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EFFECTS OF MULTI-STRAIN PROBIOTICS ON THE GROWTH AND HEMATOLOGICAL PROFILE IN JUVENILE CARP (*CYPRINUS CARPIO*, LINNAEUS 1758)

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ABSTRACT

The potential individual probiotic microorganisms to act synergistically or as an additive when mixed present a great promise for future use in the treatment of various diseases in aquaculture. Besides inhibiting pathogens and improving the immune response, multi-strain probiotics also assist in promoting the growth of the host. The experiment was performed in independent breeding units such as recirculating systems that allowed the comparative evaluation with control of the action of the three commercial probiotics with applicability in human and zootechnical consumption being tested for use as feed bio additive to grow carp juveniles. This study involved assessing the mode of action of these multiple strains of probiotics on the evolution of the nonspecific immune response as a tool to highlight their effect being used in equal concentrations of 3.2 $\times 10^9$ CFU/kg feed, the following commercial products: *BioPlus® 2B* (mixture of *Bacillus licheniformis and Bacillus subtilis in a ratio of 1: 1*), *BetaPlus®* (mixture of *Bacillus licheniformis and Bacillus subtilis* with betaine) and *Lactobact Premium* (mixture of *Bifidobacterium bifidum, Bifidobacterium breve, Bifidobacterium lactis, Lactobacillus casei, Lactobacillus paracasei, Lactobacillus plantarum, Lactobacillus rhamnosus, Streptococcus thermophilus* - lactic acid bacteria - LAB). The study aims were to evaluate the effect of multi-strains probiotics with *Bacillus* and *LAB* on the growth performance and hematological profile of juvenile carp. In conclusion, the use of multi-strain probiotics, could use a positive effect on growth performance and improved some hematological of juvenile carp.

1. Introduction

In the last two decades, there has been a massive expansion of research and the use of probiotics in aquaculture. Recent results have shown that groups of about 20 bacterial genera have been recognized as potential probiotic candidates, and most species with probiotic potential belong to the genus *Bacillus spp.* and the group of lactic acid bacteria (LAB) (Knipe et al., 2020).

Recently, the probiotic microorganisms most commonly used in aquaculture belong to *Bacillus spp.*, *Lactobacillus spp.*, *Bifidobacterium spp.*, *Enterococcus spp.*, *Streptomyces spp.*, *Carnobacterium spp*. and yeast (Van Doan et al., 2019).

Multi-strain probiotics had been recommended as a necessity for the shared use of these microorganisms to allow development in favorable conditions, such as those in the gastrointestinal tract (GIT), and dominate the specific resident microbiota. Therefore, it has also been suggested that a multispecies probiotic would be more successful than a monospecies supplement Kumar et al. 2016; Zorriehzahra et al. 2016;

Feckaninova et al. 2017; Ringø et al. 2018; Dawood et al. 2019; Ringø et al. 2019; Soltani et al. 2019; Wang et al. 2019; Kuebutornye et al. 2019b; Melo-Bolivar et al. 2020). According to previous research, multi-strain probiotics (MSP) are confirmed to be more beneficial to the host organism than the use of a single probiotic in certain aspects. Multi-strain probiotics may also be called mixed probiotic mixtures or combinations of probiotics that consist of a mixture of two or more strains or species of bacteria that have been previously demonstrated to provide various benefits for the host. Also, a combination of different species different gen, or strains of the same genus could be called probiotics with several species. The effectiveness of one probiotic varies depending on the type of host to which it is applied, and multi-strain probiotics can be used to increase their influence (Wang et al., 2019).

Probiotics, living microorganisms, confer health benefits to the host by improving the intestinal microbial homeostasis and nutrient digestion, regulating immunity, and suppressing the pathogens infection it can reduce the use of antibiotics and has received more and more attention because of its high abundance, low cost and convenient application in the aquaculture industry (Sharifuzzaman and Austin, 2017; Wang et al., 2019).

A single administration of *Bacillus velezensis*, *Bacillus cereus,* and *Lactobacillus casei* has been found to confer immunomodulatory effects and improve host health (Safari et al., 2017; Wang et al., 2019a; Yang et al., 2019). However, to the knowledge of the authors, they were not used by incorporated into the growth of any animal species still. In addition, multi-strain probiotic (MP) is much more effective in enhancing the growth and immunity of aquatic animals (Salinas et al., 2008; Wang and Xu, 2006; Wang et al., 2019b).

Probiotics have been used as an integral part of aquaculture for a long time to grow crop species. Probiotics are also considered beneficial in disease control and improving water quality in aquaculture (Aslam Hosain and Liangyi, 2020). Probiotics administered to fish can be divided into the large dominant group of $Gram^+$ bacteria such as *Lactobacillus species* (LAB), *Bacillus*, and *Bifidobacterium* and the group of $Gram^-$ bacteria (several strains of *Aeromonas, Vibrio, Pseudomonas* and *Enterobacteriaceae*). Among the strains of probiotic bacteria applied in aquaculture *Bacillus spp., Lactobacillus spp.,* and *Streptococcus spp.* are used more widely, while biomedicine contains colonies of strains of *Lactobacillus spp., Bifidobacterium spp.,* and *Sterptococcus spp*. as a mixture of feed probiotics.

