 doi:10.20944/preprints202104.0243.v1

Article

The influence of probiotics of different microbiological composition on histological structure of the organs of the gastrointestinal tract of juvenile Oncorhynchus mykiss

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Simple Summary: In this work, the effect of three probiotic was investigated on the growth processes and the histological state of the gastrointestinal tract organs of juvenile rainbow trout Oncorhynchus mykiss, the most common object of cold-water aquaculture. The action of probiotic preparations led to an increase in the growth of fish and influenced the metabolism of various parts of the gastrointestinal tract. The best effect was observed with a complex probiotic, containing two types of bacteria. Significant changes have been established in the tissues of various parts of the intestine, indicating that all probiotics used have a similar mechanism of action, which reduces inflammation and improves the absorption processes. The smallest effect was obtained with the use of lactic-acid bacteria, since the temperature of growing trout is low for the activity of bacteria of this group. It was found that the amount of the drug recommended by the manufacturer is sufficient for the formation of an active reaction in the gastrointestinal tract of trout, with the exception of the probiotic, which included lactic-acid bacteria. Complex probiotics stand out among other groups, since their effect extends to all parts of the intestine, and possibly contributes to the acceleration of lipid metabolism.

Abstract: This paper studies the influence of three probiotic preparations of various microbiological composition: Bacillus subtilis (O1); Bacillus subtilis + Bacillus amyloliquefaciens (O2); Lactobacillus acidophilus (O3) on the growth process and histological structure of the organs of the gastrointestinal tract of juvenile Oncorhynchus mykiss by morphometric parameters. The effect of the probiotic preparations led to the increase in fish growth and influenced different sections of the gastrointestinal tract. The biggest change was found in the mid intestine and the reliable difference compared to the control was obtained at the following parameters: Lamina propria width, intraepithelial lymphocytes number of prismatic epithelium and goblet cells area. The changes in the pyloric appendages were less obvious but reported as playing an important functional role. The liver preserved normal functional structure in all series of the experiment except for the probiotic group Lactobacillus acidophilus, where hepatocyte small-drop vacuolization was observed. That might be connected with the change of the digest activity resulting from a decrease in secretory activity of the intestinal exocrinocytes. The use of all the probiotic preparations led to the similar change in morphometric parameters, in all the groups it was possible to decrease the immune response.

Keywords: Oncorhynchus mykiss ;Bacillus subtilis; Bacillus amyloliquefaciens; Lactobacillus acidophilus; intestinal histology

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1. Introduction

The rainbow trout is the most widely introduced object of the cold-water aquaculture growing in many countries in the world [1,2]. This fish species is characterized by a high feed conversion due to the lifestyle and the features of the gastrointestinal tract structure. In the industrial cultivation conditions as a rule granulated rational feed is used that generates maximum growth rate, however, can cause intestine and liver functional pathology. One of the reasons for pathology states could be the lack of natural food components and the imbalance in the composition [3]. Many researchers have noticed [4] that including probiotic bacteria into the feed composition may help to restore gastrointestinal tract functions and improve the natural immunity, which is accompanied by reducing the fish death and increasing the output of the finished product. Most probiotic preparations include two bacterial groups: lactic acid bacteria used for the production of the probiotics for livestock animals and humans [5,6]; spore-forming microbes of the genus Bacillus widely spread in natural environment and belong to the natural microflora of poikilothermic animals [7,8]. The mechanism for the effect of the groups of organisms is to suppress the opportunistic intestinal microflora, as the products of its metabolism are inhibitors of pathogenic microflora [9]. Thus, probiotic bacteria can be used as an alternative of antibiotics [10]. For many fish groups the groups of organisms are proved to have a significant influence on the gastrointestinal physiology, intestinal morphology and liver structure [11,12]. However, there are specific differences of the effects of probiotic preparations expressed in the extent of immune response, proliferative activity and the secretory functions of different tissues [13]. For these reasons, individual selection of a probiotic preparation for each fish species is an actual aquacultural problem. Natural intestine microflora of fish consists of dozens of bacteria types, some of which play a vital role in homeostasis maintenance: Proteobacteria, Firmicutes Bacteroidetes [14,15], that is why the application of one component probiotic preparations could not greatly influence the ratio of bacterial community [16]. Modern probiotic preparations include several different types of probiotic bacteria of different functions, which has a positive impact on bacterial colonization [17]. Proceeding from the above, the aim of the study was to assess the condition of the gastrointestinal organs of Oncorhynchus mykiss on histological level using three types of probiotic bacteria of different microbiological content and to evaluate their impact on the growth rate. The key hypothesis of the study was the assumption that probiotics with different types of microbiological cultures will contribute to improvement of the morphological condition of the gastrointestinal organs better than probiotics with monoculture.

