Brazilian Journal of Biology®

ISSN 1519-6984 (Print)
ISSN 1678-4375 (Online)

Original Article

Evaluation of secretory activity in carp pancreatocytes (*Cyprinus carpio L.*) when using lactobacillus-based chelate and probiotic supplemental feed complexes

Avaliação da atividade secretora em pancreatócitos de carpa *(Cyprinus carpio L.)* ao usar quelato à base de lactobacilos e complexos suplementares de alimentação probiótica

D. Nikiforov-Nikishin[a], S. Antipov[b,c*], N. Kochetkov[d], A. Nikiforov-Nikishin[d] and T. Bychkova[e]

[a]Moscow State University of Technologies and Management – FCU, Department of Ecology and Nature Management, Moscow, Russia
[b]Moscow State University of Technologies and Management – FCU, Research Center of Molecular Foundations of Biotechnology of Living Systems, Moscow, Russia
[c]Voronezh State University – VSU, Department of Biophysics and Biotechnology, Voronezh, Russia
[d]Moscow State University of Technologies and Management – FCU, Department of Biology and Ichthyology, Moscow, Russia
[e]Moscow State University of Technologies and Management – FCU, Department of Biotechnology of Meat and Dairy Raw Materials Processing, Moscow, Russia

Abstract

The experimental research was carried out on the juvenile carp (*Cyprinus carpio L.*). The impact from supplemental feeds consisting of variable concentrations of chelate compounds, biogenic trace elements and probiotic lactobacillus-based product *Bacillus subtilis* VKPM B-2335 was evaluated. Optical and qualitative parameters of the lactobacillus base were studied in order to identify the major group of substances potentially able to influence the end result. The purpose of this research was to identify changes in the structure of the zymogen granules and their dimensions at which supplemental feeds produce a stimulating effect on the synthesis of zymogens in exogenous cells of the secretory part of pancreas. At the outcome of the study, for the first time, it was possible to prove that the integrated action of chelates and lactobacillus-based probiotics complemented each other. Metal chelate compounds contributed to enlargement of the zymogen granules, if compared to the control values. The bacterial products accelerated production of the zymogen granules in acinar cells diffusely located in carp hepatopancreas.

Keywords: supplemental feeds, chelates, probiotics, optical specifications, Bacillus subtilis, *Cyprinus carpio*, hepatopancreas, zymogen.

Resumo

A pesquisa experimental foi realizada em carpas juvenis (*Cyprinus carpio L.*). O impacto de suplementos alimentares consistindo em concentrações variáveis de compostos quelatos, oligoelementos biogênicos e produto probiótico baseado em lactobacilos *Bacillus subtilis* VKPM B-2335 foi avaliado. Parâmetros ópticos e qualitativos da base de lactobacilos foram estudados a fim de identificar o maior grupo de substâncias potencialmente capazes de influenciar o resultado final. O objetivo dessa pesquisa foi identificar mudanças na estrutura dos grânulos de zimogênio e suas dimensões nas quais alimentos suplementares produzem um efeito estimulante na síntese de zimogênios em células exógenas da parte secretora do pâncreas. No resultado do estudo, pela primeira vez, foi possível comprovar que a ação integrada dos quelatos e dos probióticos à base de lactobacilos se complementava. Compostos quelatos metálicos contribuíram para o aumento dos grânulos de zimogênio, se comparados aos valores de controle. Os produtos bacterianos aceleraram a produção dos grânulos de zimogênio nas células acinares localizadas difusamente no hepatopâncreas da carpa.

Palavras-chave: suplementos alimentares, quelatos, probióticos, especificações ópticas, Bacillus subtilis, *Cyprinus carpio*, hepatopâncreas, zimogênio.

*e-mail: antipov.mgutu@inbox.ru
Received: March 29, 2021 – Accepted: June 4, 2021

1. Introduction

Over recent years, the introduction of pond fishes to diets (Antipova et al., 2016) demonstrates definite positive dynamics. This inevitably leads to extensive use of supplemental feeds represented by chelate compounds of trace elements and probiotic products based on the bacteria of *Bacillus* genus (Vlasov, 2018; Bychkova et al., 2007; Ushakova et al., 2012). The initial research into these supplemental feeds and their use was undertaken in livestock husbandry (Dunkel et al., 2008), and later the feeds were introduced to fish farming. These supplemental feeds prove effective at the early stages of fish ontogenesis. Their use increases the rate of survival at the early stages of growth, enhances immunity, and stimulates mass gain in the breed (Trifonova, 2008). In particular, their use is important in regions with the temperature parameters being significantly higher than the optimum ones, and, consequently, acceleration in development of pathogenic microflora in the intestine of carp species is observed (Sklyarov et al., 2013). Nonetheless, the greater scope of research into influence of these supplemental feeds on the fish was carried out with the focus on one component only. Most papers discussed in detail either the effect of chelates on the biological indicators of acinar cells, or the effect of probiotics on the digestive function of fish (Alam Sarker et al., 2005; Apines-Amar et al., 2004; Lim et al., 1996; Li and Robinson, 1996; Simakov et al., 2020). Previously, no comprehensive studies were conducted to evaluate the joint action of the chelate compounds of biogenic elements and probiotic products. The fact that the lactobacillus-based supplements have different modes of action and, most likely, can complement each other in order to substantially increase their mutual effect in fish farming, determines the relevance of this research study.

One of the specifics of this research study is that the intensity of zymogen granule formation in carp pancreatic acinar cells was revealed at the histological and cytological levels. The zymogen granules were counted, and their morphometric parameters were examined under the action of variable concentration of components in supplemental feeds. The granulometric composition of the source milk was examined optically by means of the dynamic light diffusion method (Turishchev et al., 2016). Nowadays, some research papers are available that highlight the effects of individual components incorporated in the course of studies into supplemental feeds on the morphology of the zymogen granules and their amount in exocrine cells of fish hepatopancreas (Kadhim et al., 2020; Rašković et al., 2016; Mokhtar, 2015); still, their overall effect remains hard to comprehend. Evaluation of the zymogen formation intensity in pancreatocytes can be done based on the histological sections. In this scenario, the diffused areas of pancreas in the hepatopancreas of fish, when staining the sections with hematoxylin-eosin morphology, allow to detect the oxyphilic stains in the zymogen granules. Consequently, their detection requires no specific histochemical techniques (Stegniy and Fedyshin, 2019).

