Effect of Probiotsid-Phyto and UDP Zn on the intestinal microbiome of Cyprinus carpio

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Abstract. The system analysis of the phytobiotic "Probiocid®-Phyto" and zinc ultradispersed particles (Zn UDP) effect on growth dynamics, chemical composition of muscle tissue and structural microbiome of the common carp gut (Cyprinus carpio) was carried out in this article. The use of modern genetic methods of investigation (polymerase chain reaction with specific 16S prokaryotic primers and with Nextera XT protocol adapters and indices) allowed to determine that the main phylogenetic groups entering the intact group gut microbiome are Proteobacteria (40.49 %), Actinobacteria (28.67 %), Firmicutes (19.01 %) and Bacteroidetes (10.28 %). The most significant effect on the carp microbiome was exerted by Zn UDP, which was expressed by an increase in the specific weight of two types – Proteobacteria 66.64 % and Fusobacteria - 31.18 %, while in the bivalent application group the biodiversity of types was represented as follows: 57.08 % – Proteobacteria, 14.17 % – Firmicutes, 11.86 % – Actinobacteria, 7.85 % – Fusobacteria and 7.43 % – Bacteroidetes. The most significant effect of the preparations on the carp productivity was observed when the compared preparations were fed together; in this case, by the end of the experiment, the increase in carp live weight was 18.45 %. The research allows recommending the combined use of "Probiocid®-Phyto" (2 g/kg feed) and Zn UDP (10 mg/kg feed) as growth activators for common carp (Cyprinus carpio).

1 Introduction

The symbiotic microbiota has a significant impact on the host organism. In this case, one of the main roles in the organism functioning is played by the intestinal microflora, which provides not only metabolic processes and is involved in the formation of humoral immunity and also allows the host to cope flexibly with changing environmental conditions. The biodiversity of the intestinal microbial community is influenced by a variety of factors. The microbiome is significantly influenced by nutrition, metabolic rate and host life stage. The species diversity of gastrointestinal tract microorganisms is represented by different microbial communities (microbiome) which are unique to the host symbiont, driven by taxonomic, ecological, phylogenetic and dietary characteristics [1]. In aquaculture vertebrates, the structural microbiome is significantly influenced by environmental

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interactions, the degree of digestion, the nutritional and energy value of feed components. A number of studies provide evidence that during the initial post-embryonic stage, the host fish body is virtually sterile and the structural gut microbiome is formed as a result of the initial colonization by environmental microorganisms, that in turn could be hypothesized to form the gut microbial community by regulating environmental factors. The science has accumulated extensive data on the marked dependence of microbiome biodiversity on environmental factors, both at the individual and population levels.

Host fish organisms demonstrates the autonomous selection of structural microbiota, which is hypothesized to be due to the evolution of an innate and adaptive immune system that regulates not only species diversity but also microbial population.

The analysis of the meta-analytical literature shows a marked species diversity of the structural gut microbiome according to fish species, diet type (predators, omnivores, herbivores), geographical distribution and environmental conditions [2]. However, it should be noted that despite the biodiversity high level of the intestinal normoflora, some certain groups of microorganisms are identified. These groups form the bulk of the biomass, such as Proteobacteria, Bacteroidetes and Firmicutes, with dominant families, including Vibrionaceae and Clostridiaceae, which are specific to all the studied aquaculture representatives. It should be noted that dermal mucus communities are also dominated by Proteobacteria and other less common types such as Bacteroidetes, Actinobacteria, Firmicutes and Verrucomicrobia.

One of the main factors in the formation of the gut microbiome is the use of various feed additives that can stimulate and inhibit the growth of microbes that have already been found in the gut (residents). The response of gut residents can be related to both changes in community structure and metabolic cascades associated with food breakdown. Bacteria and enzymes specialized in the catabolism of complex carbohydrates may be present in abundance in the early stages of digestion, creating products that can be used as energy by different microorganisms in the later stages of digestion [3].

Based on the above, the objective was to assess the impact degree of phytobiotic "Probiocid®-Phyto", zinc ultradispersed particles (Zn UDP) preparation and their combined use as feed additives on the gut microbiome, growth dynamics and chemical composition of the muscle tissue of common carp (Cyprinus carpio).