2. Materials and Methods

2.1. Object of study

This Juvenile Oncorhynchus mykiss, of the original size 13±0,3 cm and weight 30±1,3 gr was divided into four groups one of which was the control group. During the whole experiment period the fish were kept in experimental pools with a capacity of 1000 l ofrecirculating aquaculture system facilities with the mechanical and biological filtering and replacing 10 percentage of the water. Water temperature was 15±1 °C, pH 6.8±0.4, the oxygen content remained above 8 mg/l. For the experiment the juvenile fish were selected of the uniform size without visible injury. The experiment lasted 30 days. The тsize/weight control was carried out every 7 days.

The study was complied with the guidelines of the Local Ethics Commission of the Institutional Review Board of Moscow State University of Technology and Management (approval number 3, 14/7/2020).

2.2. Experemental diets

The juvenile Oncorhynchus mykiss was fed with production granulated feed of a well-known European manufacturer. The composition of the experiential diet is presented in Table 1.

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Table 1. The composition of the granulated feed used in the experiment.

Ingredients	Control diet	Diet O1	Diet O2	Diet O3
Crude protein	42	42	42	42
Cride Fat	13	13	13	13
Crude Fibre	2.39	2.39	2.39	2.39
Crude Ash	7.3	7.3	7.3	7.3
Phosphorust	0.85	0.85	0.85	0.85
Calcium	1.1	1.1	1.1	1.1
Sodium	0.2	0.2	0.2	0.2
Vitamin A (IU/kg)	1000	1000	1000	1000
Vitamin D3 (IU/kg)	2274	2274	2274	2274
Iron chelat (mg/kg)	60	60	60	60
Iodine (mg/kg)	5	5	5	5
Mangaense chelat (mg/kg)	20	20	20	20
Copper chelat (mg/kg))	5	5	5	5
Zinc chelat (mg/kg)	60	60	60	60
Selenium (mg/kg)	0.2	0.2	0.2	0.2
Propulgallate (mg/kg)	53	53	53	53
Butylated hydroxyanisole (mg/kg)	53	53	53	53
Bacillus subtilis (CFU/kg)	-	$12*10^7$	$12*10^7$	-
Bacillus amyloliquefaciens (CFU/kg)	-	-	$10*10^9$	-
Lactobacillus acidophilus (CFU/kg)	-	-	-	$20*10^7$

Probiotic preparations were used in the experiment after microbiological studies confirming the composition and the accordance of quantity of bacteria with the stated numbers. The control group of species received feed without additives, the experimental groups received feed with probiotic preparations of various microbial composition in the number determined by other studies on trout and other species of fish [10, 18, 21, 22].

The introduction of the probiotic preparations in accordance with the stated concentrations (Table 1) into the ready-made feed was based on standard methods [19]. The preparation on a lactose substrate was diluted in distilled water. The lactose substrate preparation was evenly distributed on the feed moisturized up to 40% in sterile conditions, and then the feed was dried to the initial values of humidity using a drying cabinet (Binder FDL 115, Germany) and was kept in conditions preventing the development of the outside microflora. The feed was prepared for use every three days to ensure the preservation of the activity of probiotic preparations

The fish was fed three times a day. The feed was given in equal dozes determined by the generally accepted feed tables for the particular species and size of fish.

2.3. Histology

Fish was killed with the help MS-222 in the dose of 100 mg/l [20]. Selection of samples of liver, mid intestine and pyloric appendages was carried out in sterile conditions for three fish in each group (n = 3) at the end of the experiment. The tissue samples were fixed in 4% neutral formalin during 24 hours, and then they were dehydrated among graduated alcohols and got in paraffin wax. The slices (4 μm) were stained with hematoxylin and eosin (H&E) and examined under a light microscope.

2.4. Morphometry

Fish Histological slices were studied through light microscope Olympus BX53 («Olympus Corporation», Japan) with ocular attachment Carl Zeiss ERc 5s («Zeiss», Germany) and software ZEN lite («Zeiss», Germany). For measuring morphometric characteristics, the program ImageJ (National Institutes of Health, USA) with an open source was used. The nucleus area (n = 20), the number of vacuoles per 1000 microns of liver parenchyma (n = 30) and the area of vacuoles were measured in the middle liver section

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in two fish of one group according to the n of measurements on one slice. The height of endothelin (n = 60), the thickness of muscular layer (n = 30), the number of goblet cells per 100 microns of epithelium (n = 20), the area of goblet cells (n = 30), the thickness of lamina propria (n = 40), the number of intraepithelial lymphocytes per 100 microns of epithelium (n = 20) were measured in the mid intestine and pyloric appendages in two fish of one group according to the studies [1,2,25].