Chelate compounds of metals represent the first component of the complex product. They stimulate effective growth through the elimination of trace element deficiency and intensification of digestive system performance, and, as a result, through the cost-effectiveness of fish production when this component is added to granular feed (Zhou et al., 2019). The fish feed is often unbalanced in vitamins, trace elements and some amino acids (Li et al., 2017). In order to compensate for deficiency in trace elements, inorganic salts are usually added to the fish farming feeds, but they cannot always be well absorbed and accumulated in a particular way by various organs. The imbalance of this type of diet can result in foodborne pathologies and decrease in growth dynamics in fish, which, eventually, impacts the quality of fish products (Kononenko et al., 2016). The use of feedstuff with mineral salts is particularly dangerous for the juvenile fish because this leads to the disruption in metabolic processes, morphogenesis, and the decrease in survival rate of the juvenile carp. The early stages of trace elements deficiency are hard to detect against the changing morphological parameters. Nonetheless, they can reveal themselves through the slow-down in growth parameters, foodborne pathologies of the musculoskeletal system, and, as a result, through the increase in discarded fish produce when the intensive growing technique is employed. With that in mind, it is of interest to look for the components to add to supplemental feeds in order to compensate for the insufficient amount of trace elements required in feedstuff of the unbalanced composition. The combination of trace elements with chelate complexes can become the right component for the supplemental feeds. Trace elements in chelate complex compounds possess high bioavailability because of their better structural stability and lower molecular weight, if compared to the inorganic salts (Simakov et al., 2020). Chelate compounds, given their specification, protect trace elements by limiting their interaction with other components of the feedstuff.

The second component, which is studied alongside chelate supplements, is a spore-based probiotic of bacteria representing the strain of *Bacillus subtilis* VKPM B-2335. This strain boosts the recovery of microflora, prevention and correction of dysbacteriosis, and reduction of endogenous intoxication level. The powder probiotic based on this strain is very effective, and is used to prevent bacterial diseases in the gastrointestinal tract. The effect of the product is supported by the action of these bacteria aiming at suppression of pathogenic and conditionally pathogenic intestinal microflora occurrences, and, consequently, by creating the favorable growth conditions for healthy flora representatives (bifidobacteria, lactobacilli, E. coli). The integration of probiotic bacteria to the supplemental feeds stimulates resistance of the body system to viral and bacterial infections in the juvenile carp (Yukhimenko, 2019), and qualitatively and quantitatively balances flora compositions in the digestive tract (Pereira et al., 2020). At that, the presence of the lactobacillus components can enhance such effect: on the one hand, by means of the nutrients content, and on the other hand, by means of the membrane formation on the surface of the feedstuff granules.

Nowadays, the use probiotics in aquaculture is vast in carp cultivation, and displays positive results (Trifonova, 2008). It is noteworthy, that bacterial probiotic supplements in carp cultivation act as preventive products to stop

the bacterial hemorrhagic septicemia development (Juhimenko et al., 2009). Consequently, the objective of this research is to study the complex effect of the chelate compounds of biogenic metals and probiotic products of the *Bacillus subtilis* VKPM B-2335 strain upon the performance of digestive function in the juvenile carp; as well as the selection of optimum ratios of compositions and their physical and chemical properties as detected at the cellular level through the changes in morphometric properties of the zymogen granules in pancreatocytes of the fish subjects under study.

2. Materials and Methods

The series of experiments was carried out on the premises of the Nordic-Russian Center for Research and Innovation in Aquaculture at Razumovsky Moscow State Institute of Biotechnology and Fisheries.

The integrated effect from supplemental feeds was studied in the juvenile carp (*Cyprinus carpio L.*) throughout the persistent experiment lasting 30 days. At the experiment outcome, the studied individuals of carp of 4 ± 0.3 cm in length and weighing 3 ± 0.2 g were divided into groups, and were kept in the 40-liter aquariums with forced aeration (partial water flushing was done once every 2 days), with 20 individuals in each. Feeding was accomplished with the standard and universal granular feedstuff by a well-known brand (with the size of granules of 1.2 mm). Table 1 gives the feedstuff composition as per the manufacturer's certificate.

The feed additive of metal chelates developed by the scientists in charge was produced on the premises of Jupiter LLC (Russia) with the use of *Bacillus subtilis* VKPM B-2335 strain, as well as the product based on it, i.e. Sub-Pro (manufactured by VektorEvro LLC, Russia).

The liquid solution of chelate compounds of metals was added to the water-based standard granular feedstuff at the rate of 0.5; 1.0 and 2.0 mL/kg of the feedstuff. The content of trace elements for these solution components is given in Table 2.

The selection of trace elements concentrations as given above was done on the grounds of the studies focusing on application of the chelate compounds in feedstuff for different fish species (Alam Sarker et al., 2005; Apines-Amar et al., 2004; Lim et al., 1996; Li and Robinson, 1996). Application of the product onto feedstuff granules by way of the liquid solution was carried out after designing the diet. Upon that, the probiotic product in powder containing lactobacillus substrate and used as the auxiliary substance was added to the wetted feedstuff. Dosing of the product was calculated on the basis of supplements most commonly used in carp farming, i.e. 0.5 and 1.0 g/kg of the feedstuff (Vlasov, 2018). By experiment, employing inoculation in the selective environment in the Petri dish (SPL, China), the quantity of B.subtilis bacteria in 1 mg of the product used in the experiment was evaluated; then, by calculation, the quantity of bacterial cells in 0.5 mg was defined. Later, the feedstuff was dried to its initial wetness.

The granulometric studies of the lactobacillus components were performed by means of the dynamic light

Table 1. Basic components of granular feedstuff used for the purposes of the experiment.

Type of component	Protein	Fat	Ash	Fiber	Phosphorus
Content, %	46	10	7.2	2.6	1.24
Type of component	Vitamin D	Vitamin E	Vitamin C	Vitamin A	Omega-3
Content, %	7.2	7.2	2.6	7.2	-*

*Data not available.

Table 2. Content of trace elements in supplemental feed per 1 kg of feedstuff in variable chelate concentrations, probiotic values Coe/mg and Doses of components (X1-X6, mg/kg).

Doses of components	Fe mg/kg	Mn mg/kg	Zn mg/kg	Se mg/kg	I mg/kg	Cu mg/kg	*B.subtilis* Coe/mg
X_1	5.0	7.5	17.5	0.15	0.55	1.5	-
X_2	10.0	15.0	35.0	0.3	1.1	3.0	-
X_3	20.0	30.0	70.0	0.6	2.2	6.0	-
XS_1	5.0	7.5	17.5	0.15	0.55	1.5	$2.5*10^7$
XS_2	5.0	7.5	17.5	0.15	0.55	1.5	$5.0*10^7$
XS_3	10.0	15.0	35.0	0.3	1.1	3.0	$2.5*10^7$
XS_4	10.0	15.0	35.0	0.3	1.1	3.0	$5.0*10^7$
XS_5	20.0	30.0	70.0	0.6	2.2	6.0	$2.5*10^7$
XS_6	20.0	30.0	70.0	0.6	2.2	6.0	$5.0*10^7$

diffusion method using Zetasizer Nano device (Malvern Instruments, UK) that enables to detect the molecule diameter in the range of 0.3 nm to 10.0 μm, with the sensitivity to protein concentration ranging from 0.1 mg/mL and above (Turishchev et al., 2016). The helium neon laser with a wavelength of 632.8 nm and a maximum power of 4.0 mW was employed as the transmission source. The findings were processed by Malvern Instruments software (Malvern Instruments, UK).