2 Materials and methods

The research was conducted in the conditions of the aquarium stand of the "Biotechnology of animal raw materials and aquaculture" Department of the Orenburg State University. The research objects were carp yearlings reared under the conditions of LLC "Irikla-fish" (Orenburg region).

The scheme of the experiment

Four groups (n=20) were formed for the paired-analysis studies. After the preparatory period (7 days) the groups were transferred to the conditions of the reference period (56 days). The control group received the basic ration (BR), the experimental group I - BR + phytobiotic "Probiocid®-Phyto" in the amount of 2 g/kg of feed, group II - BR + Zn UDP (10 mg/kg of feed), group III - BR + phytobiotic "Probiocid®-Phyto" (2 g/kg of feed) + Zn UDP (10 mg/kg of feed).

The daily feeding rate was determined weekly depending on fish weight, water temperature and dissolved oxygen values. Feeding was carried out 6 times a day. Live weight was monitored weekly by individual weighing in the morning before feeding (±1 g) during the reference period.

3 Results and discussion

The main taxonomic groups of the fish gut microbiome of the control group (Fig. 1) were Proteobacteria (40.49 %), Actinobacteria (28.67 %), Firmicutes (19.01 %) and Bacteroidetes (10.28 %).
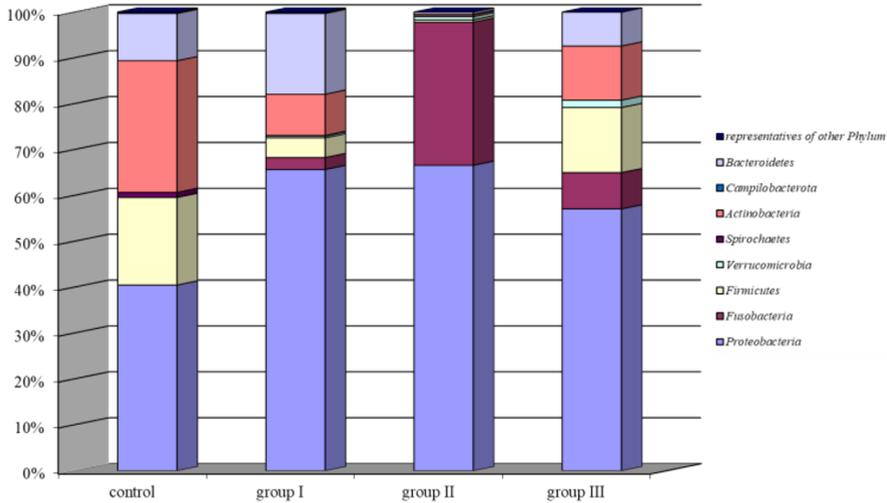

Fig. 1. Phylogenetic Distribution of the Structural Gut Microbiome of the Study Groups

The main Proteobacteria are Polynucleobacter, Schlegelella and Pseudaeromonas, which accounted for 17.56 %, 7.29 % and 4.96 % of the total biomass of the intestinal microbiome. Actinobacteria are represented mainly by Aurantimicrobium (23.90 %), Bacteroidetes genus Hydrotalea (7.66 %), respectively. It should also be noted that the microbiome structure of the intact group contained a small amount (less than 1 %) of Cyanobacteria, Ascomycota and Chytridiomycota.

Using the "Probiocid®-Phyto" feed additive the species diversity of the microbiome has undergone some changes, as in the intact group, the basis of the microbiome consists of Proteobacteria with 65.67 % (Fig. 2), represented mainly by the genera Aeromonas (38.41 %) and Pseudaeromonas (14.74 %).

