ФИЗИОЛОГИЯ ЧЕЛОВЕКА И ЖИВОТНЫХ

HUMAN AND ANIMAL PHYSIOLOGY

DOI: 10.12731/2658-6649-2025-17-2-1429 **EDN: TROYTR**
UDC 639.371.5:639.3.043.13

Original article

EFFECTS OF PHYTOBIOTIC FEED ADDITIVES ON PRODUCTIVITY AND GUT MICROBIOTA OF COMMON CARP

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Abstract

Background. The use of phytobiotics in feeding may be a promising approach to control animal diseases without antibiotics.

The aim of our study was to evaluate the effect of phytobiotic feed additives on the growth performance and on gut microbiome of common carp.

Materials and methods. The paper presents the results of a study on the use of phytobiotic feed additives in carp feeding: "Intebio" – an additive based on a mixture of essential oils (garlic, lemon, thyme and eucalyptus) and "Butitan" - a balanced microencapsulated combination of ellagotannins (sweet chestnut wood extract).

Results. When the studied additives were included in the diet of fish, a growth-stimulating effect was established: with the inclusion of "Butitan" by 11.7% ($P \leq 0.05$), and with "Intebio" by 8.8% ($P \leq 0.05$), relative to the control. The introduction of phytobiotic feed additives "Butitan" and "Intebio" into the diet of carp had a significant effect on the gut microbiome of fish. A decrease in the number of bacteria of phylum *Actinomycetota*, *Bacillota* and *Bacteroidota* and an increase in the content of microorganisms of taxa *Pseudomonadota* and *Fusobacteriota* (genus C*etobacterium*) were found, which was reflected in the change in the number of microorganisms of *Microbacteriaceae*, *Chitinophagaceae*, and *unclassified_Bacillota* families. The analysis of the sequencing results showed that the impact of "Intebio" led to a change in the dominant genera of bacteria in the gut microbiota of fish.

Numerous groups were bacteria of the genus *Aeromonas*, the genus *Vibrio* and the genus *Cetobacterium*.

Conclusion. The results obtained showed that the inclusion of "Butitan" and "Intebio" in the diet of carp has a positive effect on the indicators of body weight gain and can potentially be used as a basis for drugs to modify the gut microbiota.

Keywords: microbiome; fish; feeding; phytobiotics

For citation. Miroshnikova, E. P., Yausheva, E. V., Arinzhanov, A. E., & Kilyakova, Y. V. (2025). Effects of phytobiotic feed additives on productivity and gut microbiota of common carp. *Siberian Journal of Life Sciences and Agriculture*, *17*(2), 11–29. https://doi.org/10.12731/2658-6649-2025-17-2-1429

Научная статья

ВЛИЯНИЕ ФИТОБИОТИЧЕСКИХ КОРМОВЫХ ДОБАВОК НА ПРОДУКТИВНОСТЬ И МИКРОБИОТУ КИШЕЧНИКА КАРПА

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Аннотация

Обоснование. Использование фитобиотиков может стать многообещающим подходом для контроля заболеваний животных без использования антибиотиков.

Цель работы – оценить влияние фитобиотических кормовых добавок на показатели роста и микробиом кишечника карпа.

Материалы и методы. В работе представлены результаты исследования по использованию в кормлении карпа фитобиотических кормовых добавок: «Интебио» – добавка на основе смеси эфирных масел (чеснока, лимона, чабреца и эвкалипта) и «Бутитан» – сбалансированная микрокапсулированная комбинация эллаготанинов (экстракта древесины сладкого каштана).

Результаты. При включении в рацион рыб исследуемых добавок установлен ростостимулирующий эффект: при введении «Бутитан» на 11,7 % ($P \leq 0,05$), а с Интебио на 8,8 % ($P \leq 0,05$), по сравнению с контролем. Использование фитобиотических кормовых добавок «Бутитан» и «Интебио» в рационе карпа оказало значительное влияние на микробиом кишечника рыб. Установлено снижение числа бактерий филумов *Actinomycetota*, *Bacillota* и *Bacteroidota* и повышение содержания микроорганизмов таксонов *Pseudomonadota* и

Fusobacteriota (род *Cetobacterium*), что отразилось в изменении количества микроорганизмов семейств *Microbacteriaceae*, *Chitinophagaceae*, и *unclassified_Bacillota*. Анализ результатов секвенирования показал, что введение «Интебио» приводило к смене доминирующих родов бактерий в микробиоте кишечника рыб. Многочисленными группами являлись бактерии рода *Aeromonas*, рода *Vibrio* и рода *Cetobacterium*.

