionally, the protection provided by SM against paracetamol-induced liver toxicity can be attributed to its beneficial properties, including free radical scavenging, increased cellular GSH content, and regulation of membrane permeability (Sabi et al., 2015). Moreover, in this case, the combination of SM with BBR at a concentration of 100 mg/kg of feed potentiated this effect due to the antioxidant properties of BBR.

5.3.5. Evaluation of Oxidative Stress, Antioxidant Capacity and Lysozyme Activity

Figures 5.9 to 5.12 present the oxidative stress parameters and lysozyme activity after the 64 experimental days, as well as after the paracetamol test.

After the 64 experimental days, the plasma MDA (malondialdehyde) concentration showed significant differences among the six experimental variants (Figure 5.9).

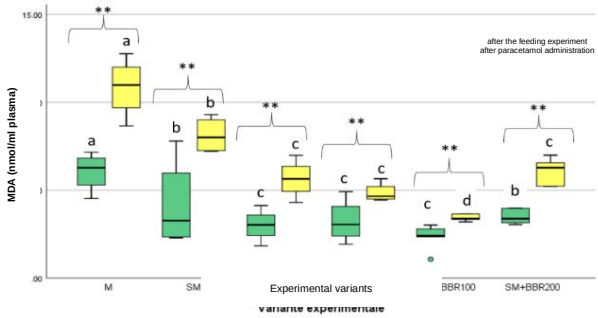

Figure 5.9. *MDA variation - box plot (median, minimum and maximum values, and quartiles)*

Values with different letters indicate significant differences (ANOVA, p<0.05) between experimental variants. Values with * indicate nonsignificant differences (t-dependent, p>0.05) after the paracetamol test. Values with ** indicate significant differences after the paracetamol test (t-dependent, p<0.05).

The lowest concentration of MDA was recorded in the SM+BBR100 variants (2.34±0.67 nmol/mL plasma), BBR100 (3±0.93 nmol/mL plasma), BBR200 (3.24±1.24 nmol/mL plasma), SM+BBR200 (3.72±0.88 nmol/mL plasma). The highest concentration was observed in the M variant (6.06±1.11 nmol/mL plasma), while in the SM variant, the MDA value was 4.15±2.56 nmol/mL plasma. MDA is a commonly used indicator for assessing lipid peroxidation. In our study, the increased plasma MDA level in the control variant may indicate a slight imbalance between the generation and elimination of reactive oxygen species (ROS). The results obtained showed that supplementation of the diet with BBR and SM reduced oxidative damage in the plasma by reducing MDA production.

Regarding the MDA values in the liver, there were no significant differences (p>0.05) observed both after the feeding experiment, after the test with paracetamol, and when comparing the MDA values after the 64-day period with the MDA values after the test with paracetamol (p>0.05). However, from the analysis of the obtained data, it can be observed that in the M variant, the MDA values are slightly higher, both after the feeding experiment (15.10±2.71 nmol/mL plasma) and after the test with paracetamol (10.48±2.20 nmol/mL plasma) (Figure 5.10).

After the 64-day feeding period, the total antioxidant capacity in the plasma did not show significant differences (p>0.05) among the six experimental variants (Figure 5.11). However, a slight increase in TAC values can be observed in the control variant.

After the administration of a single dose of paracetamol, significantly higher values were recorded in the variant where SM was administered, followed by the

SM+BBR200, BBR100, BBR200, and SM+BBR10 variants. The lowest MDA value was recorded in the M variant.

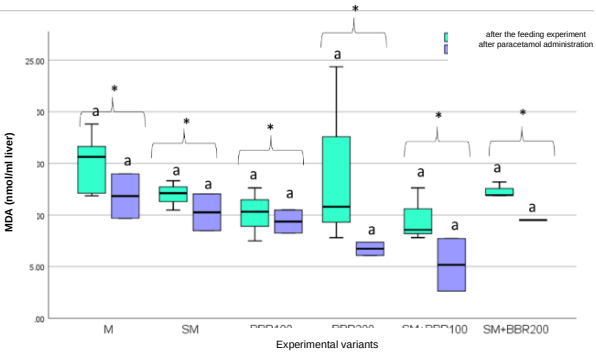

Figure 5.10. *MDA liver variation – box plot (median, minimum and maximum values, and quartiles)*
Values with different letters indicate significant differences (ANOVA, p<0.05) between experimental variants. Values with * indicate non-significant differences (t-dependent, p>0.05) after the paracetamol test. Values with ** indicate significant differences (t-dependent, p<0.05) after the paracetamol test.