Feeding aquatic organisms with acceptable amounts of probiotics incorporated in the administered feed modified the intestinal microflora by replacing pathogens with microorganisms beneficial. In addition, they could promote enzyme digestion, improve the immune system response, and growth promotion (Wang et al. 2002; Hoseinifar et al. 2017; Sookchaiyaporn et al. 2020; Doan et al. 2020).

The direct incorporation of the probiotic in granulated feed is one of the most important and applicable methods of their administration in feeding. Probiotics are applied directly in this form of spores in feed pellets (Assefa and Abunna, 2018).

According to Melo-Bolivar et al. (2021), to obtain optimal efficiency of probiotics in aquaculture fish it is necessary to determine the correct dose, the time of administration, and the stage of development of the fish during administration, and the method of administration.

Most probiotic mixtures have been tested only once, which could make it difficult to

determine the beneficial effects that could be replicated in other studies (Melo-Bolvar et al., 2021). Only three mixtures of probiotics were replicated in other articles, namely*: B. subtilis* and *B. licheniformis (Bio-Plus 2B*; Chr. Hansen A/S; Merrifield et al. 2010a; Merrifield et al.2010b); *B. subtilis, B. licheniformis* (*BioPlus 2B*; Chr. Hansen A/S) and *E. faecium* (*Lactosan* GmbH & Co. KG; Merrifield et al.2010a; Merrifield et al. 2010b); *and Bacillus sp., Enterococcus sp., Pediococcus sp*. and *Lactobacillus sp*. (*AquaStar, Biomin* GmbH; Ramos et al. 2013; Ramos et al. 2015).

2. Materials and methods

2.1. Materials

2.2.1. Experimental design the fish rearing.

To determine the effect of multi-strains probiotics, the units of the growth were populated with an equal number of 10 specimens, being comparable biomass, so that there is the possibility of comparison between the four experimental variants. The breeding system was populated with 4.65 kg of juvenile carp with an average individual weight of 38.82 g/specimen (*Cyprinus carpio*) aged three months, from the Brateş farm, Institute for Research and Development in Aquatic Ecology, Fishing, and Aquaculture from Galați. The experimental variants and multi-strains of commercial probiotics that were used in this study to test their effect on the physiological state and growth performance of carp juveniles are the following:

I. Control variant (V1) - batch fed with granulated feed treated only with the probiotic fixing binder (but without probiotic);
II. *BioPlus® 2B* probiotic variant (V2) - added in a concentration of 3.2×10^9 UFC/kg feed; *BioPlus® 2B* (veterinary use), is represented by a mixture of *Bacillus licheniformis* (DSM 5749) and *Bacillus subtilis* (DSM 5750) in a ratio of 1: 1;
III. *BetaPlus®* probiotic variant (V3) - added in a concentration of 3.2×10^9 CFU/kg feed; *BetaPlus®* (veterinary use), consists of *BioPlus® 2B* (*Bacillus licheniformis* (DSM 5749) and *Bacillus subtilis* (DSM 5750) in a ratio of 1: 1) and betaine (nitrogenous substance);
IV. *Lactobact premium* probiotic variant (V4) - added in a concentration of 3.2×10^9 CFU/kg feed; *Lactobact Premium* (human use) is a mixture of equal proportions of *Bifidobacterium bifidum, Bifidobacterium breve, Bifidobacterium lactis, Lactobacillus casei, Lactobacillus paracasei, Lactobacillus plantarum, Lactobacillus rhamnosus, Streptococcus thermophilus* (the product does not contain lactose, gluten, and yeast).

The *BioPlus® 2B* and *BetaPlus®* probiotics used in this experiment came from the Biochem company in Lohne, Germany, through the Romanian subsidiary Biochem Animal Health and Nutrition affiliated with the one in Lohne and located in Cluj-Napoca.

Preparation of experimental diets. In this experiment, the ratio used was 3.5% of body weight (BW). The total amount of feed calculated for one day was administered in five meals that were distributed manually every two hours. The fish were fed *CLASSIC EXTRA 1P* feed - 2.5 mm pellets with a protein content of 41%. The composition of *CLASSIC EXTRA 1P* feed includes the following ingredients: fishmeal, fish oil, hemoglobin, soybean oil, wheat gluten, sunflower flour, wheat and wheat products, BTH.

The working protocol for the incorporation/addition of feed with probiotic products was updated according to the ratio administered during the period of the experiment.