Bacteroidetes accounted for 17.58 %, the species diversity of this phylogenetic group is represented mainly by the genus Hydrotalea (17.48 %), that was 99.46 % compared to 65.72 % in the intact group. The level of Actinobacteria in the study group was 8.99 %, while Aurantimicrobium accounted for only 1.32 %. The most abundant representative of the Actinobacteria phylum is the Rubrobacter genus, accounting for 2.35 % of the total intestinal population. Firmicutes accounted for 4.27 %. However, it should be noted that the absence of Cyanobacteria, Ascomycota and Chytridiomycota was registered on the background of "Probiocid®-Phyto" application.
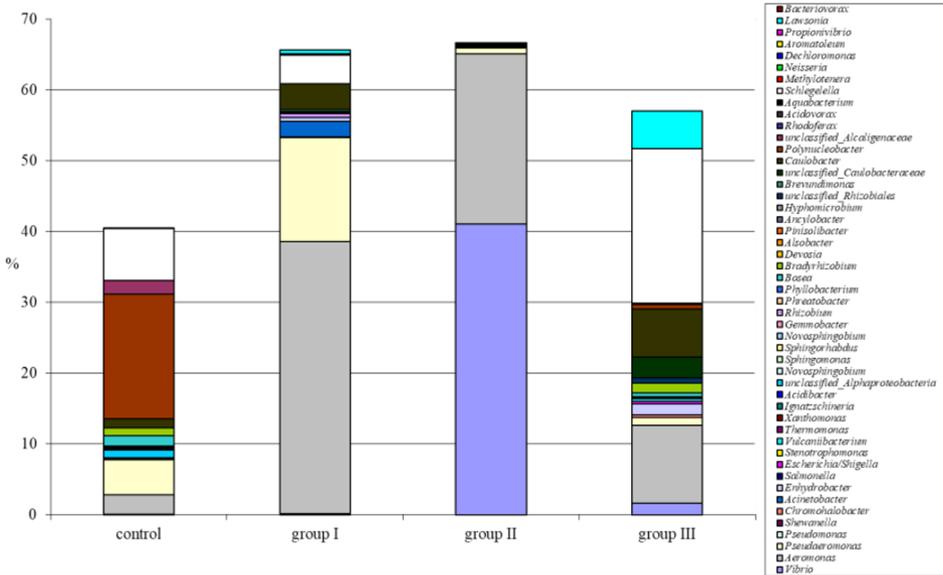
Fig. 2. Generic Distribution of Proteobacteria Representatives in the Structure of the Intestinal Microbiome of the Studied Fish

The use of Zn UDP has a significant effect on the structural microbiome. The Proteobacteria phylum, amounting to 66.64 % is represented mainly by genera Vibrio (41.09 %) and Aeromonas (24.07 %) while in the intact group their level was 0.09 % and 2.12 %, respectively.

The second main difference from the intact group was the high percentage of Fusobacteria, represented by the Cetobacterium genus, which accounted for 31.18 % of the total number of identified microorganisms, against the complete absence of this phylogenetic group in controls. It should also be noted the significant effect of Zn UDP application on Firmicutes, amounting to only 0.51 %, Actinobacteria to 0.38 % and Bacteroidetes to 0.48 %, respectively.

The combined use of "Probiocid®-Phyto" and Zn UDP has the closest to the intact group distribution of the structural microbiome with a significant stimulation of the individual representatives number of the microbial population. The main phylogenetic groups accounted for 57.08 % of Proteobacteria, 14.17 % of Firmicutes, 11.86 % of Actinobacteria, 7.85 % of Fusobacteria and 7.43 % of Bacteroidetes (Fig. 3).

The main representatives of Proteobacteria are Schlegelella genus, which accounted for 21.77 % of the total mass of identified microorganisms, Aeromonas - 11.05 %, Caulobacter - 6.83 %, Lawsonia - 5.37 %, respectively

One of the effectiveness criteria of "Probiocid®-Phyto", Zn UDP and their combined use as feed additives in carp ration was the evaluation of dynamometric indicators of growth (table 1) and the determination of chemical composition.