2.5. The growth rate

The calculation of the size/weight characteristics was made at the beginning and at the end of the experiment for species of all the studied groups (n = 30) according to the following formulas:

$$Specific\ growrh\ rate(SGR) = ln(final\ wt(g) - ln(start\ wt(g))/(n(day)) * 100 \quad (1)$$

$$Feed\ conversin\ ratio(FCR) = (dry\ feed\ intake(g))/(final\ wt(g) - start\ wt(g)) \quad (2)$$

$$Survival\ rate = (final\ N * 100)/(startN) \quad (1)$$

where: wt – fish weight, N – number of fish.

2.5. Statistical analysis

The comparison of the numerical data between different groups was made with the use of the non-parametric method of Kruskal-Wallis if the variables did not fit the normal distribution. For the comparison of the morphometric parameters complying with the normal distribution, one-way ANOVA was used with the following Tukey's test for post hoc analysis.

The level of confidence was chosen $P < 0.05$ the results were presented as the average ± SD (standard deviation).

3. Results

3.1. Growth Performance

Table 2. Fish-breeding-biological characteristics of juvenile trout during the experiment and the efficient use of feed. The value followed by the superior letters (a, b, ab) were reliably different. The value ($p < 0.05$) from one-way ANOVA with comparison using Tukey's test for post ho analysis between the experimental groups and the control.

Ingredients	Control diet	Diet O1	Diet O2	Diet O3
Initial body weight (g)	30±1.5	30±2.1	30±1.3	30±1.1
Final body weight (g)	57.9±7.8[a]	59.1±8.9[a]	66.2±11.3	65.70±9.3
Mortality (%)	0	2	0	2
FCR	1.13	1.18	0.94	0.99
SRG	2.35	2.35	2.83	2.73

During the entire time of the experiment the survivability of juvenile trout and its growth parameters met the fish-breeding standards. The use of probiotic preparations in group O2 showed a reliable ($p < 0.05$) increase in growth and decrease in feed rate (Table 2). Daily feed consumption was calculated according to low-density of fish and a quite high temperature of water, which ensured a maximum fish growth dinamic. At the end of the experiment parts of the gastrointestinal tract were studied in detail to assess the influence of probiotic preparations on histological characteristics that in turn show the efficience of digestion processeses on the cellar and tissue level.

3.2. Intestinal histology

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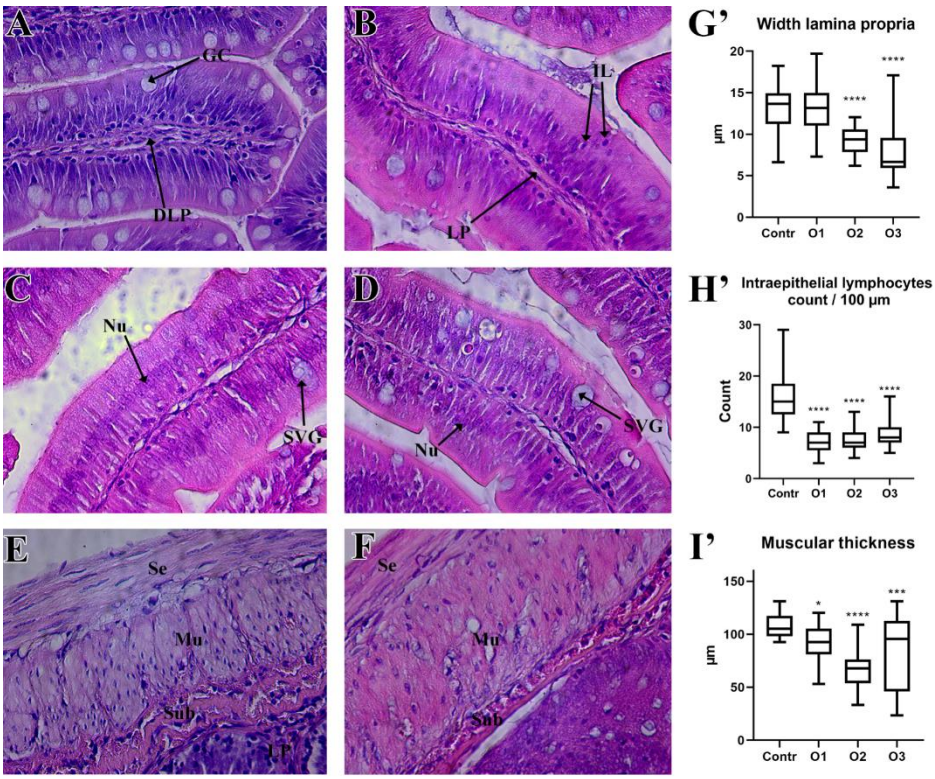