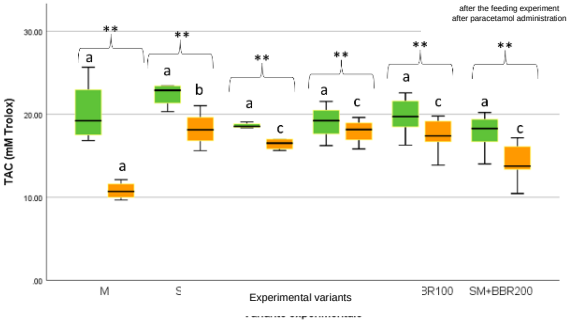

Figure 5.11. *TAC variation – box plot (median, minimum and maximum values, and quartiles)*
Values with different letters indicate significant differences (ANOVA, p<0.05) between experimental variants. Values with * indicate non-significant differences (t-dependent, p>0.05) after the paracetamol test. Values with ** indicate significant differences (t-dependent, p<0.05) after the paracetamol test.

According to the conducted studies, an inverse relationship between lipid peroxidation and total antioxidant capacity has been observed, indicating that a decrease in malondialdehyde levels leads to an increase in the percentage of

antioxidants in the body (Lupoe et al., 2011). Our results have demonstrated that in the experimental variants where additional additives were added to the diet, the concentration of MDA decreased, while TAC values increased, highlighting the positive effect of these additives in combating oxidative stress. Therefore, the addition of SM and BBR to carp diet can contribute to maintaining a balanced ratio between oxidants and antioxidants, thus preventing the occurrence of oxidative stress.

Regarding lysozyme activity, after the 64-day experimental period, ANOVA statistical analysis revealed significant differences ($p<0.05$) between the six experimental variants. The lysozyme activity in the SM+BBR100 (12.29±0.94 U/mL) and SM+BBR200 (12.7±0.74 U/mL) variants was significantly higher than in the BBR200 (11.04±1.05 U/mL), BBR100 (10.97±0.26 U/mL), and SM (9.97±0.53 U/mL) variants. The lowest lysozyme activity was observed in the control variant (9.11±0.29 U/mL).

After the paracetamol test, the statistical analysis revealed significant differences ($p<0.05$) between the experimental variants. The lowest lysozyme activity was recorded in the M variant (4.97±1.57 U/mL). The lysozyme activity values in the BBR100 (8.35±0.77 U/mL), BBR200 (9.10±0.19 U/mL), SM+BB100 (9.86±0.62 U/mL), and SM+BBR200 (8.76±1.39 U/mL) variants were significantly higher compared to the SM100 variant (7.55±0.82 U/mL).

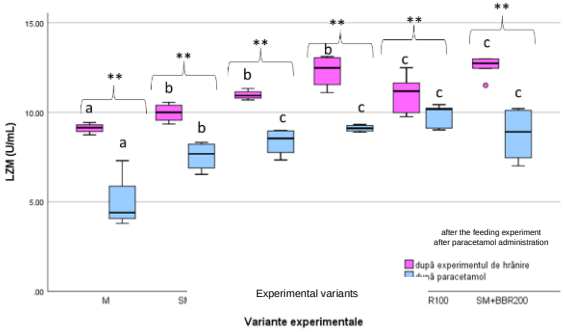

Figure 5.12. *LZM variation – box plot (median, minimum and maximum values, and quartiles)*

Values with different letters indicate significant differences (ANOVA, $p<0.05$) between the experimental variants. Values with * indicate nonsignificant differences (t-dependent, $p>0.05$) after the paracetamol test. Values with ** indicate significant differences after the paracetamol test (t-dependent, $p<0.05$).