Fish feeding was implemented by eatability and done twice a day. The data on doses of supplemental feeds added to the granular feedstuff with the final concentrations of trace elements and the strain of *Bacillus subtilis* VKPM B-2335 are given in Table 2.

Upon completion of the experiment, 5 fish individuals with no visible damage were selected from each test group of fish for further purposes of preparation of histological products. 45 fish subjects in total were selected from all the groups. The selected juvenile carp were killed by decapitation, with hepatopancreas ejected from their bodies to be preserved during the day in the 10% neutral formalin solution. Preparation of histological products from the liver was carried out in accordance with conventional guidelines (Sarkisov and Perov, 1996). The thickness of the prepared sections ranged from 5 to 7 μm. Hematoxylin eosin was used for staining (Romace, 1954; Mumford et al., 2007).

The stained products were studied using the Olympus BX45 microscope (Olympus Corporation, Japan) at magnification of 40x, 100x and 400x. Photo recording and analysis of acinar cells containing the granules of zymogen was performed by the ZEN lite software (Zeiss, Germany). The morphometric parameters of zymogenic granules (area, number, and diameter) were measured. The pancreatic zymogen activity was evaluated for each research scenario. The amount and surface area of the zymogen granules for each research scenario in variable product concentrations was evaluated in the areas of localization of the zymogen granules in the exocrine section of pancreas.

The morphometric studies were carried out in a manner comparable with that of the zymogen granules in the pancreatic exocrine cells in mammals (Dilekova, 2016). The opensource code ImageJ program (http://rsb.info.nih.gov/ij/) was used for these purposes.

The obtained digital data were analyzed using the STATISTICA software (Statsoft, Inc. (v.10), USA) employing the statistical method of one-way analysis of variance and the Newman-Keuls multiple comparison criterion. Differences within $p \leq 0.05$ were considered statistically significant.

2.1. Ethics statement

All the experiments involving animals were performed in compliance with the EC Directive 86/609/EEC and with the Russian law regulating experiments on animals.

2.2. Research outcome

In bony fishes, the pancreas is not an isolated separate organ. The glandular cells of the exocrine component (acinar cells) are found in groups of 30-50 individuals around the liver parenchyma, large vessels, or in the intestinal mesentery. Despite such random arrangement, the large protein (zymogenic) granules normally accumulate at the apical end of acinar cells. At that, in the secretory section of hepatopancreas the morphofunctional components do not differ from other vertebrates.

2.3. Control

The histological products from the control group of hepatopancreas in the juvenile carp (Figure 1) reveal the epithelial cells of a conical shape: exocrine pancreatocytes (acinar cells) in total of 8-12. The base of these cells is wide, and the apex is narrow. Each cell possesses one nucleus of a rounded or oval shape. The nucleus divides the cell into two parts: homogeneous and zymogenic. The zymogenic (apical) part of the cell contains the secretory granules of zymogen: the digestive zymogens with oxyphilic staining

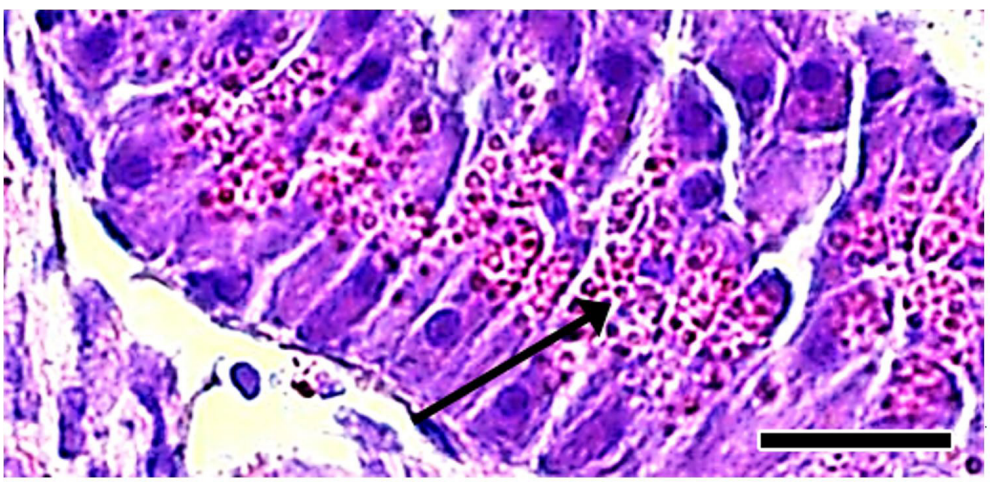

Figure 1. Control. Part of the pancreatic acinus of the juvenile carp. Zymogen granules are visible in exocrine cells. The scale bar is 20 μm. The arrow points to the zymogen granules.

are clearly isolated in the products under study. This makes it possible to calculate the amount of granules, and evaluate their size under the action of variable concentrations of the studied supplemental feeds.

The amount of granules in the exocrine cells of pancreas in the juvenile carp (control values) revealed that on average one cell contains 22 ± 2.0 granules of zymogen. The granule diameter is 0.75 µm.

2.4. Groups X_1, X_2, X_3 (without probiotic)

When only metal chelate compounds are added to the diet of the juvenile carp for 30 days, they impact the synthesis of zymogen, determined by the amount and morphometry of the zymogen granules (histological products X_2 and X_3). The lowest studied concentration of the feedstuff of 0.5 mL/kg (group X_3) produced little effect on the size of the zymogen granules, and their dimensions were close to the control values. The chelate supplement in the concentration as given did not cause any significant increase for the total of acinar cells granules when compared to the control values.

The greatest effect on the zymogen granule size was recorded when the chelate concentration added to the feedstuff reached 1.0 mL/kg (Figure 2). At that, there was some significant enlargement of the granules by 54%, if compared to the control values.

With the chelate concentration reaching 2.0 mL/kg, no particular shifts were observed in pancreatocytes performance. The effect of this concentration was less significant. Some part of the zymogen granules reduced in size (Figure 3). The total of zymogen granules was mostly similar to the control values. Consequently, the optimum added amount of the chelate metal compounds of the composition as shown above (Table 2) to the carp feedstuff is 1.0 mL/kg.

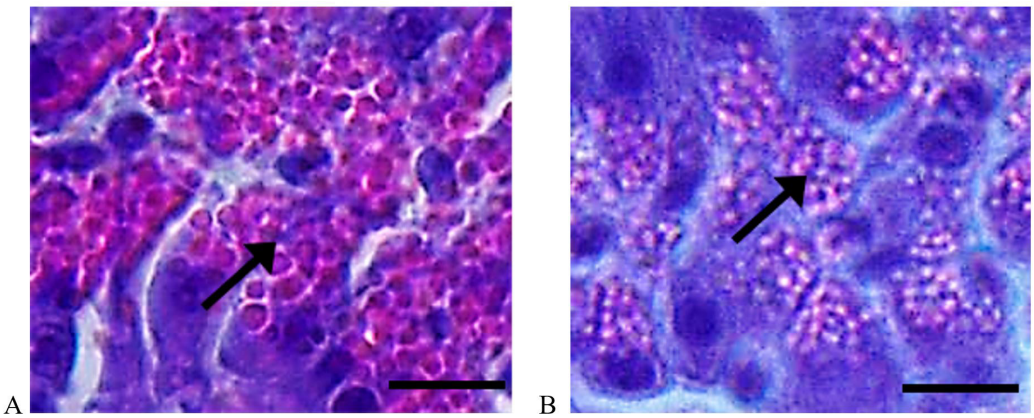

Figure 2. Hepatopancreas in carps at the exposure to the chelate compounds added to the feed in variable concentrations: (A) for 1 mL/kg (X_2) and (B) for 2 mL/kg (X_3). The scale bar is 10 µm. The arrow points to the zymogen granules.