Figure 1. Histology of the mid intestine of fish and the results of morphometric measurements; **(a)**: Control group, **(b)**: group O1, **(c)**: Group O2, **(d)**: Group O3, **(e)**: Control group, **(f)**: Group O2, **(g')**: Width of lamina propria, **(h')**: The number of intraepithelial lymphocytes per 100 of prismatic epithelium, **(i')**: Muscular thickness. GC – goblet cell; DLP – distended lamina propria; IL – intraepithelial lymphocytes; LP – lamina propria; Nu – endotheliocyte nucleus; SVG –swelling goblet cell ; Se –serosa; Mu – muscular layer; Sub – submuscular. The value (* - $p < 0.05$; **** - $p < 0.0001$) from one-way ANOVA with comparison using Tukey's test for post ho analysis between the experimental groups and the control. H&E, 400x

The study of histological structure of the mid intestine did not find any serious pathological deviations in all the studied groups. The control registered a slight extension of lamina proria at some villus (Figure.1 (A)) and normal organisation of prismatic epithelium and goblet cells. In group O1 most villi had hight of their intestinal mucosa close to the control (Figure. 1 (G')). The number of the intraepithelial lymphocytes was reliably ($P < 0.001$) less than in the control group (Figure. 1 (H')). Also the group was noted to have the most area of goblet cells (Figure. 4 (C)), reliably different from the control ($P < 0.001$), at the same time their number per 100 microns (Figure. 3 (B)) of prismatic epithelium was also reliably different ($1 < 0.001$) from the control.

The thickness of lamina propria (Figure. 1 (G')) in-group O2 was reliably less ($P < 0.001$) than in the control. The villus structure in the group was not different from normal, it was possible to distinguish separate nucleus of prismatic epithelium and goblet cells that were distended at some villi (Figure. 1 (C)). It was noted that the group had the least thickness of goblet cells (Figure. T (B)) but their area was close to the control. The structure of the mid intestine in group O3 could also be characterised as normal. The parametre of lamina propria thickness is esposed to the maximum variation, although the average value of this indication is reliably less than in the control group ($P < 0.001$). As well as in other experimental groups group O3 showed a decrease in thickness of goblet cells by 100 µm of prismatic epithelium ($P < 0.001$) (Figure. 4 (B)), compared to the control. But their area remained unchanged (Figure. 4 (C)).

The study of histological preparations allows to conclude that in all the experimental groups the structure of the muscular layer of the mid intestine does not have visible

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injures that may influence the process of digestion. (Figure. 1 (E, F)). All the experimental groups showed the decreased trend in thickness of the muscular layer of the middle part of the intestine (Figure. 1 (I')), though at some parts of the preparation in group O3 it was thickened to normal values but the average value was reliably lower than the control ($P < 0.01$).

3.3. Pyloric appendages histology

The study of histological slices of pyloric appendages did not find any visible pathological deviations in all the studied groups. The thickness of goblet cells per 100 μm of prismatic epithelium was equal in all the groups (Figure. 4 (E)). In the control group a dilatation of its mucous coat (Figure. 2 (A)) at most part of villi and a big number of intraepithelial lymphocytes was fixed (Table 3). Absorbent vacuoles were distributed across the entire area of prismatic epithelium. Group O1 was characterized by a normal intestine tissue structure (Figure. 2 (B)): the most part of prismatic epithelial cells had distinct nucleus, the width of its mucous coat (Figure. 2 (H')) was close to the control value, the area of goblet cells (Figure. 3 (F)) was reliably less than in the control ($P < 0.001$).

