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Original Article

Histological examination of the young *Oncorhynchus mykiss* intestines using the feed with chelated and probiotic supplements

Exame histológico do intestino jovem de *Oncorhynchus mykiss* utilizando a ração com suplementos quelatados e probióticos

Y. G. Simakov[a]*, A. K. Ponomarev[a], D. L. Nikiforov-Nikishin[a] and N. I. Kochetkov[a]

[a]Faculty of Biotechnology and Fisheries, Moscow State University of Technologies and Management (First Cossack University), Moscow, Russia

Abstract

Macronutrient and micronutrient deficiencies in the fish diet can affect fish growth rate, ability to resist disease, and fillet quality. Determination of effective dose of developed feed additives consisting of chelate compounds of biogenic elements and probiotic preparation based on *Bacillus subtilis*. Young rainbow trout were divided into four groups consisting of 100 fish each. The control group received standard food; the experimental groups received food with chelates and probiotics in different concentrations. At the end of the experiment (30 days), the middle intestine histology and morphometric parameters were studied. The use of chelated compounds alone affected the state of the intestinal villi and the infiltration of the epithelium by lymphocytes. The addition of a probiotic had a positive effect on the migration of immune cells in the intestinal villi; however, the probiotic in high concentrations contributed to a decrease in the number and area of goblet cells. Synergistic effects of chelated compounds and probiotic preparations on the morphometric parameters of the medium intestine were observed. In the proper doses, the studied feed additives might be recommended for rainbow trout farming under industrial aquaculture conditions.

Keywords: feed additives, chelates, intestinal morphology, rainbow trout, aquaculture.

Resumo

As deficiências de macronutrientes e micronutrientes na dieta dos peixes podem afetar a taxa de crescimento dos peixes, a capacidade de resistir a doenças e a qualidade do filé. Determinação da dose efetiva de aditivos alimentares desenvolvidos constituídos por compostos quelatos de elementos biogênicos e preparação probiótica à base de Bacillus subtilis. As trutas arco-íris jovens foram divididas em quatro grupos de 100 peixes cada. O grupo controle recebeu ração padrão; os grupos experimentais receberam alimentos com quelatos e probióticos em diferentes concentrações. Ao final do experimento (30 dias), a histologia do intestino médio e os parâmetros morfométricos foram estudados. O uso de compostos quelados sozinho afetou o estado das vilosidades intestinais e a infiltração do epitélio por linfócitos. A adição de um probiótico teve um efeito positivo na migração de células imunes nas vilosidades intestinais; entretanto, o probiótico em altas concentrações contribuiu para a diminuição do número e da área das células caliciformes. Efeitos sinérgicos de compostos quelados e preparações probióticas sobre os parâmetros morfométricos do intestino médio foram observados. Nas doses adequadas, os aditivos alimentares estudados podem ser recomendados para a criação de trutas arco-íris em condições de aquacultura industrial.

Palavras-chave: aditivos alimentares, quelatos, morfologia intestinal, truta arco-íris, aquicultura.

1. Introduction

Fish is an integral part of the human diet. The growing demand for seafood has led to an increase in fish production. The rainbow trout (*Oncorhynchus mykiss*) is the most common freshwater aquaculture species (MacCrimmon, 1971). It is grown everywhere in the world, with sources of clean, cold (FAO, 2018). The high cost of fillets and caviar makes their production one of the most profitable in the field of industrial aquaculture (Davidson et al., 2014).

Interest in fish and shellfish nutrition has increased markedly over the past two decades, mainly due to the global growth in aquaculture production. In different countries, the maximum economic effect is achieved

*e-mail: simakov.pku@inbox.ru
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using feeds made from the most accessible and cheap components while maintaining a suitable qualitative and quantitative composition of feed for mass rearing. However, the key disadvantage of such feeds is the imbalance of mineral and vitamin composition (Craig, 2014; Dulina, 2018). The quality of the diet is considered one of the important factors in determining the ability of fish to resist disease. Macronutrient and micronutrient deficiencies in the fish diet can affect fish growth rate, as well as fillet quality (Lall, 2000). Another problem of fish farming in aquaculture is the increase in summer temperatures outside the native habit, which increases the likelihood of nutritional diseases and a decrease in the function of the immune system (Brander et al., 2017). Thus, the need for a complete diet to improve the health and prevent diseases of cultured aquatic animals is widely recognized.