This protocol goes through the following steps:

a. Dissolve the probiotic in 4 (6.8) ml distilled water;

b. Stirring the solution for 10 minutes;

c. Preparation of 2% gelatin solution on a water bath;

d. Cooling the gelatin solution to 30 ^{0}C;

e. Mixing probiotics and gelatin solutions in a ratio of 2: 1 (4 (6.8) ml probiotic solution: 2 (3.4) ml gelatin solution/variant);

f. Spraying the final solution on the surface of the feed granules by continuous stirring;

g. Drying in oven T^0 = 20 ^{0}C, for 12 hours.

The study falls into the category of experimental investigations, conducted in Pilot Research Stations of the Department of Food Science, Food Engineering, Biotechnology and Aquaculture, Faculty of Food Science and Engineering, University "Dunărea de jos" of Galati. From a constructive point of view, the growth system in which the experiment took place consists of the following parts: growth units - represented by four aquariums made of glass with a thickness of 10 mm (figure 1). Each aquarium has the dimensions of 40 × 50 × 100 cm and was divided into three enclosures equal to the dimensions of 40 × 50 × 32 cm and a volume of 45 l/subvariant (figure 2).

Figure 1. Growth system units intensive for testing multi-strain probiotics.

Figure 2. Compartmentation of growth units in three enclosures/rehearsals.

Water quality recirculation and conditioning equipment at the level of each subvariant was represented by *Tetratec EX 400* filters (figure 3). Physico-chemical parameters represented by dissolved oxygen, temperature, and pH were monitored with the help of the portable instrument *HANNA* instruments type HI 7100042. Also, nitrogen compounds ($N\text{-}NO^-_2$, $N\text{-}NO^-_3$, $N\text{-}NH^+_4$) were determined with the Spectroquant Nova 400 spectrophotometer using Merk compatible kits.

Figure 3. Water quality recirculation and conditioning equipment.

2.2. Methods

2.2.1. Growth performance and conversion ratio.

At the end of the experiment, after the fish were weighed and measured, the following parameters were calculated: weight gain, feed conversion factor, specific growth rate, and efficiency of protein utilization using the following equations:

a) Weight gain (WG) = Final weight (Wt) – Initial weight (W0) (g);
b) Food conversion ratio (FCR) = Total feed (F)/Total weight gain (W) (g/g);
c) Specific growth rate (SGR) = 100 x (ln Wt – ln W0) / t (% BW/day);
d) Protein efficiency ratio (PER) = Total weight gain (W)/amount of protein fed.

2.2.2. Microbiological analyzes.

Sampling for microbiological analysis was performed from the water of the breeding units and from the feed to verify the viability of multi-strains probiotics. The protocol used for the cultivation and determination number of germs is described in the paper "Bacteria from Fish and Other Aquatic Animals - A Practical Identification Manual" By Nicky B. Buller 2009.

2.2.3. Blood sample and hematological analysis.

The collection of blood samples for hematological determinations was performed at the end of the experimental period to identify the changes between the control variant (control) and the variants with different probiotics. For an accurate assessment of the hematological indicators, blood samples were taken from 6 fish/growth units (representing 85% of the biomass) totaling a total of 72 blood samples.

Before blood sampling, fish were anesthetized with 2-phenoxyethanol in order to reduce handling stress. Research has shown that 2-phenoxyethanol anesthetic had no effect on the hematological profile (Velíšek et al., 2007). Blood analysis was performed by a method used in fish hematology described by Bocioc et al., 2015. This analysis consisted of the determination of red blood cells count - RBCc ($\times 10^6/\mu l$), hemoglobin - Hb (g/dl), and hematocrit – PVC (%). For the determination of erythrocyte, the number was used the Neubauer hemocytometer. The hematocrit was performed by duplicate using capillary tubes centrifugated for 5 minutes at 12000 rpm in a microhematocrit centrifuge. The hemoglobin concentrations were measured spectrophotometrically with SPECORD 210 Analytikjena at λ-540 nm, using Drabkin reagent. Then, using standard formulas described by Svobodova, (2001) were calculated the erythrocyte constants: mean corpuscular volume - MCV (μm^3), mean corpuscular hemoglobin - MCH (pg), mean corpuscular hemoglobin concentration - MCHC (g/dl).

The relative and an absolute number of leukocytes were obtained by microscopic examination of 200 leukocytes on blood smears (two per fish), using Zeiss Axio Imager microscope and immersion objective (10 oc. X 100 ob.). The absolute number of circulating blood leukocytes and thrombocytes was determined in comparison with 1000 erythrocytes counted on a hemocytometer, per blood volume unit. The blood smears were colored with May-Grünwald Giemsa panoptic method (MGG) and the type of leukocytes was determined based on identification characters listed by Svobodova et al. (1991).

2.2.4. Statistical analysis.

Data were analyzed by the one-way analysis of variance (ANOVA). All statistical analysis of data was performed using SPSS for Windows version 20.0 (SPSS Inc., Chicago, IL, USA) and the program PRIMER 7.