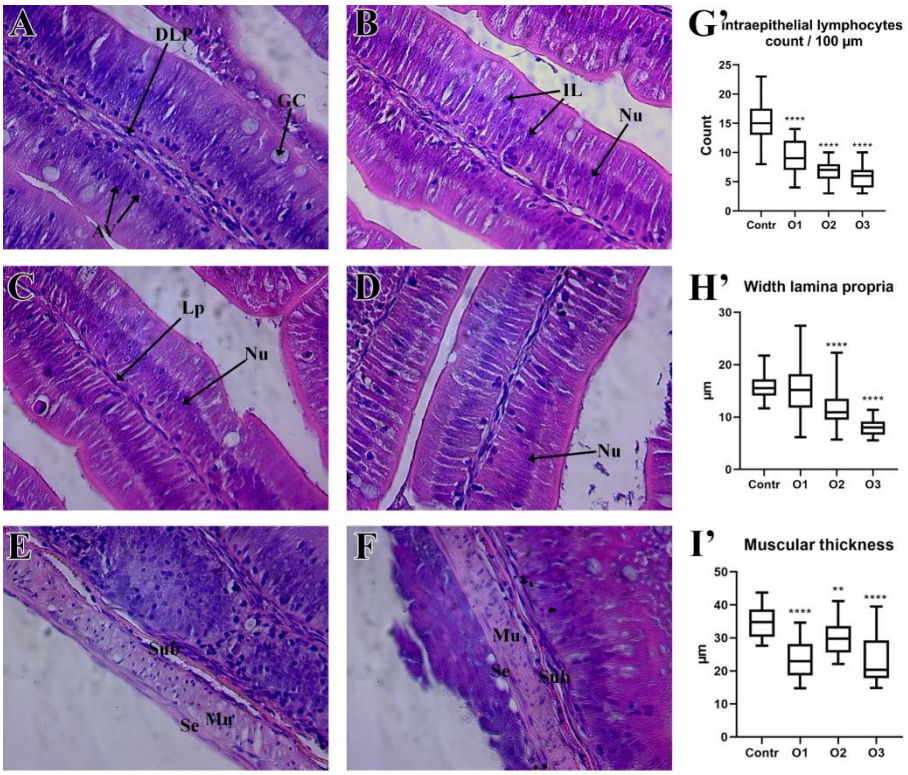

Figure 2. Histology of pyloric appendages of fish and the results of morphometric measurements; **(a)**: Control group, **(b)**: Group O1, **(c)**: Group O2, **(d)**: Group O3, **(e)**: Control group, **(f)**: Group O2, **(g')**: The number of intraepithelial lymphocytes per 100 of prismatic epithelium, **(h')**: width of lamina propria, **(i')**: muscular thickness. GC – goblet cells; DLP – distended lamina propria; AV – absorbing vacuoles; IL – intraepithelial lymphocytes; LP – lamina propria; Nu – endotheliocyte nucleus; SVG – swelling goblet cells ; Se –serosa; Mu – muscular layer; Sub – submuscular. The value (* - $p < 0.05$; **** - $p < 0.0001$) from one-way ANOVA with comparison using Tukey's test for post ho analysis between the experimental groups and the control. H&E, 400x.

This group noted to have a reliable decrease in intraepithelial lymphocytes ($P < 0.001$) comparing to the control. Lamina propria of group 3 was reliably less ($P < 0.001$) than without using probiotic preparations (Figure. 2 (G')). There was also a reliable difference

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from the control in values (P < 0.001): the prismatic epithelium height of villi (Figure. 2 (A)) and the

number of intraepithelial lymphocytes (Figure. 2 (G')), both values were less than the control parameters. The least number of intraepithelial lymphocytes was fixed in the control group 3 (Figure. 2 (G')) reliably different from the control values (P < 0.001). It was possible to note that the most part of the villi of the group had a normal structure of prismatic epithelium (Figure. 2 (D)) with well stained nuclei and also close to the normal width of the mucous coat, reliably less than in the control (P < 0.001). Adding probiotics based on Lactobacillus acidophilus into the feed did not influence the area of goblet cells (Figure. 2 (F)), however, led to a considerable decrease (P < 0.001) of the epithelium height (Figure. 2 (D)). Walls of pyloric appendages has a normal histological structure on all the studied preparations (Figure. 2 (E, G)): three layers could be distinguished including srosa, muscularis and submuscularis. The thickness of muscular layer in all the experimental groups was reliably less than the control (P < 0.01).

Table 3. The results of morphometric measurements of different histological parameters of control and experimental fish. The value is presented by the average ± SD. The value that is followed by the letters of the superscript (a, b, ab) was reliably different. The value (p <0.05) from one-way ANOVA with comparison using Tukey's test for post ho analysis between the different experimental groups and control.

3.4. Liver histology

Parameter	Contr	O1	O2	O3	p value
		Mid itestines			
Villus endothelium height (μm)	42.93±8.18^{a}	43.11±8.35^{a}	52.07±8.11	46.77±9.35^{a}	<0.0001
Muscular thickness (μm)	108.4±11.96	91.03±16.4^{a}	66.76±20^{ab}	83.73±36.65^{a}	0.0004
Goblet cells/ 100 μm	4.571±1.74	2.238±0.88a	0.9524±0.8^{ab}	1.524±1.25a	<0.0001
Goblet cells area (μm^2)	81.58±15.99^{a}	150.6±56.68	122.1±61.99	120.1±49.83	0.0002
Width lamina propria (μm)	13±2.76	13.07±2.86	9.232±1.59^{a}	8.272±3.38^{a}	<0.0001
Intraepithelial lymphocytes count / 100 μm	15.95±5.04	7.19±2.18^{a}	7.524±2.18^{a}	8.857±2.67^{a}	<0.0001
		Pyloric appendages			
Villus endothelium height (μm)	60.31±8.13	45.05±7.45^{ab}	53.6±6.83^{a}	44.18±5.14^{ab}	<0.0001
Muscular thickness (μm)	34.74±4.87	23.41±5.48^{ab}	30.08±5.14^{a}	23.23±6.74^{ab}	<0.0001
Goblet cells/ 100 μm	1.619±1.24	1.238±0.76	1±0.7746	1.476±1.03	0.1759
Goblet cells area (μm^2)	128.9±40.33	51.13±18.68^{ab}	64.87±16.6^{ab}	104.9 56.19	<0.0001
Width lamina propria (μm)	15.78±2.27	14.94±4.536	11.93±4.03^{ab}	8.118±1.68^{ab}	<0.0001
Intraepithelial lymphocytes count / 100 μm	15.52±3.8	9.286±3.02^{a}	6.762±1.7^{ab}	5.81±1.8^{ab}	<0.0001
		Liver			
Hepatocyte nucleus area (μm^2)	30.66±6.44^{a}	31.41±5.53^{a}	27.37±2.26^{a}	41.08±8.63	<0.0001
Number of vacuoles amount / 100 μm^2	1.55±1.66^{a}	0.5±0.68^{a}	0.1±0.3^{ab}	4.45±2.78	<0.0001
Hepatocyte vacuole area (μm^2)	22.18±7.92^{a}	47.79±19.7	17.84±13.2^{a}	26.29±8.48^{a}	0.0002