To increase the volume of fish products grown, a series of measures are commonly taken to expand the production of existing fishery enterprises. Among others, one of the most effective ways to intensify aquaculture is providing feed additives that can significantly change the quality of the nutrition used. The addition of mineral supplements and probiotic bacteria to feed can positively affect the digestibility of food and indirectly affect the immune function of intestinal cells (Ramos et al., 2015).

In standard feeds, the high content of mineral premixes may result in an ionic imbalance and the composition of its own microbiota, which adversely affects the growth of fish and the digestibility of feed (Rimoldi et al., 2018). However, a lack of minerals such as iron can lead to stunted fish growth and loss of productivity. Iron plays an important role in cellular respiration, oxygen transport, and mitosis (Antony Jesu Prabhu et al., 2016). The composition of the prepared feed is best adjusted by using metal chelates (Fe, Mg, Zn, Se, I, Cu) due to their high bioavailability, which allows them to be used in much lower concentrations than mineral salts (Shao et al., 2010; Buentello et al., 2009; Apines et al., 2003).

Studies conducted on various fish species (*Clarias gariepinus, Oncorhynchus mykiss, Cyprinus carpio*) showed both acceleration of growth rate and changes in intestinal morphological parameters (goblet cell size, absorbent epithelium thickness) (Simakov et al., 2020; Nikiforov-Nikishin et al., 2021, 2022).

Thanks for the comments. Corrections were made in lines 123, 126-127, 157-158. Studies conducted on various fish species (Clarias gariepinus, Oncorhynchus mykiss, Cyprinus carpio) showed both acceleration of growth rate and changes in intestinal morphological parameters (goblet cell size, thickness absorbent epithelium) (Simakov et al., 2020; Nikiforov-Nikishin et al., 2021, 2022).

A high stocking density brings upon the likelihood of infection with bacterial and parasitic diseases (Zhou et al., 2010a). The use of medications such as antibiotics in aquaculture is highly regulated because their use may pose a risk of spreading antimicrobial resistance. Simultaneously, the risk of diseases caused by the consumption of seafood increases (Gatesoupe, 2010). There is a need for alternative sustainable therapies. Various treatments have been proposed, but knowledge of the microbiota in the digestive tract of cultured fish is still very limited (Martínez-Porchas and Vargas-Albores, 2017). Probiotics are especially noteworthy for researchers looking for alternatives to traditional growth promoters in animal nutrition. Probiotics are introduced into the diet to maintain the balance of animals' intestinal flora, prevent diseases of the gastrointestinal tract, and improve, prevent diseases of the gastrointestinal tract, improve the digestibility of feed, leading to an increase in animal zootechnical parameters (Azevedo and Braga, 2012). Numerous scientific studies show the positive effect of supplementation with probiotic strains in the diet of poultry, pigs, cattle, fish, crustaceans, clams, and amphibian (Azevedo and Braga, 2012). The genus *Bacillus* is a component of the natural microflora of bony fish involved in the formation of immunity and maintenance of the intestinal barrier function (Llewellyn et al., 2014). The use of *Bacillus spp.* probiotics demonstrated an increase in the resistance of their organism to diseases of a bacterial nature in many species of fish reared under aquaculture conditions (Raida et al., 2003; Ramos et al., 2017; Ferguson et al., 2010). It is possible that at low temperatures of rainbow trout cultivation, the effect of its use will not be so significant.

The purpose of this study is to determine the effective dose of the developed feed additives, consisting of biogenic elements chelate compounds and a probiotic additive, for growing juveniles of *Oncorhynchus mykiss*. These premixes were tested at the histological level in other studies on other fish species, proving their effectiveness in changing the state of intestinal tissues (Simakov et al., 2020).

2. Methods

2.1. Experimental design

Young rainbow trout (*Oncorhynchus mykiss*) were kept in the "Trout Paradise" fish farm (Shebekino, Belgorod region, Russia), in $8 m^3$ basins at a water temperature of 8 ± 1 °C. Fish with a weight of 30 ± 5 g and length of 11 ± 1.3 cm were divided into four groups (three experimental and one control) of 100 individuals of the same size and weight composition in each group. The groups were seated in pools with a volume of $8 m^3$. During the pilot study, all applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Before starting the experiment, the fish were fed with standard compound feed produced in the Russian Federation (RF). The composition of the feed is shown in Table 1.