3. Results and discussions

3.1. Growth performance

One of the objectives of this experiment was to evaluate the effect of multi-strains of the three commercial probiotic products on the technological performance of juvenile carp. For this purpose, the rearing system was populated with 4.65 kg of juvenile carp and an average individual weight of 38.82 g/fish. The four rearing units corresponding to the experimental variants, each with three repetitions, were randomly populated with many ten fish/repetition. The initial and final values of the culture biomass from each experimental variant are presented in table 1. The statistical analysis of the weight data at the end of the experimental period showed a uniformity at the point of the average weights of the fish at the level of each variant (the averages of the three replicas did not differ statistically).

Regarding the results obtained at the end of the growth period in Table 1, a significant variation was observed between the experimental variants ($p<0.05$), where the data analysis showed a difference between the control variant (V1) and the *BioPlus®2B* probiotic variant (V2) also confirmed by Duncan test which revealed the existence of 2

homogeneous subsets. In figure 4 highlights the distribution of the averages of the individual weights from the four experimental variants.

Table 2 summarizes the technological performance indicators in the four experimental variants. The data obtained are closely related to the quality of the culture medium and the living space provided by the growing units. The physicochemical parameters of the water were monitored and maintained within the optimal limits for growth by changing the water volume of 50%.

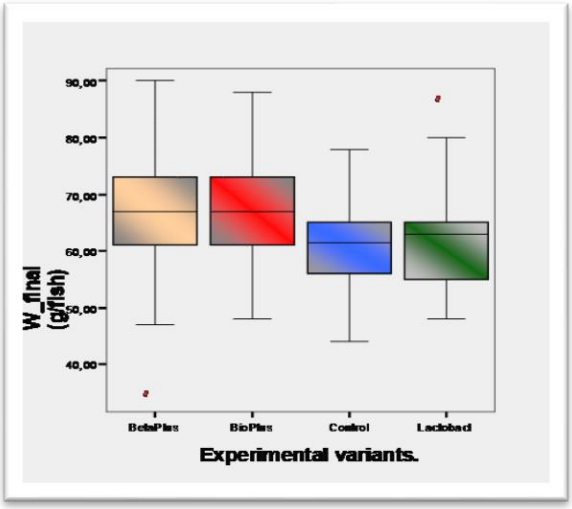

Figure 4. Individual average weight distribution/variant at the end of the experimental period.

Table 1. Variation of the average individual weights at the level of replicas/variants in the experimental period.

Experimental variant	The biometric parameter	Replication	Mean	Stdev.
V1 (Control)	W_initial (g/fish)	1	37.80	5.92
		2	38.30	4.16
		3	40.30	7.32
	W_final (g/fish)	1	62.00	6.23
		2	59.90	9.74
		3	61.70	5.80
V2 (*BioPlus® 2B*)	W_initial (g/fish)	1	40.00	6.93
		2	40.10	4.86
		3	37.50	7.85
	W_final (g/fish)	1	67.90	8.75
		2	69.80	6.68
		3	64.00	11.90
V3 (*BetaPlus®*)	W_initial (g/fish)	1	38.90	8.29
		2	38.00	6.99
		3	39.10	6.74
	W_final (g/fish)	1	67.10	11.48
		2	66.30	9.66
		3	65.20	12.54
V4 (*Lactobact premium*)	W_initial (g/fish)	1	38.70	6.46
		2	38.00	4.92
		3	39.10	6.23
	W_final (g/fish)	1	63.20	9.13
		2	62.20	9.99
		3	62.40	8.64

Table 2. The technological performance indicators of carp treated with multi-strains of probiotics.