Заключение. Полученные результаты показали, что включение «Бутитан» и «Интебио» в рацион карпа оказывает положительное влияния на показатели прироста живой массы и потенциально могут быть использованы в качестве основы для препаратов по модификации микробиоты кишечника.

Ключевые слова: микробиом; рыба; кормление; фитобиотики

Для цитирования. Мирошникова, Е. П., Яушева, Е. В., Аринжанов, А. Е., & Килякова, Ю. В. (2025). Влияние фитобиотических кормовых добавок на продуктивность и микробиоту кишечника карпа. *Siberian Journal of Life Sciences and Agriculture*, *17*(2), 11-29. https://doi.org/10.12731/2658-6649-2025-17-2-1429

Introduction

Production intensification in the aquaculture sector and reduction of resistance to pathogens among fish involves the search for new feed additives that can meet the growing needs for fish products and improve health indicators [23; 25]. Despite the fact that antibiotics remain the main means of combating fish pathogens, their use leads to ecosystem pollution and has a negative impact on metabolic processes in the fish body [6; 11]. Potential drugs that solve these issues are phytobiotic additives [1; 2; 3]. The introduction of various phytobiotic additives in fish feeding shows a positive effect on the growth and physiological parameters of the fish body. At the same time, the use of phytobiotic additives requires a thorough study of their effect on metabolic processes in the body, including on the gut microbiota of fish.

The microbiota associated with macroorganisms is a diverse population of microorganisms that play various important roles in the biology of multicellular hosts. Microorganisms of the digestive system play an extremely important role in the body [7; 10]. It has been established that the microbiota of the gastrointestinal tract affects not only the digestive process, but also the immune response, energy homeostasis, hormone secretion and other metabolic processes [13; 26]. In particular, for some fish species the influence of changes in the composition of the microbiota on many biosynthetic pathways, lipid, amino acid and carbohydrate metabolism has been shown [20; 24; 28]. In this regard, the control of changes in the composition of the gut microbiota is an integral part of the study of the use of new generation feed additives in fish feeding. It has been noted

that the introduction of food additives from a phytonutrients class in fish feeding can positively affect microorganisms with probiotic properties and reduce the number of potential pathogens in the gut microbiota [12].

The *aim* of our study was to evaluate the effect of phytobiotic feed additives on the growth performance and on gut microbiome of common carp.

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» (Russia, Orenburg).

Three groups (n=20) were formed to conduct research using the method of pair-analogues. After the preparatory period (7 days), the groups were transferred to the conditions of the accounting period (56 days). The fish of the control group received the main diet (MD) - compound feed «KRK-110» of JSC «Orenburg Feed Plant» (Russia, Orenburg). The experimental groups received additional phytobiotics in the diet: Group I – MD + "Butitan" at a dose of 0.5 g/kg of feed, Group II – MD + "Intebio" at a dose of 0.5 g/kg of feed.

"Intebio" is a feed additive based on a mixture of essential oils: garlic, lemon, thyme and eucalyptus (BIOTROF Ltd., Russia). "Butitan" is a balanced microencapsulated combination of ellagitannins (sweet chestnut wood extract) with calcium butyrate (SIVETRA-AGRO Ltd., Russia).

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 during the reference period.

Animal care and experimental studies were carried out in accordance with the instructions and recommendations of Russian regulations (1987; Order of the Ministry of Health of the USSR No 755 of 12.08.1977 «On measures to further improve the organizational forms of work using experimental animals») and «Guide for the Care and Use of Laboratory Animals» (National Academy Press, Washington, D.C., 1996). During the research measures were taken to ensure a minimum of animal suffering and to reduce the number of experimental samples studied.

Extraction of total DNA bacteria and archaea

Samples were homogenised on a TissueLyser LT (Qiagen, Hilden, Germany) with a Lysing Matrix Y (MP Biomedicals, Solon, USA). DNA extraction from samples was performed using QIAamp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer instruction. The quality

of the extracted DNA was assessed with electrophoresis in 1% agarose gel and a Nanodrop 8000 (Thermo Fisher Scientific, Waltham, MA, USA). The DNA concentration was quantified using a Qubit 4 Fluorometer (Life Technologies, Carlsbad, CA, USA) with dsDNA High Sensitivity Assay Kit (Life Technologies, Carlsbad, CA, USA).