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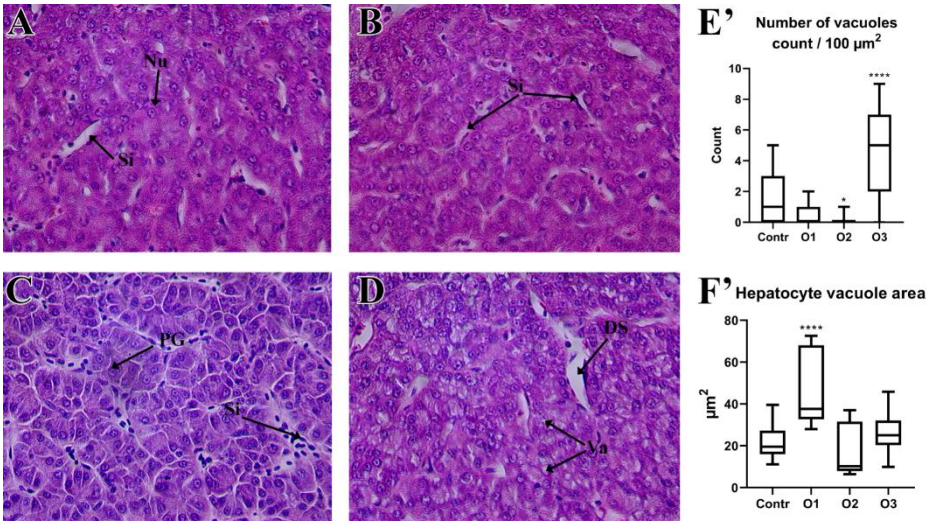

Figure 3. Histology of fish liver and the results of the morphometric measurements; **(a)**: Control group, **(b)**: Group O1, **(c)**: Group O2, **(d)**: Group O3, **(e')**: The number of vacuoles per 100 μm 2 of liver parenchyma, **(f')**: The area of vacuoles of hepatocyte. Nu – hepatocyte nucleus; Si – sinusoid capillary; PG – polyploid hepatocyte; DS – distended sinusoid capillary; PG – polyploidy hepatocyte; DS – distended sinusoid capillary; Va – vacuolization. The value (** - p <0.01; **** - p <0.0001) from one-way ANOVA with comparison using Tukey's test for post ho analysis between the different experimental groups and control. H&E, 400x.

In the control and all the series of the experiments the liver retained normal histological structure. In the control the structure of hepatocytes did not have any deviations (Figure. 3 (A)), which nuclei could be distinguished without signs of necrosis and also a slightly distended sinusoid capillary. Using probiotic preparations based on Bacillus subtilis (group 2) signs of the initial degree of vacuolization begin to appear and the vacuoles have the reliable (P < 0.001) largest size (Figure. 3 (F')) in all the studied groups. In addition, liver sinusoids in this group had a normal size. (Figure. 3 (B)). In group O2 liver parenchyma mostly complied with the standards: separate hepatocytes with normal nuclei may be distinguished, hepatic cords are clearly visible and there is a sinusoid blood circulation (Figure. 3 (C)). Some polyploid hepatocytes were also found. In the group using preparation based on Lactobacillus acidophilus (group O3) the liver has signs of small drop vacuolization. The number of vacuoles per 1000 μm 2 (Figure. 3 (E')) reliably more than the control (P < 0.001). In the group there is also a reliable (P < 0.001) increase in the hepatocyte nucleus area (Figure. 4 (E)).