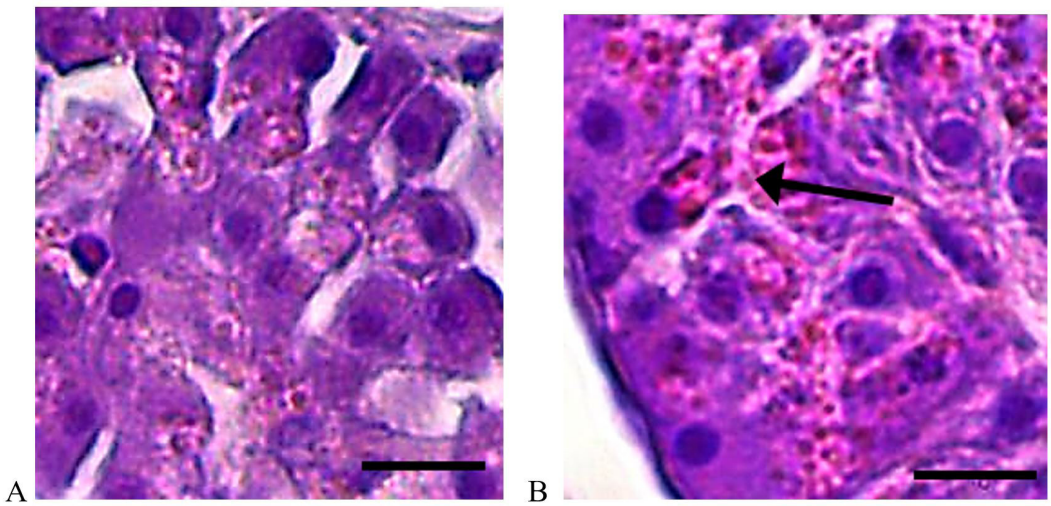

Figure 3 Formation of zymogen granules in carp pancreatocytes in Groups XS_1 (A) and XS_2 (B)**.** The scale bar is 20 µm. The arrow points to the zymogen granules.

2.5. Groups XS_1 and XS_2 (in the lowest concentrations of probiotic and chelate compounds)

When 0.5 mL/kg of the studied supplemental feeds are added to the feedstuff of carps (in the lowest concentration of chelates) in combination with two concentrations of probiotic (0.5 and 1.0 g/kg respectively), the concentrations of the studied supplemental feeds produce no effect on the amount of the zymogen granules in acinar cells, or on the granules size. The recorded morphometric indices of the zymogen granules are close to the control values.

2.6. Groups XS_3 and XS_4

When adding 1.0 mL/kg of the chelates and 0.5 g/kg of the probiotics to the granular feedstuff of carps, the significant enlargement of the zymogen granules in the acinar cells is observed. The zymogen granules increase in size by 57%, and this becomes similar to the scenario with only metal chelates added to the feedstuff. (Figure 4A).

With the concentration of probiotic product getting higher in feedstuff and reaching 1.0 mg/l, the size of granules slightly decreases (upto 1.45 µm in diameter and 0.86 µm^2 of the surface area). Besides, the action of the VKPM B-2335 subtilis strain of *Bacillus subtilis* in the concentration of 1.0 g/kg reveals one particular pattern: a dark basophilic zone appears at the core of oxyphilic granules. This most likely points to the biochemical changes taking place in the composition of zymogen.

2.7. Groups XS_5 and XS_6 (in the highest concentrations of probiotic and chelate compounds)

The final scenario of the supplemental feed complexes is specific in the way that the chelates were added in its higher concentration, i.e. 2 mL/kg, and the probiotics in concentrations of 0.5 and 1.0 g/kg. When adding to the feedstuff, there observed the return of the pattern when the probiotic product in the concentration of 0.5 g/kg does not produce any effect upon synthesis of the zymogen granules in acinar cells. The principal effect on the zymogen granules size is produced by the chelate supplement causing the enlargement of the granules (Figure 5A). When the probiotic content reaches 1.0 g/kg, the pattern returns with the basophilic zone appearing at the core of the granule. Meanwhile, there observed the decrease in the size of zymogen granules under the action of probiotic (Figure 5B).

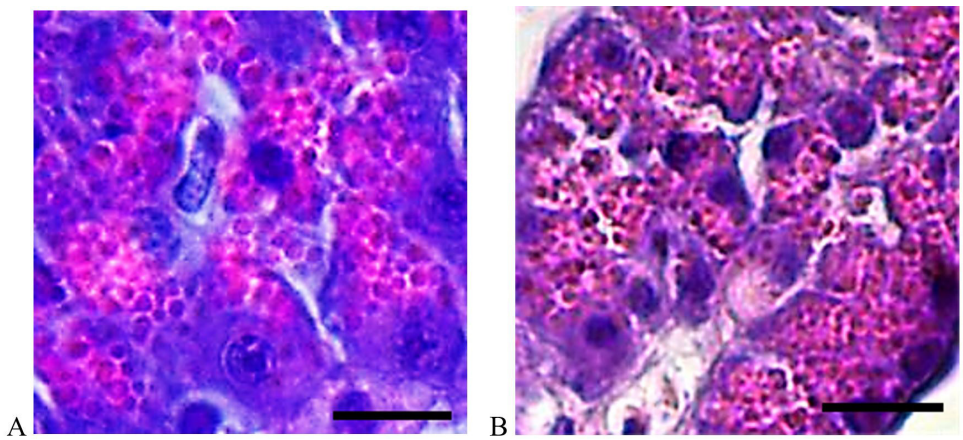

Figure 4. Increased synthesis of zymogen granules in carp pancreatocytes in Groups XS_3 (A) and XS_4 (B). The scale bar is 10 µm.