Table 1. Growth dynamics of experimental fish on the background of the studied feed additives, g

Timing of measurements, one week	Group			
	Control	**I**	**II**	**III**
Background measurement	15.0±1.2	15.1±1.2	15.1±1.0	15.0±1.3
1	17.3±1.4	17.4±1.3	17.4±1.3	18.2±1.5
2	20.6±1.7	20.7±1.6	21±1.8	22.2±1.8
3	23.8±2.0	24.8±1.9	25.4±2.0	26.7±2.1
4	26.3±2.4	29.2±2.5	29.0±2.7	30.9±2.7*
5	30.6±3.1	34.0±3.1*	34.3±3.2*	36.4±3.3*
6	34.6±3.7	40.6±3.6*	39.9±3.7*	42.3±3.6*
7	40.6±4.2	45.5±4.2*	44.2±4.0*	48.8±4.1*
8	47.7±4.7	52.4±4.7*	52.5±4.6*	56.5±4.9*
*p≤0.05 - I, II, III in comparison with control				

The data presented in Table 2 show significant differences in growth rates in all studied groups in relation to the same indicator of the intact group. Thus, against the background of "Probiocid®-Phyto" application on the eighth week of the experiment, the experimental group indicators exceeded the control values by 9.85 % ($p≤0.05$), in the group with Zn UDP by 10.06 % ($p≤0.05$) and in the group of combined application of the tested additives by 18.45 % ($p≤0.05$), respectively. It should be noted that significant differences in monovalent application were recorded from the fifth week of the experiment ($p≤0.05$), while bivalent application significantly increased weight from the fourth week of the study ($p≤0.05$).

The correlation analysis between the indicators of the main phylogenetic groups of microorganisms isolated in the intestinal microbiome with growth dynamics and chemical composition of muscle tissue of common carp showed a significant direct relationship between the level of Proteobacteria with Verrucomicrobia, live weight, total mineralization level (ash residue) ($p≤0.01$), and Fusobacteria ($p≤0.05$). There was also a significant positive dynamic relationship between Fusobacteria and dry matter ($p≤0.05$), Firmicutes and Actinobacteria ($p≤0.01$), Verrucomicrobia with live weight ($p≤0.01$) and ash content ($p≤0.05$), Spirochaetes with fat content in muscle tissue ($p≤0.01$) and Actinobacteria level ($p≤0.05$), Bacteroidetes with moisture content and protein in fish meat ($p≤0.05$). Live weight values had positive correlation indicators with protein concentration ($p≤0.05$) and ash ($p≤0.01$) in the studied samples, the moisture level in muscle correlated with protein content ($p≤0.05$).

The analytical study also found significant negative correlations between Proteobacteria with Spirochaetes ($p≤0.01$) and fat content ($p≤0.05$) in the samples studied, Fusobacteria with Firmicutes, Spirochaetes, the moisture level ($p≤0.05$), Actinobacteria and Bacteroidetes ($p≤0.01$). The inverse correlation was recorded between Firmicutes with ash

content ($p \leq 0.05$), Verrucomicrobia with Spirochaetes ($p \leq 0.05$), Spirochaetes, the ash level and live weight ($p \leq 0.01$), Actinobacteria with ash content in tested samples ($p \leq 0.01$). The analysis of the relationship between the chemical parameters of meat and live weight revealed an inverse relationship of dry matter content with moisture ($p \leq 0.05$), fat with the ash level, protein and live weight ($p \leq 0.01$) in the samples obtained from fish of the experimental groups.

Against the background of using feed additives, no reliably significant differences in the chemical composition of muscle tissue were found in all the studied groups. It hypothetically indicates that there is no effect on the nutritional value of fish meat. At the same time, it should be noted that the application of the "Probiocid®-Phyto" and Zn UDP combination significantly reduces the feed conversion ratio, which is one of the key economic indicators in modern fish farming.

Summarizing the above, it should be noted that the use of feed additives "Probiocid®-Phyto", Zn UDP and their combination has a significant impact on the biodiversity level of the gut microbiome, but it should be noted that in all studied groups the phylum Proteobacteria is of key importance, ranging from 40.49 % to 66.64 %. It was also experimentally determined that the bivalent use of the tested additives contributed to the significant increase in the identified forms of microorganisms in all studied groups by 29.90 % relative to the intact group, by 42.86 % for "Probicide®-Phyto" and by 31.10 % for Zn UDP, respectively. The presence of a significant biological effect against the representatives of the phylum Cyanobacteria, Ascomycota and Chytridiomycota was experimentally established, that in turn could hypothetically be due to the presence of a significant antifungal action, as well as the stimulation of the body defence functions.