Experimental variants	**Control (V1)**				**BioPlus® 2B (V2)**				**BetaPlus® (V3)**				**Lactobact premium (V4)**			
Indicator/ growth unit	**R_1**	**R_2**	**R_3**	**Unit 1**	**R_1**	**R_2**	**R_3**	**Unit 2**	**R_1**	**R_2**	**R_3**	**Unit 3**	**R_1**	**R_2**	**R_3**	**Unit 4**
Probiotic concentration (CFU/kg feed)	0.00	0.00	0.00	0.00	3.2×10^9	3.2×10^9	3.2×10^9	0.00	3.2×10^9	3.2×10^9	3.2×10^9	0.00	3.2×10^9	3.2×10^9	3.2×10^9	0.00
Survival rate (%)	100	100	100	100.00	100	100	100	100.00	100	100	100	100.00	100	100	100	100.00
Initial Biomass(g)	266	275	276	817.00	288.00	288.00	266.00	842.00	282.00	278.00	274.00	834.00	271.00	260.00	272.00	804.00
Final Biomass (g)	448	418	433	1299.00	481.00	492.00	438.00	1411.00	478.00	461.00	451.00	1390.00	443.00	434.00	435.00	1312.0
Biomass gain (g)	182	143	157	482.00	193.00	204.00	172.00	569.00	196.00	183.00	177.00	556.00	171.00	174.00	163.00	508.00
Mean initial weight (g/fish)	38.00	39.00	39.00	38.66	41.00	41.00	38.00	40.00	40.00	40.00	39.00	39.66	39.00	37.00	39.00	38.33
Mean final weight (g/fish)	64.00	60.00	62.00	62.00	69.00	70.00	63.00	67.33	68.00	66.00	63.00	65.66	63.00	62.00	62.00	62.33
Individual weight gain (g)	26.00	20.00	22.00	22.66	28.00	29.00	25.00	27.33	28.00	26.00	25.00	26.33	24.00	25.00	23.00	24.00
Daily growth rate (g/day)	5.20	4.09	4.49	4.59	5.51	5.83	4.91	5.41	5.60	5.23	5.06	5.29	4.89	4.97	4.66	4.84
Specific growth rate (%/day)	1.49	1.20	1.29	1.32	1.47	1.53	1.42	1.47	1.51	1.45	1.42	1.46	1.39	1.46	1.34	1.39
Feed conversion ratio (g feed/g biomass gain)	1.74	2.22	2.02	1.99	1.64	1.55	1.84	1.67	1.62	1.73	1.79	1.71	1.85	1.82	1.94	1.87
Protein efficiency ratio (g/g)	1.40	1.10	1.21	1.23	1.48	1.57	1.32	1.45	1.51	1.41	1.36	1.42	1.32	1.34	1.25	1.30

The most significant technological indicators are the specific growth rate (SGR) and the feed conversion ratio (FCR). These indicators recorded the best values in the experimental variant *BioPlus® 2B* (V2). In the control variant (V1), an average value of SGR of 1.32 g%/day and an FCR of 1.99 g feed/g growth increase was obtained, while variant *BioPlus® 2B* (V2) recorded an SGR value of 1.47 g%/day and an FCR of 1.67 g feed/g growth increase. In figure 5 we can see the inverse correlation established between the evolution of SGR and FCR. Also, the feed conversion factor (FCR) varied inversely with the protein efficiency coefficient (PER) in all experimental variants. From the analysis of the variants treated with probiotics, it was found that the best PER coefficient was registered for the basins of the *BioPlus® 2B* variant where it varied between 1.32-1.57 g/g, while the treatment with *Lactobact premium* led to lower values 1.25-1.34 g/g (average 1.27).

Figure 5. Variation in specific growth rate (SGR) and feed conversion ratio (FCR) between experimental variants.

The authors, Yanbo and Zirong (2006) found that *Bacillus spp.* used alone or in combination with an unidentified photosynthetic bacterium has improved the growth performance of juvenile common carp. Mukherjee et al. (2019) demonstrated that a mixture of *Bacillus methylotrophics* and *Bacillus licheniformis* compared to single strains contributed to disease resistance against *Aeromonas hydrophila*, immune response, and rohu carp growth performance. In the present study, juvenile carp, *Cyprinus carpio* fed diets containing supplementation with multi-strains probiotics of *Bacillus* and *LAB* had better average weight gain compared to the control diet with fed fish. Supplementing the fodder diet with multi-strains of probiotics consisting of *Bacillus* and *LAB* can improve the growth performance of juvenile carp. Compared with our study, previous studies on fish such as canal catfish (Thurlow et al., 2019), Pengze crucian carp (Cao et al., 2019; Yang et al., 2019), grass carp (Tang et al., 2019), carp fingerlings (Amit et al., 2021) also demonstrated the positive effect of *B. cereus*, *B. subtilis* and *L. plantarum* on growth performance.

3.2. Evaluation of microbiological parameters.

During this experiment, microbiological testing was performed by determining the total number of germs in the water, and analyzing the evolution of its quality. The test was performed at an interval of 8 hours after receiving the water and even before replacing the volume of water (2 samples), at an interval of one week to have a picture of the diurnal evolution of the total number of germs in the water culture. The dynamics of the microbial load of water are shown in figure 6 highlighting, as expected, the depreciation quality of water from a microbiological point of view with the accumulation of catabolism products in pool water.

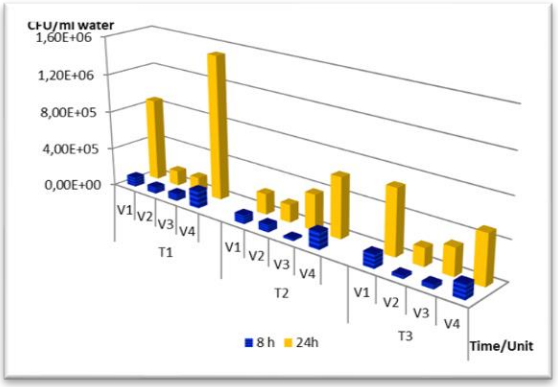

Figure 6. Dynamics of the total number of germs in the water of the growth units.