Preparation, sequencing and bioinformation processing of DNA

Preparation of the DNA libraries was performed according to the Illumina protocol (Part #15044223, Rev. B.) with primers targeting the V3–V4 regions of the SSU ribosomal RNA (rRNA) gene, S-D-Bact-0341-b-S-17 (5′-CCTACGGGNGGCWGCAG-3′) as the forward primer and S-D-Bact-0785-a-A-21 (5′-GACTACHVGGGTATCTAATCC-3′) as the reverse primer. The reaction mixture (25 μL) contained both primers, 0.2 μM each; 80 μM dNTPs; 0.2 U Q5 High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA, USA). The Following PCR program was used: 95 °C for 3 min, 25 cycles; 95 °C for 30 s, 56 °C for 30 s; 72 °C for 30 s; final extension 72 °C for 5 min. The DNA libraries were cleaned up using Agencourt AMPure XP beads (Beckman Coulter, Brea, CA, USA) and were validated with capillary electrophoresis on a Qiaxcel Advanced System (Qiagen, Hilden, Germany) using the QIAxcel DNA Screening Kit (Qiagen, Hilden, Germany). Paired-end 2 × 251 bp sequencing was performed on the MiSeq platform (Illumina, San Diego, CA, USA) with the Reagent Kit v.2 (Illumina, San Diego, CA, USA).

DNA libraries preparing, sequencing and bioinformatics treatments were performed in the Center of Shared Scientific Equipment "Persistence of microorganisms" of Institute for Cellular and Intracellular Symbiosis UrB RAS (Orenburg, Russia). All stages of preparation, sequencing and bioinformatic processing of DNA libraries are performed according to the previously described methodology.

Ethics Statement

The Local Ethics Committee of the Orenburg State University, Orenburg, Russia, has approved the report about this research.

Statistical analysis

The resulting OTUs, after filtering and assigning taxonomic affiliations, were used to calculate alpha (chao1, Fisher's alpha, - statistical method: ANOVA) and beta (ordination method: NMDS; distance method: Bray-Curtis index; statistical method: PERMANOVA) diversity.

Results

The introduction of various phytobiotic additives in the fish diet had a positive effect on the growth performance of carp. Productive effect was observed in all experimental groups (Table 1). By the end of the experiment (56 days), it

was revealed that the weight of the fish of the experimental group I was 11.7% (P≤0.05) higher than the control, and the difference in live weight of the fish of the control and experimental group II was 8.8% (P≤0.05).

Table 1.

Dynamics of fish growth, g

Week of the accounting period	Groups		
	Control	Group I	Group II
1	17,3±1,4	18,2±1,5	18,1±1,4
2	20,6±1,7	21±1,6	20,7±1,8
3	23,8±2,0	25,8±2,1	25,2±2,2
4	26,3±2,4	29,5±2,6	29,3±2,8
5	30,6±3,1	34,7±3,2*	33,8±3,0*
6	34,6±3,7	40,1±3,9*	38,9±3,8*
7	40,6±4,2	45±4,3*	44,2±4,4*
8	47,7±4,7	53,3± 4,6*	51,9±4,8*

Note: * - P≤0.05 compared to the control group

Analysis of fish gut microbiota showed a high diversity of taxonomic groups. Sequencing resulted in 204377 reads, with 14413 to 28919 original reads per sample. After the merging and filtering steps, 150059 reads were included in the analysis. After clustering, a total of 150 OTUs were obtained. After removing singletons, doubletons, and likely sample contaminants, 75 OTUs remained.

The obtained OTUs were taxonomically grouped from the phylum level to the genus level (Table 2).

Table 2.

The number of taxa of different levels identified in the gut microbiome of fish from experimental groups

Taxa	Groups		
	Control	Group I	Group II
phylum	6	8	9
class	13	13	17
order	27	21	28
family	37	23	38
genus	42	26	42

Discharge curves were plotted based on the sequences and OTUs obtained. The discharge curves of all samples tended to plateau to a maximum, which indicated that the sequencing depth was sufficient to characterize the fish gut microbiota in this study (Figure1).