The fish were fed with standard feed (granule size 3 mm) (Table 1). The control group (C) received standard feed without additives. The experimental group (O1) received standard nutrition with the addition of chelated compounds of biogenic elements (Table 2). Experimental groups (O2-O3) received standard feed, supplemented with chelate compounds and a probiotic, at various concentrations (Table 2). The fish were fed manually, 2 times a day. The experiment lasted for 30 days.

2.2. Feed additives production

The composition of the chelate additive was developed by a research group and manufactured based on technological production (LLC "Jupiter", Russia). The strain of probiotic bacteria *Bacillus subtilis* BKTM B-2335 was obtained from the preparation SUB-PRO (manufactured by LLC VectorEuro, Moscow, Russia). The choice of concentrations of the studied feed additives was based on the results of previous studies (Simakov et al., 2020) and literature data (Ramos et al., 2017) (Table 2). Coppens PRE GROWER 3 mm (Coppens, Netherlands) was used as the base feed. The addition of chelates and probiotic additives to granulated feed was carried out according to the following technology (Sklyarov, 2008): (i) under sterile conditions, an aqueous solution of chelate compounds was diluted in distilled water to certain experimental concentrations; (ii) by spraying, an aqueous solution of chelate compounds was applied to the granulated feed. The granules were dried using a desiccator (Binder FDL 115, Germany) to reach the initial moisture content; (iii) the probiotic preparation, on a lactose substrate, was diluted in distilled water at a temperature of 30-35 Cº, and the resulting solution was evenly distributed throughout the entire mass of the feed and dried accordingly. The amount of processed feed was rated for three days of feeding and stored in a refrigerator to preserve the properties of probiotics.

Table 1. Composition of standard feed pellets *Oncorhynchus mykiss*.

Ingredients	Amount
Crude protein (%)	42
Cride Fat (%)	13
Crude Fibre (%)	2.39
Crude Ash (%)	7.3
Phosphorus(%)	0.85
Calcium (%)	1.1
Sodium (%)	0.2
Vitamin A (IU/kg)	1000
Vitamin D3 (IU/kg)	2274
Propulgallate (mg/kg)	53
Butylated hydroxyanisole (mg/kg)	53

2.3. Histological examination

A detailed study of histology and morphometric parameters of juvenile trout midgut was carried out to identify the regularities of the combined action of organo-mineral additives and probiotics.

At the end of the experiment, 5 specimens of *Oncorhynchus mykiss* without visible damage were selected from each experimental group. According to standard methods (Humason, 1962), histological analysis was carried out at the premises of the aquatic center of the Institute of Biotechnology and Fisheries of the K.G. Razumovsky Moscow State University of Technology and Management. A sampling of tissue for histological examination was carried out from the middle part of the intestine. The tissue was fixed in a 10% formalin solution. After that, the samples were washed and embedded in paraffin. Histological sections were stained with hematoxylin and eosin (H&E). Histological images were obtained at 40x, 100x, and 400x magnification using an Olympus BX53 microscope (Olympus Corporation, Japan) with an eyepiece attachment Carl Zeiss ERc 5s (Zeiss, Germany) and ZEN lite software ("Zeiss", Germany).

2.4. Morphometric study

Identification of significant differences at the histological level was carried out through a comparison of quantitative indicators characterizing the change in the morphology of the cellular structures of the middle part of the intestine. Studying the morphometric parameters was implemented by image processing with the ImageJ software (an open platform for scientific image analysis) (Rasband, 2020). Measurements of muscle plate thickness and columnar epithelium height were performed according to the protocol described by Pirarat et al. (2011). The number and area of goblet cells, as well as the number of infiltrated lymphocytes, were calculated per 100 µm of the prismatic epithelium of the villi.

2.5. Statistical analysis

Statistical processing of the research results was carried out using the STATISTICA v.10.0 software package (StatSoft Inc., USA) and Microsoft Excel 2019 (Microsoft, USA). All parameters were expressed as means and standard deviation. Comparative analysis between groups was performed using variance (ANOVA) and Tukey's posthoc

Table 2. The composition of the studied feed additives and their distribution by groups.