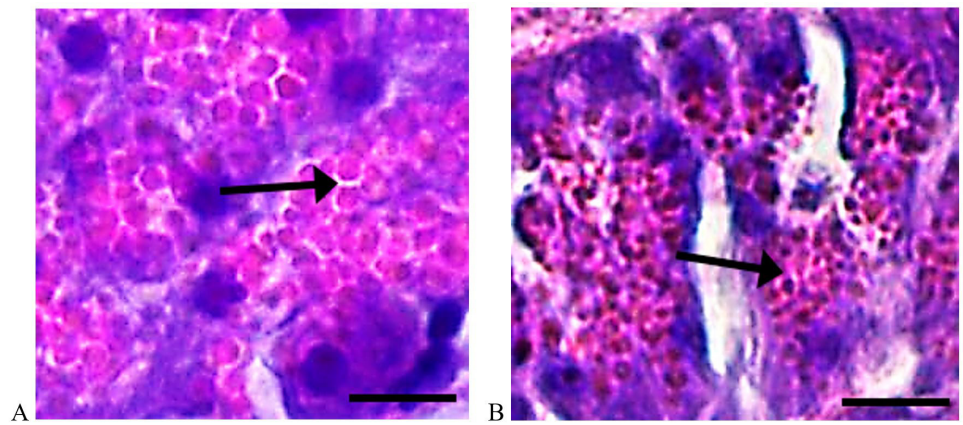

Figure 5. Zymogen granules in carp pancreatocytes in Groups XS_5 (A) and XS_6 (B). The scale bar is 10 µm. The arrow points to the zymogen granules.

2.8. Morphometric evaluation outcome for zymogen granules

For the purposes of meaningful understanding of the effect produced by supplemental feeds containing metal chelate compounds and probiotic products of the lactobacillus basis, the morphometric evaluation of the zymogen granules in pancreatic acinar cells in the juvenile carp was undertaken in the context of this research study. The diameter and surface area of the zymogen granules in pancreatocytes in the juvenile carp were measured following the 30-day integrated exposure to the studied supplemental feeds in variable concentrations.

The authors believe that the enlargement of the surface area of the zymogen granules is associated with the intensity in the secretory activity of exocrine parenchyma, and with the increase in the concentration of the digestive enzymes contained in the zymogen granules. All those aspects may represent the stimulation of digestive function in the juvenile carp (Huang et al., 2020).

The evaluation outcome of morphometric indicators for the zymogen granules in pancreatic acinar cells in in the juvenile carp are given in Table 3. The obtained values for the amount of zymogene granules per cell prove that their total, in all chelate and probiotic concentration scenarios, is close to the control values. Consequently, neither certain metal chelates nor their effect in conjunction with the probiotics in their low studied concentrations have any particular impact upon the amount of zymogen granules in pancreatocytes.

In that, it is noteworthy that one of the components of supplemental feeds is lactobacillus-based. The data on optical parameters of the lactobacillus-based component are shown in Figure 6, with protein and amino acid compositions given in Table 4.

Blue line is the source milk; green line is the milk diluted twice with the deionized water; red line is the milk diluted three times with the deionized water.

Pursuant to the data obtained by means of the dynamic light scattering method, the majority of particles found in the source milk as well as in twice or three times thin milk are around 400 nm in size. This is coherent with the general scientific knowledge and research data.

Table 3. Morphometric evaluation of zymogen granules when adding variable concentrations of metal chelates and probiotic products of the VKPM B-2335 subtilis strain of *Bacillus subtilis* basis to feedstuff for the juvenile carp. The value is presented by the average ± SD.

Doses of components per g/kg of feedstuff	Total of granules in acinar cell, items	Diameter of granules, μm	Surface area of granules, μm²
X_1	22 ±1.5	0.75±0.05	0.43±0.06
X_2	18±3.3	1.38*±0.03	1.49*±0.08
X_3	23±1.8	0.81±0.07	0.50±0.04
XS_1	19±1.2	0.67±0.03	0.34±0.07
XS_2	20±2.1	0.78±0.02	0.48±0.04
XS_3	22±2.0	1.45*±0.05	1.62*±1.10
XS_4	23±1.5	0.86*±0.06	0.59±0.07
XS_5	19±1.8	1.40*±0.12	1.54*±0.14
XS_6	25.0±2.1	1.24*-0.03	1.21*±0.09
Control	22±2.0	0.73±0.05	0.41±0.04

*Statistically significant difference in mean values versus the control (at p≤0.05).

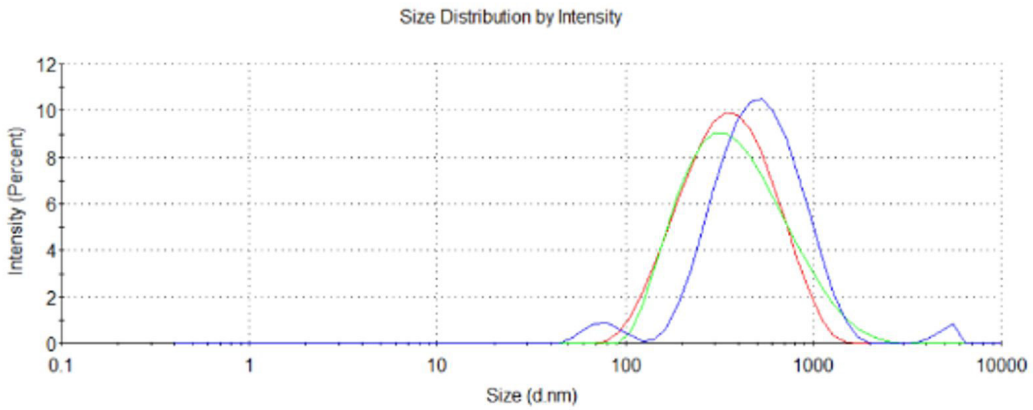

Figure 6. Distribution of particles in different sizes present in the lactobacillus-based component used in supplemental feeds preparation.

Table 4. Protein and Amino acid content in source milk.

(A) Protein composition																		
Crude protein	Casein				Serum protein	Lactoglobulin		Serum albumin	Immune serum globulin									
	Total casein	casein α	casein β	casein γ		α	β											
3.35± 0.02	2.60± 0.01	1.045±0.02	1.30±0.01	0.26±0.02	0.75±0.01	0.14±0.02	0.40± 0.02	0.08±0.004	0.12±0.006									
(B) Amino acid compositio																		
Threonine	Valine	Methionine	Isoleucine	Leucine	Phenyl-alanine	Lysine	Aspartic acid	Serine	Glutamic acid	Proline	Glycine	Alanine	Tyrosine	Histidine	Arginine			
1.45±0.06	2.17±0.09	0.87±0.06	2.1±0.1	4.05±0.06	1.72±0.07	2.95±0.2	2.25±0.02	1.6±0.09	6.38±0.03	1.48±0.06	0.52±0.03	1.0±0.02	1.42±0.09	1.1±0.03	1.32±0.06			

The experimental data on protein and amino acid content prove that the presence of lactobacillus-based component in supplemental feeds can be of consideration as yet another factor partially supporting the obtained results.