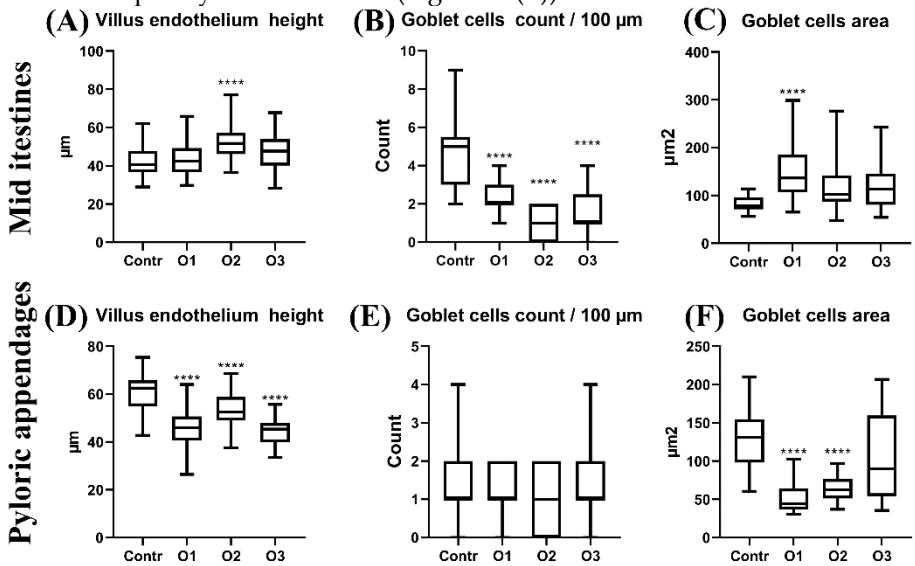

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Figure 4. Morphometric parameters of the mid intestine, pyloric appendages.

4. Results

The set of experiments allows to establish the differences in the use of probiotic preparations. The results confirm the influence of probiotics on histological structure of intestine and liver tissues. The studies of the use of probiotics on Oncorhynchus mykiss have been conducted by many scientists using various species of microorganisms [8,12,18,25,26]. According to the authors, the use of probiotics is reasonable only when natural intestinal microflora needs correcting and for improvement of digestibility of feed with unbalanced composition [3,27]. Under such conditions, probiotic effect was the strongest. Adding probiotics to high quality production feed has more likely preventive function and has a little influence on biomass accumulation process of fish, at the same time it stimulates the immune response of fish intestine increasing survivability and viability as shown in a number of works [23, 29-30].

The study demonstrates not only a positive influence of some parts of the gastrointestinal tract on morphological structure, but also the increased effectiveness of feed and growth of fish. Thus, in group O2 feed coefficient decreased by 12.9%, other groups did not demonstrate such a significant decrease. At the histological level, the appearance of the following tendencies could be noted: all the used probiotic preparations regardless their composition led to reliable changes of morphometric parameters of trout intestine. Group O1, that used preparation including one type of bacteria Bacillus subtilis was marked as keeping lamina propria width in the middle part at the control level but the number of intraepithelial lymphocytes reliably increased reflecting a microbe change of the intestinal microflora composition and the immune response stabilization. A decrease in inflammation was also observed in all the experimental groups both in the mid intestine and pyloric appendages, thus, indicating a similar nature of the mechanism of action of all the probiotic preparations and in every case, it reduces the number of intraepithelial lymphocytes. In most studies, an opposite effect of probiotic preparations was noted [32-35].

The results obtained in this paper could be explained by difference of keeping conditions, age characteristics and composition of the basic diet [34, 36]. Villus endothelium height might indicate the change of digestive activity and nutrient absorption area [36]. Using probiotic preparations consisting of two types of bacteria (group O2) prismatic epithelium height in the mid intestine achieves maximum value. As only group O2 had a reliable endothelium increase, it can be assumed that the combined effect of different microorganisms stimulates endothelial cell hypertrophy. All the rest groups didn't show a reliable difference on this indicator. In pyloric appendages, in turn the prismatic epithelium cell height reliably decreased in all the experimental groups. Accordingly, it could be affirmed that the endothelium of pyloric appendages is more sensible to probiotic preparations. It may be assumed that during intensive feeding the main pressure on absorbing epithelium is in the anterior and mid intestine and pyloric appendages play a minor functional role. Regardless, in prismatic epithelium of villus pyloric appendages in the control group a big number of absorbing vacuoles was found indicating an active searching process of digestion. In other experimental groups, this number of vacuoles was not found proving the hypothesis of less functional role of pyloric appendages using probiotic additives.

The authors according to two parameters assessed secretory activity of the intestine exocrinocytes: goblet cells density per 100 microns of prismatic epithelium and their area. The examination of mid intestine showed a reliable decrease of the number of goblet cells in all the experimental groups, though the average dense of cells increased only in group O1. A decrease of secretory activity of goblet cells in the mid intestine may be connected with stabilization of microbial intestine community and, consequently, the constancy of chemical composition of the inner intestinal environment [37]. A decrease of goblet cells number and an increase of goblet cells area could be characterized as a general tendency of the probiotic preparation effect noted in a number of works [38, 39]. The preparation

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based on Lactobacillus acidophilus (group O3) has the least influence on the exocrinocytes area, both in the mid intestine and in pyloric appendages. It can be assumed that the low temperature of trout cultivation (in the experiment 14±1 °C) inhibits the effect of this type of lactic acid bacteria and prevents from realizing their probiotic properties. However, according to Rimoldi S. et [41], trout intestinal microbiota has several types of Lactobacillus, thus, making it impossible to assert the full probiotic inhibition in group O3.