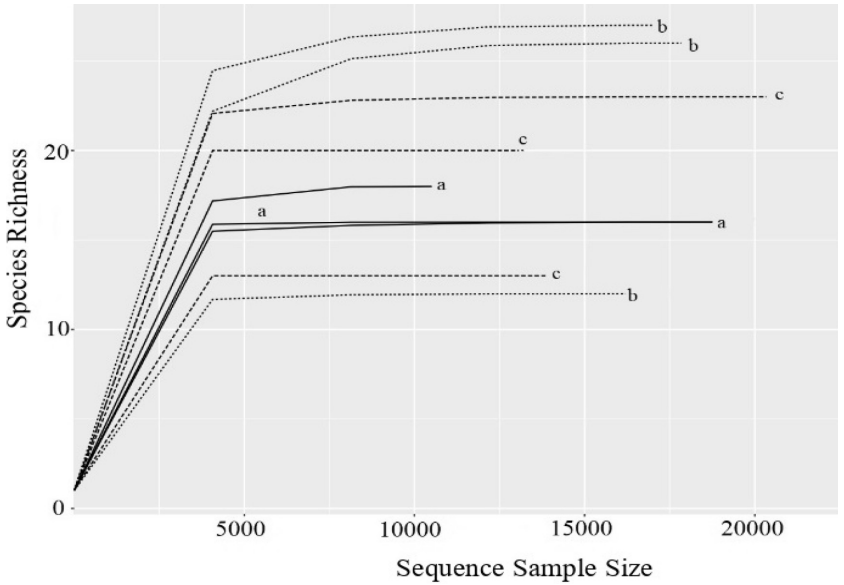

Figure 1. Sequence-based resolution curves for gut microbiota samples I (a), II (b), and control (c) groups

A study of the gut microbiota of the control group of fish showed that the main taxa at the phylum level were *Pseudomonadota* and *Actinomycetota*, which accounted for more than 70% of the total number of bacteria (Figure 2).

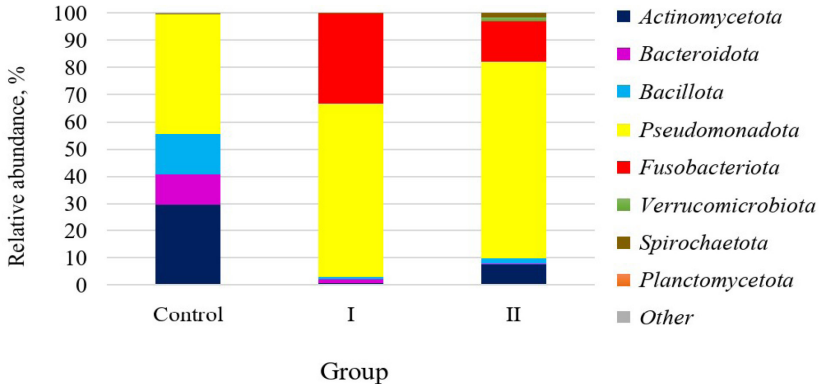

Figure 2. Relative abundance of phylum identified in samples of the gut microbiome of fish of the studied groups

Among small groups, bacteria of the phyla *Bacillota* (15%) and *Bacteroidota* (11%) were noted. At deeper taxonomic levels, the classes *Actinomycetes*, *Betaproteobacteria*, *unclassified_Bacillota*, *Chitinophagia*, *Alphaproteobacteria*, and *Gammaproteobacteria* were abundant (Figure 3). The main number of classified microorganisms belonged to the families *Microbacteriaceae* (genus *Aurantimicrobium*), *Chitinophagaceae* (genus *Hydrotalea*), *unclassified_Bacillota*, *Sphaerotilaceae* (genus *Schlegelella*), *Burkholderiaceae* (genus *Polynucleobacter*) and *Aeromonadaceae* (genus *Pseudaeromonas* and genus *Aeromonas*). The dominant group of bacteria at the genus level were bacteria belonging to the class *Betaproteobacteria*.