Our results are comparable with previous studies that have demonstrated the administration of SM or BBR in fish feed can reduce the level of free radicals while increasing the activity of antioxidant enzymes, thereby contributing to the reduction of oxidative stress.

In conclusion, the administration of SM and BBR in carp feed has led to the improvement of the immune status in fish, demonstrating their hepatoprotective effect.

5.4. Conclusions

In recent years, liver lesions have a high incidence among aquatic organisms. Fish in aquaculture farms suffer from "hepatic syndrome," characterized by a significant enlargement of the liver (up to two to three times its initial size) and a change in color. The causes of these manifestations are unclear, and no pathogenic bacteria or viruses associated with hepatic syndrome have been identified.

The xenobiotic challenge induced by the presence of pharmaceutical compounds in the aquatic environment represents one of the most important pathological causes. Thus, hepatic lesions have often been reported in various fish species exposed to different xenobiotics. Despite the fact that cells can protect themselves against toxic compounds through mechanisms such as biotransformation of xenobiotics into water-soluble metabolites by phase I enzymes and the elimination of metabolites through conjugation to excretable molecules by phase II enzymes, an efficient method for treating hepatic syndrome has not been found. This is why, in recent years, research has been directed towards identifying nutritional strategies involving effective nutraceuticals to counteract the toxic effects of xenobiotics.

In line with the scientific community's aspirations, this study aimed to investigate the effect of supplementing feeds with various potential nutraceutical products on the health status of fish biomass maintained under controlled conditions, as well as on technological performance and the quality of aquaculture products.

The first stage, which lasted 64 days, focused on assessing the effects of feeding additives on growth performance, biochemical composition, maintenance status through the evaluation of somatic indices, hematological and serum parameters, as well as the main markers of oxidative stress. Following the 64-day experimental period, the subsequent experiment aimed to evaluate the effect of the feed additives on fish immunity after the administration of an overdose of paracetamol.

The results obtained from the experimental activity highlighted the following aspects:

- From the analysis of growth performance indicators, the best Feed Conversion Ratio (FCR) was recorded in the experimental variants where SM was administered, as well as BBR at a concentration of 200 mg/kg of feed. On the other hand, poorer nutrient utilization was observed in the BBR100 variants, as well as in the variants where a mixture of SM and BBR was administered at different concentrations;
- The analysis of somatic indices did not reveal significant differences, although a slight decrease in the Hepatosomatic Index (HSI) was observed in the variants where the feed was supplemented with the selected dietary additives. This suggests a reduction in fat accumulation or toxic substances at the liver level;
- The analysis of the meat's biochemical composition revealed a slight increase in protein content and a significant decrease ($p<0.05$) in lipid content in the variants where the dietary supplements were administered. This suggests the

potential for obtaining a superior quality product with lower lipid content but a higher protein source;

- The administration of SM and BBR demonstrated hepatoprotective effects, as reflected by the values of serum parameters, which were significantly improved in these experimental variants. The administration of BBR led to a reduction in serum cholesterol and triglycerides, a slight decrease in ALT levels, and an improvement in the HDL/LDL ratio.
- After the administration of a single dose of paracetamol, a reduction in hepatotoxicity was observed in the experimental variants where the selected additives were administered, as evidenced by the values of serum parameters. It was observed that after exposure to paracetamol, the levels of ALT, AST, and ALP were not significantly affected ($p>0.05$), while in the M variant, a deterioration of the obtained values was observed.
- The addition of SM and BBR to the diet of carp brood led to a decrease in the concentration of MDA and an increase in TAC values, highlighting the positive effect of these additives in combating oxidative stress. Therefore, the inclusion of these food additives in carp's diet can contribute to maintaining an adequate balance between oxidants and antioxidants, thus preventing the occurrence of oxidative stress.