Discussion

Currently, the study of the intestinal microbiota has received much attention, due to the fact that it, along with other physiologically relevant systems, is an integral part of any living organism. Despite the large amount of bibliometric data, the information on the microbiota of both animals and fish is scarce. Understanding the factors influencing the species diversity of the digestive tract of the microbial community is necessary to manage or improve the composition of the microbial community [4]. The structural microbiome of the fish gut differs significantly from that of other animals and the microbial communities of water and soil. Numerous studies indicate that the fish gut microbiome is dominated by Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria and Fusobacteria types. Developments in metagenomics and high-throughput amplicon sequencing technologies have demonstrated that classical microbiological methods of microbiome studies cannot reflect the overall biodiversity [5].

The representatives predominance of phylogenetic groups Proteobacteria, Fusobacteria, Firmicutes and Bacteroidetes in the intestinal microbiota of freshwater fish promotes the active decomposition of plant polymers, the fermentation of organic compounds, thus providing the host with nutrients and energy. It should also be noted that a number of microorganisms, although pathogenic for some fish species, can act as a probiotic component for others, e.g. some members of the genus Aeromonas have pronounced cellulose degrading characteristics, Cetobacterium are involved in production of cyanoblamin and are abundantly present in the intestinal tract of Cyprinus caprio [6].

Bacteria of the genus Vibrio are the dominant facultative gut microbiota of a wide range of marine fish. The ability of specific bacteria to occupy the attachment sites in the larval intestine, preventing the reproduction and the colonization by opportunistic bacteria, is an important protective mechanism, especially in the early larval stages when the immune system is not yet fully developed. Vibrio populations in water have a greater influence on the composition and structure of vibrio gut populations than those in feed (Blanch et al.

2009). Several researchers have found that the presence of bacteria of the genus Vibrio significantly increases the content of some primary metabolites such as thymine, adenine, guanine, cafestol, cortisol, valine, proline, serine, lysine leucine, adenine, glutamine, proline, piperidine, guanosine and propionic acid [7]. The occurrence of symbiotic bacteria of the genera Bradyrhizobium and Mesorhizobium in the structural microbiome is usually due to fish omnivorousness, as these genera are usually associated with the roots of plants acting as food [8].

Dominant taxa contribute to the maintenance of the immune function and the metabolic activity. The importance of metabolic properties is consistent with the functions of gut bacteria in freshwater fish, which have shown relatively large numbers of genes related to cofactor and vitamin metabolism, suggesting that the availability of essential nutrients in the environment determines the choice of fish gut microbiota to correct for host nutrient deficiencies [9].

Actinobacteria are well known to produce various secondary metabolites, many of which are potent antibiotics. These bacteria are widespread in both terrestrial and aquatic (including marine) ecosystems, especially in soils where they play a key role in recycling refractory biomaterials through the decomposition and humus formation. However, this type usually accounts for a small proportion of the bacterial sequences extracted from fish guts involved in cellulose decomposition. The phylogenetic groups Firmicutes and Bacteroidetes are the primary agents responsible for bacterial degradation of cellulose in eutrophic habitats with neutral pH, whereas Actinobacteria dominate aerobic cellulose degradation in sphagnum peatlands in acidic conditions (pH) from 3.5 to 5.5. Fibrobacter spp. are the main cellulose-degrading bacteria in the rumen, while a similar function has been established for Bacillus, Vibrio, Aeromonas and Enterobacter in the gut of white amur. There is also evidence in the literature that Anoxybacillus, Leuconostoc, Clostridium, Actinomyces Streptococcus and Prevotella have cellulose-degrading activity [10].

The presence of Nitrobacter, Rhodobacter and Roseomonas in the structure of the intestinal microbiome ensures the active degradation of nitrates and pesticides. The members of the genus Paludibacter possess enzymatic metabolism and can break down oligofructose (raftylose P95), the genus Planctomycetes is involved in the polysaccharides degradation, Cyanobacteria are an important food source for some fish species, in predatory fish, Chitinophagales are involved in the degradation of chitin and some organic substances, Lactobacillus are actively involved in the breakdown of polyunsaturated fatty acids, contribute to glucose and lipid metabolism in the host fish [11].