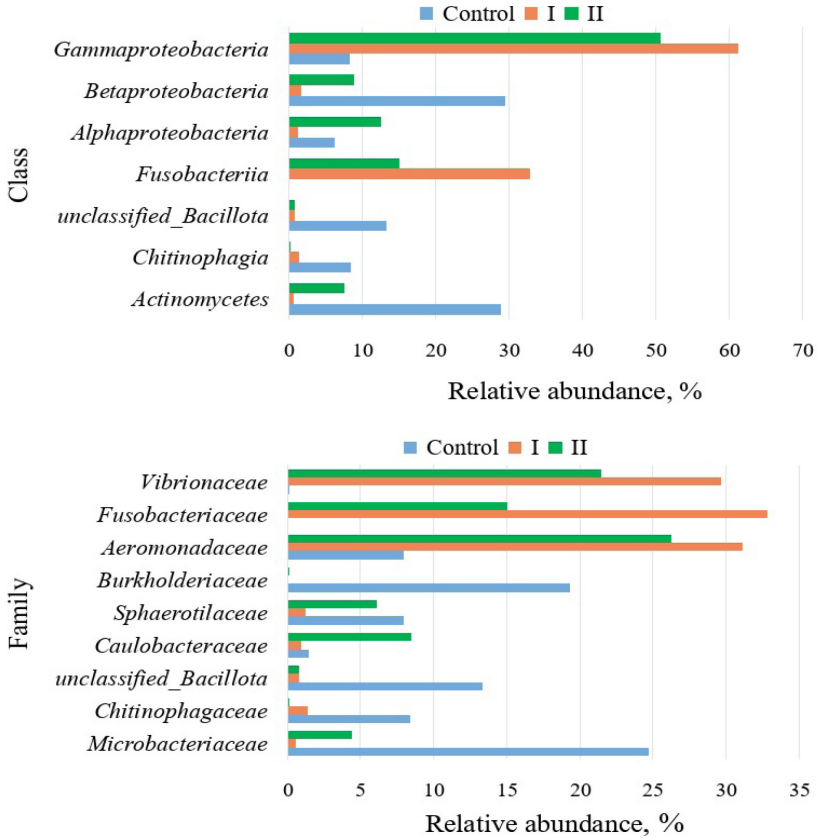

Figure 3. Comparison of the relative content of the main groups of bacteria in the gut microbiota of fish of the studied groups at the level of classes and families

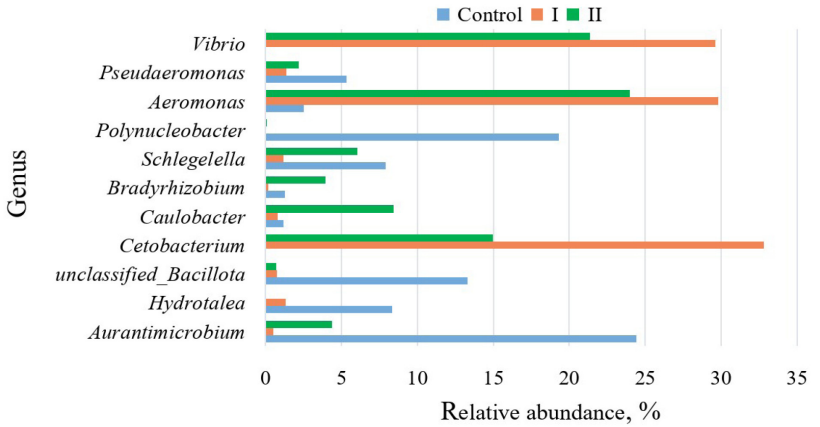

Figure 4. Relative content of the main groups of bacteria in the gut microbiota of fish of the studied groups at the genus level

The introduction of the phytobiotic feed additive "Butitan" in the diet had a significant impact on the gut microbiome of fish. There was a decrease in the number of bacteria of the phyla *Actinomycetota* (-29.1%), *Bacillota* (-14.2%) and *Bacteroidota* (-9.49%), which was expressed to a greater extent in a change in the number of microorganisms of the families *Microbacteriaceae*, *Chitinophagaceae*, and *unclassified_Bacillota*. At the same time, an increase in the number of microorganisms *Pseudomonadota* and *Fusobacteriota* (genus *Cetobacterium*) by 20.1% and 32.8%, respectively, was observed. Within the taxon *Pseudomonadota*, there was a decrease in the number of bacteria *Alphaproteobacteria*, *Betaproteobacteria* and an increase in representatives of the class *Gammaproteobacteria*, which accounted for more than 60% of the total number of detected bacteria of the phylum *Pseudomonadota*.