The maximum value determined was 1.5 x10^6 CFU/ml which indicates very impure water in all experimental variants but a significantly lower number was observed in variants V2 and V3 in which the feed was administered with *Bacillus* (10^4, 10^5 CFU/ml). The highest values were recorded in the growth unit of the variant treated with lactic acid bacteria (V4).

The verification of the existence and viability of the multiple strains of probiotics incorporated in the fodder administered to the carp juvenile during the experimental period was performed by performing the sowing on a nutritious and selective culture medium. Following the cultivation of these species of probiotic bacteria, their existence and viability in embedded feed were observed (figure 7).

Figure 7. Multi-strains probiotics in feed (a - *Bacillus subtilis, Bacillus licheniformis;* b – lactic acid bacteria).

3.3. Evaluation of hematological indicators

Blood indicators are significant tools that show us the response to physiological stress and the health of fish to nutritional and environmental changes (Kader et al., 2012). Knowledge and research of hematological parameters can facilitate the development of indicators of the health status of fish in response to changes related to nutrition, water quality, and disease.

The hematological indicators studied were hemoglobin, hematocrit, and red blood cell count. Red blood cell indices including mean corpuscular hemoglobin (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were calculated and compared among the groups.

3.1.1. Hemoglobin (Hb)

The mean hemoglobin values recorded in the four experimental variants are presented in figure 8 and it can be seen that it falls within the normal values (6.5-10.6 g/dL by Bocioc et al., 2015) of the species *Cyprinus carpio*, without significant differences between the variants with multi-strains probiotics and control. As you can see, at the end experimental period, hemoglobin (Hb) ranged from 9.59 g/dL in the control variant (V1) case to 12.85 g/dL for Lactobact variant (V4).

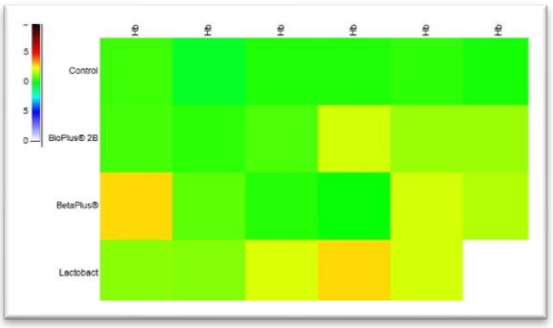

Figure 8. Matrix of mean hemoglobin values.

3.1.2. Hematocrit (PVC)

Hematocrit is the ratio of the volume of red blood cells to the total volume of blood that can be affected by the number of cells. In experimental variants with multi-strains probiotics, there was an increase in hematocrit compared to the control variant (from 29.00 % in a control group to 37.00 % in variants *BioPlus® 2B* and *Lactobact*) in general, all batches with values within the normal range (32-43.8% by Bocioc et al., 2015) for carp (figure 9).

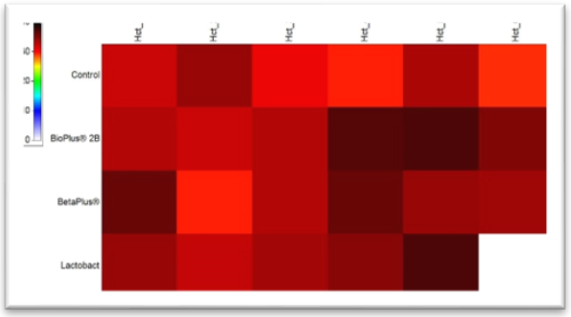

Figure 9. Matrix of mean hematocrit values.

3.1.3. Red blood cells count (RBCc)

The red blood cells count in the experimental period registered an increasing evolution in the carp specimens treated with multi-strain probiotics, the average values indicating an increase from 1.37×10^6/µL in the control variant to 1.65×10^6/µL in the *BetaPlus* variant and 1.77×10^6/µL in the variant treated with LAB (Lactobact). Statistical analysis revealed a significant difference between the control groups and *Lactobact* variant (figure 10), and the values recorded fall within the normal range of *Cyprinus carpio* ($1.10\text{-}2.20\times10^6$/µL by Bocioc et al., 2015). This evolution indicates stimulation of erythropoiesis in the variants treated with multi-strain probiotics implying positive consequences on the transport of oxygen to the tissues.

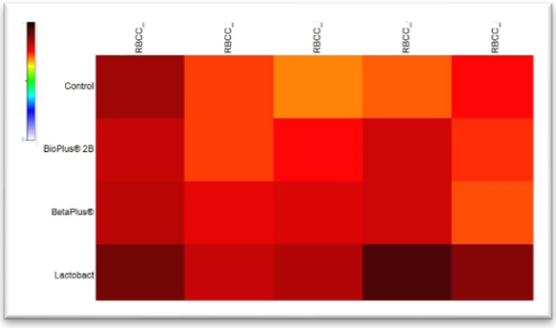

Figure 10. Matrix of mean red blood cells count values.