3. Discussion of Research Results

This research study of the zymogen granules morphometry in pancreatic acinar cells on histological sections in the juvenile carp made it possible to determine the intensity of zymogen formation in pancreatic cells under the action of variable concentrations of supplemental feeds added to the granular carp feedstuff and of variable concentrations of chelates and probiotics. It was revealed that the probiotic supplement have no impact upon enlargement of the zymogen granules. Morphometric evaluation of zymogen granules proved that the granules diameter increases almost twofold, both by adding only one chelate compound in the concentration of 1.0 mL/kg to the feedstuff and by adding additional probiotics in the concentration of 0.5 g/kg. Lowering chelate concentration no longer impacts the size of the zymogen granules formed in the acinar cells, with their values being close to the control (XS_1 scenario).

Nonetheless, with reference to the optical microscopy, the acinar cells when exposed to the integrated lactobacillus-based supplemental feeds, are more filled with zymogen than those of the control group. Most probably, this is due to enlargement of the zymogen granules, when the chelates of 1.0 and 2.0 mL/kg are added to the feedstuff. Consequently, at the later stage, particular attention shall be drawn to these specific morphological parameters of the granules under the action of variable concentrations of components in supplemental feeds.

As given in Table 3, low concentrations (equal to 0.5 g/kg), particularly for chelates and in combination with probiotics, produce no effect on the zymogen granule size when compared to those of the control group. The significant increase in the zymogen granules is recorded for the experimental scenarios in Groups X_2, XS_3 and XS_5, when the chelate compounds of metals are added to feedstuff in the concentration of 1.0 mL/kg, or used separately, or added together with probiotic products in the concentration of 0.5 g/kg. When adding the chelates in 2.0 mL/kg and probiotics in 0.5 g/kg to the feedstuff, the effect of enlargement in the granule size and the increase of zymogen in acinar cells is recorded as well. Still, the effect shall be comparable to the action of chelates in the concentration of 1,0 mL/kg, so that the excessive content of the product per 1 kg of feedstuff does not make commercial sense.

Therefore, the principal stimulating effect associated with the size of zymogen granules in supplemental feeds for the juvenile carp is produced by supplements of the metal chelates in 1.0 mL/kg without probiotics. By adding the probiotic product in 0.5 g/kg, supplements of the chelates in 1.0 mL/kg are most effective. It is obvious, that probiotics cause formation of a dark (basophilic) zone at the core of the zymogen granules, which, in fact, may be indicative of their complete maturation and presence of other enzymes.

Therefore, it is sufficient to use chelate supplement in the concentration of 1.0 mg/kg together with the lowest probiotic concentration to boost the growth of digestive enzymes. This pattern was proved effective by experiment for the juvenile carp when using the chelate supplement of

the composition formulated by the authors. It is required to correct the dose for other aquaculture species, with the same mode of exposure to chelates and probiotics, once the preliminary tests are made (Wang et al., 2019; Dunkel et al., 2008).

Pursuant to the outcome of the undertaken research studies, adding chelates to feedstuff in the concentration of 2.0 mL/kg is not cost-effective. From the point of view of toxicology, such supplemental feed may contain heavy metals in doses exceeding tolerable values (Andini et al., 2019). Consequently, 2 mL of the studied chelate supplement contain 70 mg of zinc at the tolerable bonded zinc content in the feed being 40-50 mg (Tarleva, 2014). Thus, the previously established safe content will be exceeded. Eventually, it is toxic for the juvenile carp in other ways, and, in general, it inhibits enlargement of the zymogen granules, particularly if used together with the probiotics in the concentration of 1.0 g/kg. The similar effect can be produced by adding chelates to feedstuff in the concentration of 2 mL/kg. This dose contains 6 mg of copper (ref. Table 2). The academic sources discuss that the optimum added dose of 2 g of copper per ton gives positive results (Kuzmina, 2011). Therefore, the exposure for carp to 6 mg/kg of this element through feedstuff may produce a toxic effect on the body system in grown fish (Hoseini et al., 2016). When adding chelate compounds to ensure better absorption of trace elements which are included into their composition, it is probable, that the combined action of the ions of copper and manganese shall produce a toxic effect upon the processes of absorption of iron ions by cells, and upon the integration of iron into enzymes, including those which are part of the zymogen granules (Semenova et al., 2013). This proves that the overdose in these elements when adding trace element supplements to foodstuff is not acceptable for the juvenile carp. This is yet another proof that chelates shall not be added to foodstuff in the concentration exceeding 1 mL/kg.

Nonetheless, adding the probiotics to the fish feedstuff was not neuter, and they impacted the structure of zymogen granules. The revealed pattern when adding probiotics to the feedstuff consisted in appearance of more intense basophilic staining in the form of a dark zone at the core of zymogen granules.

The research undertaken by other authors showed that more mature zymogen granules contain a dark zone at the core, because probiotics produce favorable effect upon the intestinal microflora in carps, and promote synthesis of various digestive enzymes, including zymogen (Fong and Takeda, 2008). It can be considered, that 1.0 g/kg of the probiotic product in supplemental feeds may have a positive effect on formation of pancreatic zymogens in carps. The microscopic evaluation of histological products proved the stimulating effect where the zymogenic granules were released by acinar cells into intestine. At maturation of pancreatic juice, they shall contain even more complete combination of digestive enzymes. By contrast, the content of only chelates in supplemental feeds in the concentrations of 1.0 and 2.0 mL/kg or together with probiotics in low concentrations shall stimulate formation of larger zymogen granules (with the surface area reaching 1.62 μm^2). Still chelates shall be deficient in various biologically active compounds being part of zymogens. Given this assumption, the use of supplemental feeds in the concentration of 1.0 mL/kg for chelate compounds and 0.5 g/kg for the VKPM B-2335 subtilis strain of *Bacillus subtilis* shall be regarded as optimized.

In the course of these research studies, and in order to identify the most optimum concentrations of the studied supplemental feeds in view of the secretory function of exogenous cells in fish pancreas, the authors additionally took into consideration the daily secretory activity of acinar cells (Kalhoro et al., 2018). It is known, that the secretory activity of pancreatic cells in fish is different at different times of day. Consequently, the morphological parameters of zymogen granules in pancreatocytes are related to circadian activity of the digestive system in fish. At various stages of their life cycle, carps reveal two active periods in view of the digestive system function: morning and evening (Jarzhombek, 2016). Taking that into account, decapitation of the juvenile carp, and extraction of hepatopancreas from fish with its further preservation in the formalin solution was accomplished in the evening period of their activity, when the daily rate of zymogen formation in acinar cells is high (Kuzmina, 2009; Eropkina, 1983).

Therefore, the conducted research studies of the histological products of diffusely located pancreas in the juvenile carp revealed a set of patterns associated with stimulation of zymogens formation in pancreatocytes, and contributed to definition of the most optimum ratio of chelate compounds and probiotics in supplemental feeds helping to stimulate metabolism in the digestive system.

4. Conclusion

This research enabled the solution of a number of academic and practical problems associated with the regulation of zymogens synthesis in pancreatocytes of the juvenile carp, with the studied products being chelate mineral supplements and probiotics based on the VKPM B-2335 subtilis strain of *Bacillus subtilis*.