A significant increase in the number of bacteria of the families *Aeromonadaceae* and *Vibrionaceae* was observed in the gut microbiota of fish, which was manifested in the growth of microorganisms of the genera *Aeromonas* and *Vibrio*.

The introduction of the phytobiotic feed additive "Intebio" in the diet of fish showed a similar effect on the gut microbiome of common carp, as with the introduction of "Butitan". There was a decrease in the number of bacteria of the phyla *Actinomycetota* (-22.2%), *Bacillota* (-12.8%) and *Bacteroidota* (-10.7%), and an increase in the content of microorganisms of the taxa *Pseudomonadota* and *Fusobacteriota* (genus *Cetobacterium*). Such changes were directly associated with a decrease in the number of bacteria from the families

Microbacteriaceae, *Flavobacteriaceae*, *Chitinophagaceae*, and *unclassified_ Bacillota*. Changes in the number of bacteria of the phylum *Pseudomonadota* were associated with an increase in the number of representatives of the families *Caulobacteraceae*, *Bradyrhizobiaceae*, *Aeromonadaceae*, *Vibrionaceae*, and *Moraxellaceae*. Nevertheless, with the increase in the total number of bacteria of the taxon *Pseudomonadota*, there was a decrease in the level of bacteria of the families *Burkholderiaceae*, *Alcaligenaceae* and *Sphaerotilaceae* belonging to the class Betaproteobacteria. Analysis of the sequencing results showed that the impact of "Intebio" led to a change in the dominant genera of bacteria in the gut microbiota of fish. Numerous groups were bacteria genus *Aeromonas*, genus *Vibrio* and genus *Cetobacterium*. Microorganisms genus *Schlegelella*, genus *Polynucleobacter* and *unclassified_Alcaligenaceae* belonging to the class *Betaproteobacteria* amounted to no more than 9%.

The calculations of alpha diversity indices made it possible to assess the richness, diversity and homogeneity of fish gut microbiota (Table 3). The values of Chao1, ACE and Simpson indices indicated the taxonomic richness of the gut microbiota of fish in experiment and the absence of predominance of one large OTU in the samples. At the same time, the Chao1, ACE and Simpson indices had higher values in Group II compared to the control and Group I. Similarly, the Shannon and Fisher's alpha diversity scores showed differences between the experimental groups.

Table 3.

Indices of alpha diversity of the gut microbiota of groups I and II

Index	Groups			P-value
	I	II	Control	
chao1	16,7	21,7	18,6	0,59
ACE	16,8	21,9	18,9	0,55
Fisher's alpha	1,85	2,47	2,1	0,59
simpson	0,7	0,75	0,77	0,36
shannon	1,41	1,71	1,87	0,2

PERMANOVA analysis to assess beta diversity showed a significant effect of the introduction of phytobiotic additive in the diet of fish on the Bray-Curtis distance (Figure 5). Significant differences in the organization of gut bacterial communities were observed between samples from groups I and II compared to control (p-value = 0.1).

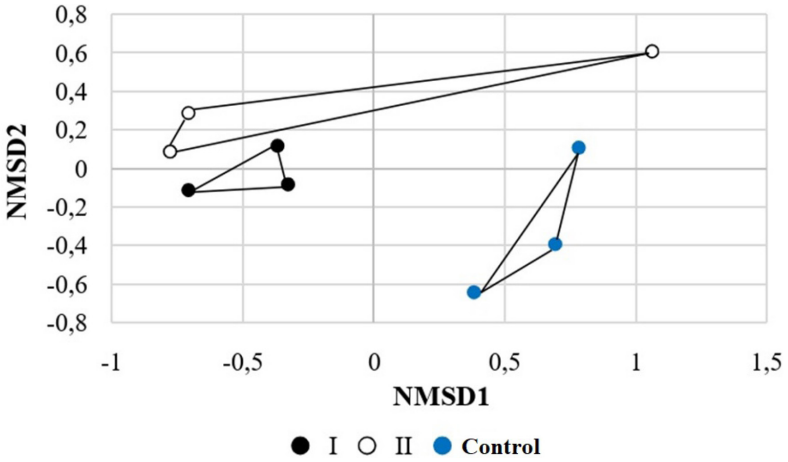

Figure 5. Beta diversity of gut microbiota in fish of studied groups using the PERMANOVA statistical method, non-metric multivariate scaling, and Bray-Curtis dissimilarity

Discussion

The positive effect of phytobiotics on the growth dynamics and physiological state of fish that we have established is consistent with our previous studies on animals and poultry [17; 18; 21]. There an increase in productive indicators has been established due to the activation of the beneficial microflora of the body, which leads to an increase in the body's resistance, to a more complete breakdown and assimilation of feed nutrients [4; 9; 16; 22].