	Control (C)	Experemental group(O1)	Experemental group(O2)	Experemental group(O3)
Fe (mg/kg)	-	10×10^{-3}	10×10^{-3}	10×10^{-3}
Mn (mg/kg)	-	15×10^{-3}	15×10^{-3}	15×10^{-3}
Zn (mg/kg)	-	35×10^{-3}	35×10^{-3}	35×10^{-3}
Se (mg/kg)	-	0.3×10^{-3}	0.3×10^{-3}	0.3×10^{-3}
I (mg/kg)	-	1.1×10^{-3}	1.1×10^{-3}	1.1×10^{-3}
Cu (mg/kg)	-	3×10^{-3}	3×10^{-3}	3×10^{-3}
B. subtilis(CFU/mg)	-	-	2.5×10^{7}	5×10^{7}

test (Tukey HSD test). If the values did not correspond to the normal distribution, the significance was calculated using the Kruskal-Wallis analysis of variance (ANOVA). A $p < 0.05$ value was taken as statistically significant.

3. Results

3.1. Histological changes in intestinal tissue

A detailed examination of the histological specimens of the intestine of the control group (C) (Figure 1A) revealed a high degree of lymphocytic infiltration of *lamina propria* and intestinal epithelium. All studied preparations showed a normal structure of the *lamina propria* and goblet cells. In the basal cell area, the darkly stained cytoplasm contained a nucleus; closer to the apical part, the basophilia significantly decreased. No pathological changes were found in the intestinal tissues of the control group.

In the experimental group O1, when fed with standard food with the addition of chelated compounds of biogenic elements, the histological structure of the intestine changed. On some of the studied preparations, desquamation phenomena were found, not accompanied by infiltration of blood cells (Figure 1B). The epithelium of some part of the villi was marked with a large number of absorbing vacuoles. Despite this, most of the villi on the studied preparations were close to normal. The minimum value of infiltrated lymphocytes, an average number of goblet cells, and the allowable width of *lamina propria* (Figure 1.(C)) do not unequivocally confirm the negative effect of chelated microelements in this study group.

In the experimental group O2, which received food with the addition of a chelate and probiotic additive (2.5×10^7 CFU / mg), histological changes were also observed, compared with the control group (C). Expanded goblet exocrinocytes were found throughout the entire area of the studied preparation. There was a slight expansion of *lamina propria* and its infiltration with lymphocytes, which are also present in the basal epithelium (Figure 1D). In the epithelium of the villi, as well as in the O1 group, there were absorbing vacuoles of a larger size than in the other groups. No signs of epithelial desquamation and its erosion were found in this group.

The state of intestinal tissues of the experimental group O3, receiving a chelate and probiotic additive nutrition (5×10^7 CFU / mg), was close to that of the control group (C). There were practically no pathological deviations noted in other experimental groups. The mucous membrane with a small number of villi was dilated. Moreover, such changes were always accompanied by significant lymphocytic infiltration (Figure 1E) compared with the control group. In general, the histological state of the intestine in this group can be characterized as close to normal. Most of the nuclei were in the basal cell area and had natural basophilia; a certain number of goblet cells were distinguished by active secretion (Figure 1F). The condition of the muscular plate of the mucous membrane in all groups did not differ from the control values and showed no pronounced pathological changes.

3.2. Morphometric changes in intestinal tissue

Morphometric changes in the structural components of the middle part of the intestine are shown in Figure 2. In the experimental groups O1, O2 and O3, a significant increase in the height of the villous epithelium was noted by 39.38% ($p < 0.05$), 52.57% ($p < 0.05$) and 13.90% ($p < 0.05$) in relation to the control group (C), respectively (Figure 2a). In all experimental groups, the thickness of the muscular plate of the mucosa was less than in the