After determining the hematological indices, erythrocyte constants of juvenile carp were calculated which helps to detect physiological lesions in the process of hemoglobin formation, and provides information about the size, shape, and hemoglobin charge of the erythrocyte.

3.1.4. Mean corpuscular volume (MCV)

The mean corpuscular volume was maintained in the range of normal values for *Cyprinus carpio* species (152-364 µm^3 by Bocioc et al., 2015) observing a statistically insignificant difference between the groups treated with multi-strains probiotics and the control group. In the variant with *BioPlus® 2B*, there was a slight increase at 255.70 µm^3 compared to the control (190.12 µm^3). This indicates that the volume occupied by a single erythrocyte is higher in the group treated with the probiotic based on *Bacillus* compared to the control group (figure 11).

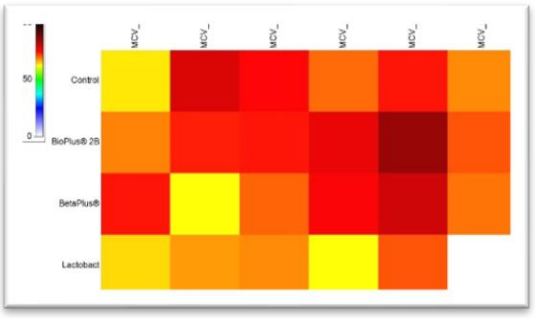

Figure 11. Matrix of mean corpuscular volume values.

3.1.5. Mean corpuscular hemoglobin (MCH)

The mean corpuscular hemoglobin registers average values that do not fall within the normal range of variation (50-63 pg by Bocioc et al., 2015) which indicates an increase in the variants fed with probiotics containing *Bacillus* species of 83.97 pg compared to 62.64 pg in the control variant. Given that hemoglobin is the major component of erythrocytes (95% of erythrocyte cytoplasmic proteins) and that MCV is higher in *Bacillus* variants (figure 12), this suggests that the adaptive response consists in stimulating the function of hemoglobin synthesis in these carp specimens.

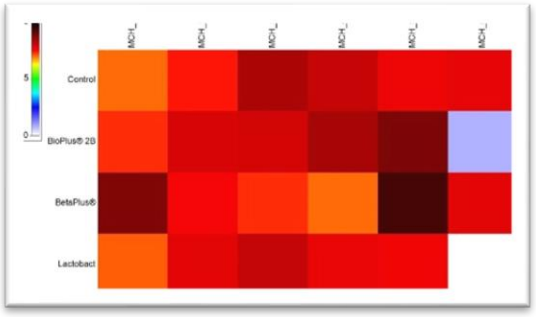

Figure 12. Matrix of mean corpuscular hemoglobin values.

3.1.6. Mean corpuscular hemoglobin concentration (MCHC)

The mean corpuscular hemoglobin concentration that measures the average Hb concentration in a given volume of erythrocytes indicates an increased response in all experimental variants compared to the normal range (15-25 g/dL by Bocioc et al., 2015), the control group recording values less than 28.19 g/dL compared to the *Lactobact* variant where 37.24 g/dL was recorded (figure 13).

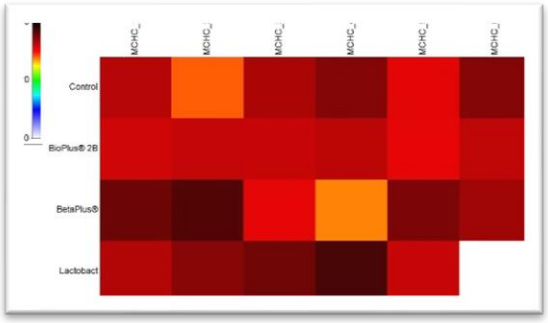

Figure 13. Matrix of mean corpuscular hemoglobin concentration values.

According to our results, the use of multiple probiotic strains determined a positive effect on hematopoiesis because red blood cell count and hematocrit were higher in the experimental groups with *Bacillus* and *LAB* compared to the control group.

The ecophysiological response of carp chicks to adaptation to new supplements of multiple strains of probiotics has consisted of some quantitative or qualitative changes in blood characteristics that suggest positive stimulation of metabolic health and immune response, especially nonspecific cellular. Similarly, feeding common carp (*Cyprinus carpio*) with a mix of *Bacillus subtilis* and *Bacillus licheniformis* could increase serum immunoglobulin levels and some immune parameters (Wang et al., 2017). Normal reference ranges of hematologic indicators are considered significant for assessing and monitoring the health status of fishes. In recent years more normal reference ranges of hematologic parameters of cultured and wild fish have been established (Witeska et al., 2016; Bocioc et al., 2015, Fazio et al., 2012b; Fazio et al., 2013a, 2013b)

3.1.7. Evaluation of leukocyte reactions in carp juveniles

A study of erythrocyte and leukocyte series on panoptical stained blood smears by the May-Grunwald Giemsa (MGG) method did not show any qualitative changes or morphological aspects in terms of their shape, size, or color. A comparative leukogram of the averages of the carp from the variants experimental is shown in table 3 and the variation in the absolute number of leukocytes in Table 4. Total WBC Count indicates a significant increase in leukocytes compared to other white blood cell types in the experimental variants with multi-strains probiotics. Lymphocytes contribute significantly to the total white blood cells. Usually, lymphocytes are responsible for the immune response. An increase in lymphocytes triggers the production of antibodies (Volbana et al., 2018). Granulocytes in fish blood are usually neutrophils. Reducing the number of granulocytes in the blood may be associated with increased disease resistance (Venkatalakshmi et al., 2015).