As a result of the research, data were obtained that allow evaluating the features of the impact of phytobiotic additives on the gut microbiota of fish.

Analysis of the data on the composition of the gut microbiota of fish showed the presence of typical taxa for samples from the control group [19; 32]. The introduction of phytobiotic additives "Butitan" and "Intebio" into the diet led to similar changes in the composition of the gut microbiota of fish. There was a decrease in the number of bacteria of the same taxa, and an increase in the number of bacteria of the same groups in the gut microbiota, regardless of which additive was used in the fish feeding. At the same time, the "Butitan" additive led to a stronger change in the number of microornagisms of such taxa as: *Actinomycetota* (genus *Aurantimicrobium*), *Bacillota* (genus *unclassified_Bacillota*), *Pseudomonadota* (genus *Aeromonas*, genus *Vibrio*) and *Fusobacteriota* (genus *Cetobacterium*). Similarly, the action of the phytobiotic additive "In-

tebio" was more pronounced in relation to bacteria of the phyla *Bacteroidota* (*Hydrotalea* genus) and *Pseudomonadota* (genus *Schlegelella*, genus *Polynucleobacter*, genus *Caulobacter*).

Significant changes in the composition of the gut microbiota of carp with the introduction of phytobiotics were associated with an increase in the number of bacteria genus *Cetobacterium*, genus *Aeromonas* and genus *Vibrio*.

Certain strains of bacteria of the genus *Cetobacterium* are the basis of post-probiotic drugs. It has been noted that metabolites, cell wall components and culture supernatants obtained from *Cetobacterium* bacteria can exhibit prebiotic features [33]. Previously, positive effects on growth performance, antioxidant defense, liver condition, and resistance to bacterial and viral infections have been described when using *Cetobacterium somerae* culture supernatant in feeding of common carp (*Cyprinus carpio*), Nile tilapia (*Oreochromis niloticus*), and *Danio rerio* fish [30; 31; 35].

Considering the ability of individual representatives of genus *Aeromonas* and *Vibrio* cause the development of an infectious process, an increase in their number is of certain interest [5; 8; 27; 34]. However, the absence of fish mortality, external signs of the disease, and the presence of an increase in the live weight of fish suggests that an increase in the number of bacteria of the genus *Vibrio* and genus *Aeromonas* in the composition of the microbiota did not lead to the development of pathological processes of an infective nature. Also, some strains of bacteria of the genus *Vibrio* and the genus *Aeromonas*, in separate studies, are considered as potential probiotics that can reduce the number of pathogenic bacteria in the gut [14; 15; 29]. These data suggest potential beneficial effects of increasing the number of genus *Vibrio* and genus *Aeromonas* bacteria in the gut microbiota of fish.

As the result of the introduction of the phytobiotic additive "Butitan", the number of bacteria genus *Cetobacterium* in the gut of fish was twice as much as when using the additive "Intebio". Similarly, the number of bacteria of the genus *Aeromonas* and genus *Vibrio* was 5.8 and 8.3% higher in the gut of fish that received the "Butitan" additive. Such changes were probably one of the factors causing a greater increase in the mass of fish when using the "Butitan" additive.

Conclusion

Thus, the results obtained showed that the introduction of phytobiotic additives in fish diet has a positive effect on live weight gain and can potentially be used as the basis for the gut microbiota modifying drugs. The increase of the number of certain groups of bacteria that can potentially have probiotic features will reduce the risk of disease outbreaks at the carp cultivation stage.

Sponsorship information. The research was made with the financial support of the Russian Science Foundation No. 23-76-10054.

Conflict of interest. The authors declare that they have no conflicts of interest.

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Поступила 05.09.2024
После рецензирования 30.10.2024
Принята 07.11.2024

Received 05.09.2024
Revised 30.10.2024
Accepted 07.11.2024