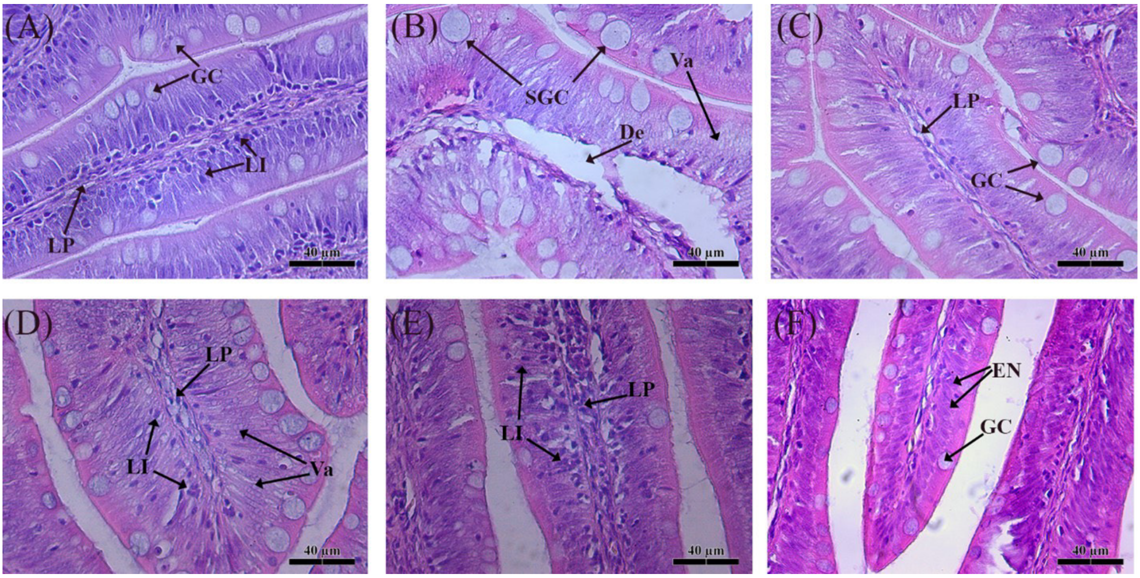

Figure 1. Histology of the middle intestine *Oncorhynchus mykiss*. (A) - control group; (B), (C) - experimental group O1; (D) - experimental group O2; (E), (F) - experimental group O3. Explanations: GC - goblet cell; LP - own lamina of the mucous membrane (*lamina propria*); LI - leukocyte infiltration; SGC, swollen goblet cell; Va - vacuolization (absorbing vacuoles); De - desquamation; EN is the core.

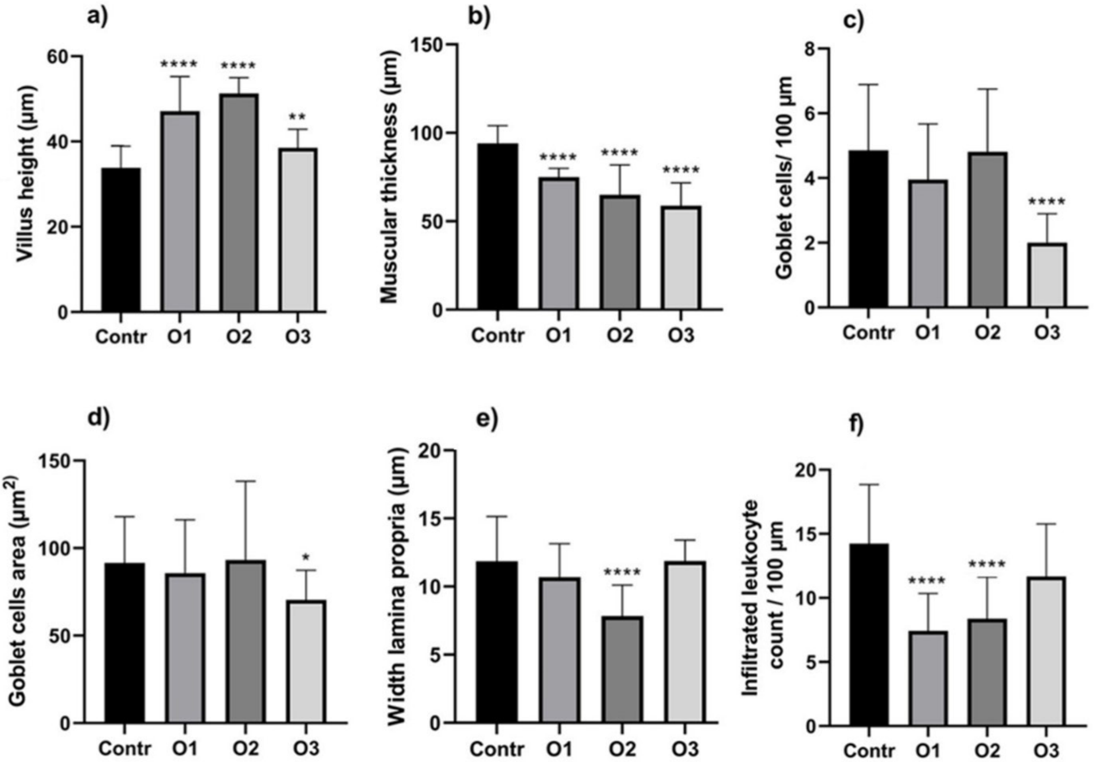

Figure 2. Morphometric parameters of the structural components of the *Oncorhynchus mykiss* middle intestine in the studied groups. The bar graph is presented as mean ± standard deviation. A significant difference compared with the control group was noted at $p < 0.05$ (*); at $p < 0.01$ (**); for $p < 0.0001$ (****)