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6. General Conclusions

Intensive aquaculture in recirculating aquaculture systems is characterized by the use of high stocking densities of fish species and intense feeding practices. However, this approach can lead to increased prevalence of stress and, consequently, an increase in the incidence of diseases within fish populations. Therefore, to prevent and treat diseases, as well as to promote growth and stimulate fish appetite, there has been a recent trend towards the use of a wide range of dietary supplements in fish feed.

The general objective of the doctoral thesis was to evaluate the influence of dietary supplements on aquatic organisms in the context of improving their health and assessing the enhancement of their resistance after exposure to antiparasitic treatments or medicinal substances.

Thus, based on the experimental results and the partial conclusions presented at the end of each chapter in the experimental part, a series of general conclusions can be highlighted, as follows:

From the first experiment "Research on the influence of krill oil on the growth performance of carp brood, the biochemical composition of the meat, and the technological comfort state", the following general conclusions can be drawn:

- ✓ *in our experiment, the addition of Krill oil to the feed led to an improvement in technological performance, even when the fish were stocked at a higher population density (8 kg/m3).*
- ✓ *the results obtained highlight that the levels of hepatic enzymes ALT, ATP, and AST were significantly lower in the groups that received a diet supplemented with KO compared to fish fed a normal diet, thus attenuating the density stress and hepatic toxicity of albendazole.*
- ✓ *albendazole should be used with caution as it can induce changes in hematological profile as well as oxidative stress in the liver.*
- ✓ *Krill oil enhances lipid metabolism and increases the capacity to eliminate free radicals.*

Therefore, Krill oil (KO) can be used to alleviate stress, especially in situations where fish are exposed to factors that disrupt homeostasis, such as high stocking densities or the administration of hepatotoxic drugs. Krill oil can be considered a complementary component in managing fish health in the aquaculture industry and can be used for the development of nutraceutical products. However, the exact mechanism by which the inclusion of Krill oil in fish diet exerts positive effects needs to be further investigated in future studies.

The results obtained from conducting the experimental activity of the second experiment "Research on the influence of silymarin and berberine on the growth performance of carp brood, the biochemical composition of meat, and technological comfort" have highlighted the following aspects:

- ➢ the administration of SM and BBR in the feed of carp brood resulted in superior growth performance, excepting the cases where the synergistic effect of these supplements was tested.
- ➢ the analysis of somatic indices demonstrated a reduction in liver fat accumulation in the experimental variants that included dietary supplements. At the same time, the analysis of meat composition revealed a slight increase in protein content and a significant decrease ($p<0.05$) in lipid content in the variants where dietary supplements were administered. These results suggest the possibility of obtaining a high-quality product with low lipid content but rich in protein.
- ➢ the results regarding the concentration of malondialdehyde (MDA) and total antioxidant capacity highlighted the effectiveness of dietary supplement administration in reducing oxidative stress, both after the feeding experiment and after the administration of the paracetamol dose.
- ➢ the results obtained regarding lysozyme activity indicate an acceleration of the phagocytic process, suggesting that the inclusion of SM and BBR in the diet of carp contributes to strengthening the immune status of the fish.

Based on the obtained results, the conclusion can be drawn that the administration of SM and BBR in the diet of carp brood leads to an improvement in growth performance, physiological status, as well as improvement in oxidative stress markers.

However, further studies could provide more information about the ideal dosages and the interaction between silimarin and berberine in the diet of carp brood. It is also important to carefully monitor the response of fish to these supplements and adjust the dosages based on the obtained results. Prolonging the administration period of the dietary supplements would allow for a more in-depth observation of their effects on the health of aquatic organisms.

7. Personal Contributions and Perspectives Regarding Further Research

Intensive fish farming in recirculating aquaculture systems involves high stocking densities and large inputs of feed, which can lead to stress and the development of severe pathologies among the fish population.

The objective of this doctoral thesis was to identify alternative solutions for preventing pathologies associated with stress caused by intensive growth, by improving the immune system of fish. Additionally, our research aimed to evaluate the influence of dietary supplements on growth performance and the nutritional quality of the biological material (*Carp - Cyprinus carpio*, Linnaeus 1758).