The histological pattern of the intestinal wall layers and pyloric appendages in all the experimental groups was close to the standard indicating the absence of catarrh inflammation. It could be concluded that the probiotic preparations effect on the tissues is quite restricted [15]. Group O3 marked the most variation of the muscular layer thickness that might be connected with the increased peristaltic activity aiming at speeding transit of hummus. In the other experimental groups, the thickness of muscular layer was reliably less than the control values. The decrease of the activity of muscular myocytes might contributes to a slower movement of chyme through the gastrointestinal tract and, consequently, improves digestion. It could also result from lower secretory activity of goblet cells [24], thus, mucin helps digestion and movement of the food coma. The muscular thickness of pyloric appendages is always less than the mid intestine regardless the used probiotic preparation, which is physiological norm for this fish species. Histological structure of the liver while using probiotic preparations in diet group O1 and O2 remained almost the same and was close to the control values.

Morphology of liver parenchyma in group O2 was characterized by the normal structure in accordance with all the evaluation criteria under consideration, which demonstrates the absence of metabolic disorder of trout and a high feed quality. Gonzalez-Felix M. L. et al. note that bacteria could lead to a reduction of glycogen and lipid accumulation in the liver [12]. However, trout liver is a very dynamic organ acting in many metabolic processes and its influence of probiotic effects needs further research. The liver of group O3 in turn was exposed to small-drop vacuolization, sinusoid capillary dilatation and a reliable increase of the hepatocyte nucleus area. All these characteristics might point to the change of lipid metabolism resulting from the change of the speed of digestion [40]. However, the accumulation of lipids and glycogen is normal when fish are kept under industrial conditions and intensively fed and is reversible [41,42,43]. This degree of change is not a sign of development of serious pathological changes and could be balanced by the change of feed composition. It follows that probiotic preparations based on lactic acid bacteria are recommended to be used in combination with other types of bacteria.

5. Conclusions

The study has shown that probiotic additives of complex microbiological composition (Bacillus subtilis + Bacillus amyloliquefaciens) in the concentrations under the research have a better effect on fish growth and histomorphometric parameters of the mid intestine, pyloric appendages and the liver. Preparations based on Lactobacillus acidophilus in this study had a mixed influence on digestive processes due to a low water temperature insufficient for activity of this group of microorganisms. Lactic acid bacteria slow down digestive processes of Oncorhynchus mykiss that, according to the author, leads to disorders of fat metabolism in the liver, which is proved by morphometric measures. The study found an unusual characteristic of decreasing the number of intraepithelial lymphocytes in prismatic epithelium in all the experimental groups. At the same time, the tendency was equally observed in the mid intestine and in pyloric appendages. The same effect was found while measuring lamina propria width and its sizes reliably decreased in all the experimental groups. Secretory activity of exocrinocytes in the mid intestine decreased as a result of the reduction of goblet cells number and the absence of change of their area except for group O2. In pyloric appendages a reliable decrease in goblet cells area was marked in the group using only the preparation based on Bacillus subtilis and in the group using complex probiotic preparation (Bacillus subtilis + Bacillus amyloliquefaciens) keeping their number according to the control level. Based on the results of this

study it could be assumed that using probiotic preparations for feeding juvenile Oncorhynchus mykiss in a recirculating aquaculture system may lead to a reliable decrease in growth rate of fish, their physiological status improvement and objective changes of histological structure of gastrointestinal organs. Doses of probiotic preparations recommended by the manufacturers demonstrated effectiveness except for Lactobacillus acidophilus which need either increasing the dose of the preparation or complementing it with other probiotics. It should be noted that all the probiotic preparations used in this study except for Lactobacillus acidophilus showed a similar mechanism of influencing tissue structures resulting in a change of goblet cells secretory activity and a decrease of a general immune response.

Author Contributions: For Conceptualization, A.N., D.N.; mythology, N.K., D.N.; formal analysis, N.K., S.S; investigation, N.K., D.N.,; resources, N.K., D.N.; data curation, D.N., S.S; writing—original draft preparation, N.K., D.N.; writing—review and editing, N.K., D.N. A.N, V.K.; visualization, N.K.. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: The study was complied with the guidelines of the Local Ethics Commission of the Institutional Review Board of Moscow State University of Technology and Management.

Conflicts of Interest: The authors declare no conflict of interest.

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 doi:10.20944/preprints202104.0243.v1