control group: by 20.37% ($p < 0.05$) in the O1 group, by 31.06% ($p < 0.05$) in the O2 group, and by 37, 47% ($p < 0.05$) in the O3 group (Figure 2b). The number of goblet cells in the O1 and O2 groups did not differ from the control group; however, in the epithelium of specimen from the O3 group, they were significantly less, by 58.82% ($p < 0.01$) (Figure 2c). The spread of goblet cells by area in the O1 and O2 groups was similar to the control group, and in the O3 group, it was significantly less, by 24.76% ($p < 0.05$) (Figure 2d). There was no clear correlation between the parameters of the width of the *lamina propria* and the number of infiltrated leukocytes in the epithelium of the intestinal mucosa. The control group (C) had the widest *lamina propria* (11.87 ± 3.27 µm) and the largest number of intraepithelial lymphocytes, blood cells (14.24 ± 4.6 pcs / 100 µm). Only the experimental group O3 was able to approach the values of the width of the *lamina propria* and the number of intraepithelial lymphocytes in the control group (C). In groups O1 and O2, the values of these parameters were significantly different. The width of the *lamina propria* in the O2 group was less by 33.91% ($p < 0.05$) compared to the control (C). Moreover, the number of infiltrated lymphocytes in the O1 and O2 groups was less by 47.89% ($p < 0.05$) and 41.14% ($p < 0.05$) compared with the control group, respectively.

4. Discussion

Minerals are a group of microelements that fish need. Nutrition is the main route of supplying fish with minerals. When grown in industrial aquaculture, fish can also receive the necessary microelements in the form of feed additives. However, imbalance in commercial feed can cause inflammation of the intestinal mucosa. The onset of the inflammatory process may be indicated by areas infiltrated by lymphocytes found on the preparations of the control group (C). Inflammation can also be promoted by the instability of the biocenosis of the intrinsic microflora of the trout intestine (Lyons et al., 2017), which, given a fish size, has not yet been fully formed and cannot compensate for the negative impact of environmental factors. The mucoid coat plays an essential role in intestinal protection. Its density depends on the secretory activity of goblet cells (Pirarat et al., 2011), which maximum degree of development of which noted in control.

Many studies indicate the positive effect of feeds with the addition of chelated microelements on the organization of intestinal tissues of industrially farmed fish (Antony Jesu Prabhu et al., 2016). The researchers note that chelates have a higher bioavailability degree than common inorganic salts (Apines et al., 2003). In some cases, the addition of chelate compounds to the diet led to an increase in intestinal absorption area due to proliferation and hypertrophy of endothelial cells, and an increase in lymphocytic activity, which is interpreted as an immune response enhancement (Shao et al., 2010; Buentello et al., 2009). The results of this study indicate that the use of premixes in the form of chelated microelements (Fe, Mg, Zn, Se, I, Cu) at the indicated concentrations have a positive result. It causes an increase in the height of the villous endothelium and promotes an increase in the intestinal absorption area, and also reduces the concentration of lymphocytes in the epithelial layer of the intestine. Similar results were obtained by other researchers using chelates (Pirarat et al., 2011; Pierri et al., 2021; Romano et al., 2015). An increase in the number of absorbing vacuoles in experimental groups O1 and O2 indicates an increase in the metabolic activity of endothelial cells.

However, in the experimental group O1, which received a standard diet with the addition of chelates, pathological abnormalities were revealed, such as desquamation of prismatic epithelium and erosion of the apical part of the intestinal villi. Most likely, these pathological abnormalities are a consequence of the toxic effect of excess Fe ion, which can react with the formation of free radicals and damage intestinal cells (Pirarat et al., 2011). It is noted that the appearance of pathological abnormalities in the intestines of fish most often occurs during a long period of feeding with chelated compounds (Valko et al., 2006). The use of organic compounds in the feeding of rainbow trout (*Oncorhynchus mykiss*) may cause higher absorption than inorganic compounds (Apines-Amar et al., 2004). This fact requires decreasing the proportion of chelates in feed since large doses can manifest a toxic effect. It is also worth noting that under yearlings fish can directly absorb copper ions from water when they lack it in food (Yamamoto et al., 1981).