Our innovative research on supplementing the diet of carp brood with krill oil, silimarin, and berberine has yielded relevant conclusions and highlighted the benefits of these dietary supplements in achieving superior growth performance, combating high-density stress, enhancing immune function, and improving liver function even under the influence of antiparasitic treatments or pharmaceutical pollutants. This research provides an original contribution to the field of aquaculture research. The use of krill oil in carp feed, as well as the unique combination of dietary supplements (silimarin + berberine), has not been previously investigated by other researchers. This demonstrates its promising potential in improving the growth and health of carp brood. The obtained results will contribute to the development of knowledge in the field and provide valuable information for optimizing sustainable nutritional strategies in the aquaculture sector. The goal is to optimize fish growth, health, and the efficiency of recirculating aquaculture systems.

Based on the results obtained in the doctoral thesis, new research directions emerge for further investigating the impact of dietary supplements on aquatic organisms, including:

- Optimization of dietary supplement dosages: it is necessary to explore a wide range of optimal dosages for krill oil, silimarin, and berberine to achieve the best results in terms of fish growth and physiological state. Further studies can explore the synergy between these dietary supplements and identify the optimal combinations and concentrations.
- Evaluation of long-term effects of supplements: to gain a comprehensive understanding of the influence of dietary supplements on aquatic organisms, it is important to conduct long-term studies, tracking the effects as fish grow and develop. This will enable the evaluation of long-term effects on growth, health, and immune system.
- Assessment of mechanisms of action in dietary supplements: future research can explore the mechanisms by which dietary supplements influence aquatic organisms. This may involve molecular, biochemical, and immunological

studies to better understand how these supplements interact with the organism and determine the observed effects.

8. Dissemination of the Research Results

A. Articles published in ISI-rated journals

1. Năstac, L., Dediu, L., Crețu, M., Rîmniceanu, C., Docan, A., Grecu, I., Vizireanu, C. (2023). The Protective Effects of Korill Product on Carp FingerlingsReared in HighDensitiesandChallengedwithAlbendazoleTreatment. *Fishes*, 8(3), 153. Factor de impact 3,17.

C. Articles published in BDI-rated journals

1. Năstac, L. G., Crețu, M., Dediu, L., Docan, A. I., Rîmniceanu, C., & Vizireanu, C. (2023). Effect of KrillOilSupplementationandStockingDensity on Growth Performance, ProximateComposition, andOrgano-somatic Indices of *Cyprinuscarpio. European Journal of BiologyandBiotechnology*, 4(1), 1-6.

B. Papers communicated at international scientific events

1. Năstac, L., Crețu, M., Docan, A., Grecu, I., Dediu, L., Vizireanu, C. (2023). *Effects of dietaryberberine, silymarinandtheirassociation on lipidandglucose metabolism in common carp Cyprinuscarpio,* SCDS-UDJG 2023 The EleventhEdition of theScientificConference of the Doctoral Schools of Dunărea de Jos, University, 8th-9th of June 2023.
2. Năstac, L.,Crețu, M., Docan, A., Dediu, L., Vizireanu, C., (2022). *Effect of Albendazole on the oxidative stres markersandhematologicalparameters in tissues of Cyprniuscarpio,* SCDS-UDJG 2022 The Ten Edition of theScientificConference of the Doctoral Schools of Dunărea de Jos, University, 9-10 June, 2022.
3. Năstac, L., Crețu, M., Constatin, O., Docan, A., Dediu, L.,Vizireanu, C (2021). *Krilloildietprotectsagainstdensityinduced oxidative stress in Cyprinuscarpiofingerlingsreared in a recirculatingaquaculturesystem*. Nine Edition of theScientificConference of the Doctoral Schools of Dunărea de Jos, University, 10-11 June 2021.
4. Năstac, L., Crețu, M., Istrati, D., Grecu, I., Dediu, L., Vizireanu, C. (2021). *Evaluatinghematological status of fishreared in recirculatingaquaculturesystemsaftersupplementingtheirdietwithkrilloil.* Nine Edition of theScientificConference of the Doctoral Schools of Dunărea de Jos, University, 10-11 June 2021.