The interaction between probiotics and the host's immune system depends on several aspects, namely: source, type, strain, and species of probiotics. Therefore, there is the probability that when a probiotic strain was singularly supplemented in feeding to a particular host, may not have a positive effect on the host's

immune system. On the contrary, the combination of different genera and species of probiotics in a multi-strain probiotic can act synergistically and improve the host's immune response (Nayak, 2021).

Abdulrahman and Ahmed (2015) reported that white blood cells and lymphocyte levels were improved by supplementing diets with synbiotics for common carp. Similarly, the effects of different IMBO symbiotic concentrations, such as *Enterococcus faecium* (as a probiotic) and FOS (as a prebiotic) on the survival, growth performance, and digestive enzyme activities of the common carp juvenile (Dehaghani et al ., 2015).

Table 3. Variation of carp leukocyte formula at the end experimental period.

Leukogram (%)		**V1**	**V2**	**V3**	**V4**
Lymphocytes	Min	91.00	94.50	91.50	89.50
	Max	98.50	99.50	99.00	99.00
	X±SD	95.14±1.94	94.59±1.45	96.38±2.05	97.13±2.27
Monocytes	Min	0.00	0.00	0.00	0.00
	Max	3.00	0.50	0.50	1.00
	X±SD	0.47±0.75	0.013±0.12	0.15±0.23	0.13±0.29
Neutrophils	Min	1.50	0.50	1.00	1.00
	Max	6.50	4.50	8.50	10.00
	X±SD	4.23±1.64	2.16±1.13	3.44±1.99	2.63±2.17
Eosinophils	Min	0.00	0.00	0.00	0.00
	Max	1.00	2.50	0.50	1.50
	X±SD	0.15±0.34	0.22±0.65	0.02±0.12	0.10±0.38

Table 4. Variation in the absolute number of leukocytes at the end experimental period.

		V1	**V2**	**V3**	**V4**
Leukocyte ($x10^3$cell/µL)	Min	46.02	50.65	59.78	77.87
	Max	96.50	117.82	147.83	147.55
	X±SD	63.55±14.05	85.90±17.23	102.93±24.47	99.41±19.93
Lymphocytes ($x10^3$cell/µL)	Min	43.26	50.14	57.69	70.73
	Max	92.64	113.69	139.70	144.60
	X±SD	60.54±13.77	83.78±16.68	99.12±23.27	96.68±20.08
Monocytes ($x10^3$cell/µL)	Min	0.00	0.00	0.00	0.00
	Max	1.47	0.48	0.74	0.91
	X±SD	0.26±0.38	0.03±0.12	0.15±0.27	0.11±0.26
Neutrophils ($x10^3$cell/µL)	Min	0.84	0.50	1.05	0.90
	Max	4.60	4.12	10.50	7.90
	X±SD	2.64±1.04	1.87±1.11	3.62±2.63	2.50±1.69
Eosinophils ($x10^3$cell/µL)	Min	0.00	0.00	0.00	0.00
	Max	0.83	2.62	0.44	1.66
	X±SD	0.10±0.23	0.21±0.67	0.02±0.10	0.11±0.42
Thrombocytes ($x10^3$cell/µL)	Min	0.02	0.01	0.01	0.01
	Max	0.07	0.05	0.09	0.11
	X±SD	0.05±0.01	0.02±0.01	0.03±0.02	0.03±0.02

4. Conclusions

In the present study, we investigated the effects of multi-strain probiotics from three different commercial products with probiotics on the growth and hematological indicators of juvenile carp for 35 days. According to the results of this study, the use of *Bacillus subtilis, Bacillus licheniformis*, and *LAB* caused a positive increment in growth performance and hematological factors of juvenile carp. The highest growth and feed utilization were recorded in a fish group with *BioPlus® 2B* compared to control fish. The analysis of the results regarding the hematological profile shows stimulation of erythropoiesis (increase in the number of erythrocytes in the circulating blood); hemoglobin synthesis; leukopoiesis (increase in the absolute number of leukocytes) by a significant increase in the absolute number of lymphocytes (lymphocytosis) and a slight decrease in the number of neutrophils (neutropenia) in a fish group with multi-strain probiotics compared to control experimental variant.

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