In other experimental groups that received probiotics in addition to feeding, no similar pathological abnormalities were found. Thus, it can be assumed that the probiotic bacterium *Bacillus subtilis* leads to the activation of natural mechanisms of protection of intestinal tissues from excessive accumulation of metals in the intestinal lumen by increasing the secretory activity of goblet cells. The presence of such mechanisms in bony fish was previously mentioned in the work of Pierri et al. (2021), which investigated the effect of chelates of trace elements on the characteristics of the intestines of the Nile tilapia (*Oreochromis niloticus*). The researchers also noted that utilization of probiotics, in particular bacteria of the genus *Bacillus subtilis*, increases lysosomal activity and expansion in the number of goblet cells on the intestinal villi, which contributes to an increase in mucin secretion and intestinal protection from poor-quality feed and pathogens (Ramos et al., 2017; Hoseinifar et al., 2018). Some authors suggest that certain probiotic bacteria can directly affect the proliferative activity of goblet cells (Harper et al., 2011). The most frequent consequence of probiotics in the intestines of fish is an increase in the activity of lymphocytes in the *lamina propria* of the intestinal mucosa and epithelium of the intestinal villi, which leads to an increase in the immune response (Irianto and Austin, 2003; Aly et al., 2008).

The gastrointestinal tract structure in predatory fish such as *O. mykiss* differs from the structure of the intestine of omnivorous fish (Simakov et al., 2020). For this reason, the bulk of the intestinal bacterial microflora is concentrated in the pyloric appendage's and its influence on the processes of food assimilation is lower than in cyprinids and sturgeons. Therefore, it can be assumed that the effective doses of probiotic preparations should be higher. However, in the experimental group O3, which received the largest amount of probiotic, a significant decrease in the secretory activity of goblet cells was observed concerning other experimental groups and controls (C). In the case of using probiotics on rainbow trout (*O. mykiss*), the absence of their effect on the number and secretory activity of goblet cells has been repeatedly noted (Ramos et al., 2013; Harper et al., 2011; Ramos et al., 2015). The results obtained by this study once again confirm the thesis that the secretory part of the intestines of rainbow trout is less susceptible to probiotic bacteria, in contrast, for example, from channel catfish (*Ictalurus punctatus*) (Queiroz and Boyd, 1998) and Nile tilapia (*Oreochromis niloticus*) (*Oreochromis niloticus*) (Zhou et al., 2010a).

The revealed regularity of a decrease in the degree of leukocytic infiltration of prismatic epithelium and its mucous membrane in the experimental groups during the use of mineral-chelate compounds and a probiotic additive indicates the suppression of its microflora by a high level of metal ions deposition. In the O2 and O3 groups, this effect is partially compensated by the introduced probiotic microflora. Moreover, the higher the concentration of the probiotic preparation, the higher the measured degree of infiltration. The presence of blood cells in the epithelial layer of the villi, according to many authors, is a manifestation of the intestinal immune response (Ramos et al., 2017). Thus, maintaining homeostasis of the fish's intestinal microflora is a key factor in supporting its health (Zhou et al., 2010b). It should be noted that although the thickness of the muscle plate tends to decrease, it is not accompanied by pathological changes. Perhaps the change in the state of myocytes occurs due to a decrease in the rate of their renewal.

5. Conclusion

This article presents the results of the effect of a mineral-organic supplement and a probiotic preparation on the morphometric parameters of the middle intestine of juvenile rainbow trout (*Oncorhynchus mykiss*). Studies have shown that the use of chelates alone can affect the state of the intestinal villi and the degree of epithelial infiltration by lymphocytes, associated with the suppression of microflora and an increase in the intestinal absorption area due to an increase in the size of the prismatic epithelium. These changes in the intestine can be characterized as positive, affecting the digestion processes, and negative due to decreased immune response. The addition of probiotics at various concentrations positively affected the migration of immune cells in the intestinal villi without affecting the secretory function. However, in the experimental group O3, which received the probiotic in high concentrations, a significant decrease in the number and area of goblet cells was observed. According to the research results, the maximum positive effect was obtained in the O2 group, in which feeds were used combined with a mineral chelate additive and a probiotic in medium concentrations. The dose of the studied feed additives can be recommended for the practical application of growing rainbow trout (*Oncorhynchus mykiss*) in industrial aquaculture.

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