The integrated action by these two components used in supplemental feed complexes in variable concentrations upon the secretory function of exogenous pancreatic cells is brought to discussion for the first time. The histological examination and morphometry of zymogen granules in the digestive acinar cells served grounds to evaluate the integrated effect of zymogen granules in supplemental feeds upon the juvenile carp.

This research study revealed as follows:

1. Metal chelate compounds activate secretion of zymogen granules in pancreatic acinar cells in the juvenile carp when added to feedstuff in the concentrations of 1.0 and 2.0 mL/kg. In lower concentrations, no stimulation of synthesis of zymogen granules occurs. At that, the amount of granules does not get bigger, still the size of granules increases by 57%. It is proved, that enlargement in the surface area of the zymogen granules occurs even with no probiotics added;
2. Optimum concentration of chelates in supplemental feeds shall be of 1.0 mL/kg because in the concentration of 2.0 mL/kg the size of the zymogen granules increases, as

well as when using chelates in the concentration of 1.0 mL/kg. Still, the content of heavy metals like manganese, copper and zinc when adding 2.0 mL/l of metal chelates to feedstuff becomes potentially harmful to fish;

3. The probiotic products, acting on the intestinal microflora, promote the synthesis of different enzymes that are included in the zymogen granules and, consequently, accelerate the synthesis of granules. The maturation rate of the zymogen granules is defined by basophilic staining of the zone at the core of granules. This coloring was detected on the histological products as well, with probiotics in the concentrations of 0.5 and 1.0 g/kg used together with metal chelates in the concentrations of 1.0 and 2.0 mL/kg;
4. The undertaken research studies enable practical implementation of the examined supplemental feed complexes containing the above discussed components in the concentration of 0.5 g/kg of probiotics and 1 mL/kg of metal chelate compounds to activate the zymogen synthesis in exogenous pancreatic cells. As a result, this stimulates digestion in the juvenile carp.

Acknowledgements

This work was supported by the Ministry of Science and Education of the Russian Federation (Grant Agreement 075-15-2020-774).

References

ALAM SARKER, S., SATOH, S. and KIRON, V., 2005. Supplementation of citric acid and amino acid-chelated trace element to develop environment-friendly feed for red sea bream, Pagrus major. *Aquaculture*, vol. 248, no. 1-4, pp. 3-14. http://dx.doi.org/10.1016/j.aquaculture.2005.04.012.

ANDINI, N.S., ANSHARY, H., WAHYUNI., PUTRA, A. and SARI, D.K., 2019. Histopathological study of hepatopancreas and kidney of butini fish (Glossogobius matanensis) in Matano Lake, South Sulawesi, Indonesia, caused by metal contamination. *IOP Conference Series: Earth and Environmental Science*, vol. 343, no. 1, pp. 012033. http://dx.doi.org/10.1088/1755-1315/343/1/012033.

ANTIPOVA, L.V., DVORYANINOVA, O.P. and SOKOLOV, A.V., 2016. Pond fishes in improvement of the structure of population's nutrition: hygienic aspects. *Gigiena i Sanitariia*, vol. 95, no. 1, pp. 84-90. http://dx.doi.org/10.18821/0016-9900-2016-95-1-84-90..

APINES-AMAR, M.J.S., SATOH, S., CAIPANG, C.M.A., KIRON, V., WATANABE, T. and AOKI, T., 2004. Amino acid-chelate: a better source of Zn, Mn and Cu for rainbow trout, Oncorhynchus mykiss. *Aquaculture*, vol. 240, no. 1-4, pp. 345-358. http://dx.doi.org/10.1016/j.aquaculture.2004.01.032.

BYCHKOVA, L.I., YUKHIMENKO, L.N. and KHODAK, A.G., 2007. Probiotic product Sub-Pro (Subalin): prevention and treatment of bacterial diseases in fish. *Fish Farming*, vol. 2, pp. 33-35.

DILEKOVA, O.V., 2016. Morphometric parameters of granules of zymogene the pancreas of mammals in postnatal ontogeny. *International Research Journal*, vol. 34, no. 12, pp. 46-48.

DUNKEL, Z., KLUGE, H., SZPILKE, J., EDER, K. 2008. *Use of chelates in animal husbandry*. Germany: Martin Luther University, pp. 77-80. no. 1.

EROPKINA, E.M., 1983. Secretory activity of pancreatic acinous cells at different times of the day and the sequence of maturation processes for secretory granules. *Archives d'Anatomie, d'Histologie et d'Embryologie Normales et Expérimentales*, vol. LXXXIV, no. 1, pp. 67-71.

FONG, G. and TAKEDA, K., 2008. Role and regulation of prolyl hydroxylase domain proteins. *Cell Death and Differentiation*, vol. 15, no. 4, pp. 635-641. http://dx.doi.org/10.1038/cdd.2008.10. PMid:18259202.

HOSEINI, S.M., HEDAYATI, A., TAHERI MIRGHAED, A. and GHELICHPOUR, M., 2016. Toxic effects of copper sulfate and copper nanoparticles on minerals, enzymes, thyroid hormones and protein fractions of plasma and histopathology in common carp Cyprinus carpio. *Experimental and Toxicologic Pathology*, vol. 68, no. 9, pp. 493-503. http://dx.doi.org/10.1016/j.etp.2016.08.002. PMid:27555376.

HUANG, L., YANG, L., LIU, J. and CAO, X., 2020. Comparative histological analysis of intestines of loach, grass carp and catfish provide insights into adaptive characteristics in air breathing fish. *Ribarstvo*, vol. 78, no. 2, pp. 91-98. http://dx.doi.org/10.2478/cjf-2020-0009.

JARZHOMBEK, A.A., 2016. *Lifestyle and behavior of commercial fish*. Moscow: Publishing House VNIRO, 200 p.

JUHIMENKO, L.N., BYCHKOVA, L.I., ZYUKIN, A.N. and KLIMOV, A.V., 2009. Prevention of bacterial homorrhagic septemia using *Bacillus subtilis* (case study of Biserovsky fish factory). *Fisheries*, no. 1, pp. 86-88.

KADHIM, K.H., KARIM, A.J. and KADHIM, K.K., 2020. Histological and histochemical study of the liver and gall bladder of adult male common carp *Cyprinus carpio*. *Plant Archives*, vol. 20, suppl. 1, pp. 438-442.

KALHORO, H., TONG, S., WANG, L., HUA, Y., VOLATIANA, J.A. and SHAO, Q., 2018. Morphological study of the gastrointestinal tract of Larimichthys crocea (Acanthopterygii: perciformes). *Zoologia*, vol. 35, pp. 1-9. http://dx.doi.org/10.3897/zoologia.35.e25171.

KONONENKO, S.I., YURINA, N.A., MAXIM, E.A. and CHERNYSHOV, E.V. 2016. Innovative supplemental feeds in growing juvenile fish. *Bulletin of Gorsky State Agrarian University*, vol. 53, no. 1, pp. 30-34.

KUZMINA, V.V., 2009. The influence of diet and biochemical composition of food on eating behavior of the carp *Cyprinus carpio*. *Questions of Ichthyology*, vol. 49, no. 1, pp. 111-116.