In our study it was found that the use of phytobiotic "Probiocid®-Phyto" and Zn UDP as feed additives, both as a single component and as their complex, has a significant effect on the species diversity of the gut structural microbiome of common carp (Cyprinus carpio). Thus, in the intact group the major types forming the normoflora are Proteobacteria (40.50 %), Actinobacteria (28.67 %), Firmicutes (19.07 %) and Bacteroidetes (10.28 %) while the phylogenetic group of Proteobacteria was represented mainly by genera Polynucleobacter and Schlegella which comprised 17.56 % and 7.29 %, respectively. Actinobacteria is mainly represented only by the genus Aurantimicrobium, accounting for 23.90 % of the total number of identified species. The type Firmicutes is represented by unclassified group Firmicutes constituting 15.61 % from all biodiversity of intestine. The genus Hydrotalea belonging to the phylum Bacteroidetes constituted 7.66 %. It should be noted that the populations inhabiting the intestine also included Cyanobacteria, Ascomycota, Chytridiomycota and Fungi incertae sedis [12].

Against the background of the "Probiocid®-Phyto" application there was a slight decrease in intestinal biodiversity by 14.58 % relative to the intact group, mainly due to the suppression of Cyanobacteria, Ascomycota, Chytridiomycota and Fungi incertae sedis.

Here we should note, that the maximal colonization rate of intestine, as well as in controls, was registered in phylum Proteobacteria (65.67 %) represented mainly by genera Aeromonas (38.41 %) and Pseudaeromonas (14.74 %). The second most numerous phylogenetic group is Bacteroidetes (17.58 %), represented by the genus Hydrotalea, which comprised 99.46 % of the total number of isolated representatives of this type. Among the main types, Actinobacteria (8.99 %), represented by genera Corynebacterium (1.90 %), Aurantimicrobium (1.32 %), Micrococcus (1.67 %) and Rubrobacter (2.35 %) should also be noted [13].

The maximum differences in species diversity were recorded in the experimental group in which Zn UDP were included in the diet. The Proteobacteria type (66.64 %) was represented mainly by the genus Vibrio and it comprised 41.09 % of the total number of species isolated, that is radically different from the figures of other groups, where representatives of this genus constituted from 0.09 % to 1.61 %, the second most numerous genus in this phylum was Aeromonas (24.07 %). Also we should note high percentage of Fusobacteria representatives (31.18%) represented by genus Cetobacterium (31.17%), which is one of the main differences from the intact group in samples of intestines of which this phylogenetic group was absent. The other groups accounted for less than 1% each [14].

The combined application of "Probiocid®-Phyto" (2 g/kg food) and Zn UDP (10 mg/kg food) had the most beneficial effect on the formation of structural microbiome without cardinally significant changes in the indicators of biological diversity of the main phylogenetic groups in comparison with the intact parameters, that along with maximum growth dynamics indexes ($p \leq 0.05$) and minimal differences in chemical composition of muscle tissue, manifested only by reliably significant decrease of fat level by 16.36 % ($p \leq 0.05$), allows to recommend this complex as a growth activator in conditions of commercial carp (Cyprinus carpio) feeding. The species diversity of identified gut microorganisms of this group was represented by Proteobacteria (57.08 %), Firmicutes (14.17 %), Actinobacteria (11.86 %), Fusobacteria (7.85 %) and Bacteroidetes (7.43 %). It should also be noted that this group has the highest rate of isolated specimens, exceeding the intact values by 42.65% [15].

4 Conclusion

Summarizing the above, it should be noted the presence of positive growth dynamics in all experimental groups in relation to the intact indicators ($p \leq 0.05$), which hypothetically may be due, firstly, to the presence of significant inhibitory effect of compounds tested in the experiment in relation to individual representatives of microbiome Cyanobacteria, Ascomycota, Chytridiomycota and Fungi incertae sedis, that in turn may indicate the presence of antifungal action. Secondly, this positive effect of the studied food additives in activating fish growth indices can be caused by disturbance of intercellular communication (Quorum Sensing) of separate representatives of the microbial community, which results in higher adaptation and antagonistic characteristics (determined by resistance to the tested food additives) of other microbiome species with higher metabolic and enzymatic activity indices.

5 Acknowledgements

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