KUZMINA, V.V., 2011. The influence of zinc and copper on the latency period for feeding and the food uptake in common carp, *Cyprinus carpio L. Aquatic Toxicology*, vol. 102, no. 1-2, pp. 73-78. http://dx.doi.org/10.1016/j.aquatox.2010.12.018. PMid:21371614.

LI, M.H. and ROBINSON, E.H., 1996. Comparison of chelated zinc and zinc sulfate aszinc sources for growth and bone mineralization of channel catfish (*Ictalurus punctatus*) fed practical diets. *Aquaculture*, vol. 146, no. 3-4, pp. 237-243. http://dx.doi.org/10.1016/S0044-8486(96)01388-9.

LI, S., JI, H., ZHANG, B., ZHOU, J. and YU, H., 2017. Defatted black soldier fly (*Hermetia illucens*) larvae meal in diets for the juvenile Jian carp (*Cyprinus carpio* var. Jian): growth performance, antioxidant enzyme activities, digestive enzyme activities, intestine and hepatopancreas histological structure. *Aquaculture*, vol. 477, pp. 62-70. http://dx.doi.org/10.1016/j.aquaculture.2017.04.015.

LIM, C., SEALEY, W.M. and KLESIUS, P.H., 1996. Iron methionine and iron sulfate as sources of dietary iron for channel catfish Ictalurus punctatus. *Journal of the World Aquaculture Society*, vol. 27, no. 3, pp. 290-296. http://dx.doi.org/10.1111/j.1749-7345.1996.tb00610.x.

MOKHTAR, D.M., 2015. Histological, histochemical and ultrastructural characterization of the pancreas of the grass

carp (*Ctenopharyngodon idella*). *European Journal of Anatomy : Official Journal of the Spanish Society of Anatomy*, vol. 19, no. 2, pp. 145-153.

MUMFORD, S., HEIDEL, J., SMITH, C., MORRISON, J., MACCONNELL, B. and BLAZER, V., 2007. *Fish histology and histopathology*. Amerika Serikat: US Fish and Wildlife National Conservation Training Center, 357 p.

PEREIRA, S.A., JESUS, G.F.A., PEREIRA, G.V., SILVA, B.C., SÁ, L.S., MARTINS, M.L. and MOURIÑO, J.L.P., 2020. The Chelating mineral on organic acid salts modulates the dynamics and richness of the intestinal microbiota of a silver catfish *Rhamdia quelen*. *Current Microbiology*, vol. 77, no. 8, pp. 1483-1495. http://dx.doi.org/10.1007/s00284-020-01962-z. PMid:32236647.

RAŠKOVIĆ, B., ĆIRIĆ, M., KOKO, V., STANKOVIĆ, M., ŽIVIĆ, I., MARKOVIĆ, Z. and POLEKSIĆ, V., 2016. Effect of supplemental feeds on liver and intestine of common carp (Cyprinus carpio) in semi-intensive rearing system: histological implications. *Biologia*, vol. 71, no. 2, pp. 212-219. http://dx.doi.org/10.1515/biolog-2016-0017.

ROMACE, B., 1954. *Microscopes.* Moscow: Gostekhizdat , 718 p.

SARKISOV, D.S. and PEROV, Y.L., eds., 1996. *Microscopes.* Moscow: Medicina, 554 p.

SEMENOVA, E., KUNINA, M. and STUKLOV, N., 2013. The role of copper and manganese in iron metabolism. *Doctor*, vol. 12, pp. 47-52.

SIMAKOV, G., NIKIFOROV-NIKISHIN, A.L., NIKIFOROV-NIKISHIN, D.L., BEKETOV, S.V., KOCHETKOV, N.I. and KLIMOV, V.A., 2020. Histological changes in the liver, intestines and kidneys of Clarias gariepinus when using feed with chelated compounds. *International Journal of Pharmaceutical Research*, vol. 12, no. 3, pp. 2380-2391. http://dx.doi.org/10.31838/ijpr/2020.12.03.331.

SKLYAROV, V.Y., BONDARENKO, L.G., KOVALENKO, YU.I., PETRASHOV, V.I., KASHIRIN, A.V. and CHERNYKH, E.N. 2013. Aquaculture of the Russian South: development trends. *VNIRO Papers*, vol. 150, pp. 50-56.

STEGNIY, N. M. and FEDYSHIN, P. M., 2019. *Morphological features of the liver and kidney in carps.* Publishing House VNIRO.

TARLEVA, A.F. 2014. The influence of zinc and copper coming with food on eating behavior of the juvenile *Cyprinus carpio. Problems of Biology in Productive Animals*, vol. 2, pp. 50-56.

TRIFONOVA, E.S., 2008. New approach towards the increase in biosecurity and productivity (experience of using Sub-Pro (Subalin) in fisheries). *Fish Farming and Fisheries*, vol. 7, pp. 20-22.

TURISHCHEV, S.Y., ANTIPOV, S.S., NOVOLOKINA, N.V., CHUVENKOVA, O.A., MELEKHOV, V.V., OVSYANNIKOV, R., SENKOVSKII, B.V., TIMCHENKO, A.A., OZOLINE, O.N. and DOMASHEVSKAYA, E.P., 2016. A soft X-ray synchrotron study of the charge state of iron ions in the ferrihydrite core of the ferritin Dps protein in *Escherichia coli*. *Biophysics*, vol. V61, no. 5, pp. 705-710. http://dx.doi.org/10.1134/S0006350916050286.

USHAKOVA, N.A., NEKRASOV, R.V., PRAVDIN, V.G., KRAVCOVA, L.Z., BOBROVSKAJA, O.I. and PAVLOV, D.S., 2012. New generation of probiotic feed preparations. *Fundamental Research*, vol. 1, pp. 184-192.

VLASOV, V.A., 2018. Use of biologically active supplements in fish feeding. *Fish Farming and Fisheries*, vol. 6, pp. 14-18.

WANG, Z., YU, H., XIE, J., CUI, H. and GAO, X., 2019. Effect of pectin oligosaccharides and zinc chelate on growth performance, zinc status, antioxidant ability, intestinal morphology and short-chain fatty acids in broilers. *Journal of Animal Physiology and Animal Nutrition*, vol. 103, no. 3, pp. 935-946. http://dx.doi.org/10.1111/jpn.13076. PMid:30801843.

YUKHIMENKO, L.N. 2019. *Experimental studies of the effects of feedstuff components on aquaculture facilities under RAS research conditions: research report: branch for freshwater fisheries of FSBNU VNIRO (VNIIPRH); manuscript by P.P. Golovin; person in charge.* Publishing House VNIRO, 14 p.

ZHOU, C., XU, D., CHEN, L., ZHANG, S., SUN, C., YANG, X. and WANG, Y., 2019. Evaluation of fish feeding intensity in aquaculture using a convolutional neural network and machine vision. *Aquaculture*, vol. 507, pp. 457-465. http://dx.doi.org/10.1016/j.aquaculture.2019.04.056.