



Alteration in Gut Microbiome of Common Carp (*Cyprinus carpio* L., 1758) Mediated by Probiotics and Yeast Prebiotic [†]

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Abstract: The objective of the present study was to examine the impact of dietary supplementation with probiotics and yeast cell wall prebiotics on the intestinal microbiota of common carp (*Cyprinus carpio*). A total of 96 carp, with an average body weight of 932 ± 161 g, were distributed into 12 fish tanks (800 L), with 8 fish/tank. The fish were fed a variety of experimental diets, including a basal diet only (CD) or a basal diet supplemented with the probiotic *Pediococcus acidilactici* (PA), the yeast probiotic *Saccharomyces cerevisiae* (SC), or the yeast cell wall prebiotic (YANG) at a concentration of 0.1% (1 g/kg) for a duration of 42 days. At the end of the trial, fish digesta were withdrawn, and the total bacterial community of the gut of common carp was analyzed using Illumina's NGS targeting the 16S rRNA gene. A Krona phyla richness pie chart showed that 11 bacterial phyla were recorded in fish fed YANG, with the top three phyla being Fusobacteria, Firmicutes, and Proteobacteria. In addition, 10 phyla were identified in fecal samples from carp fed PA, with the top three phyla being Proteobacteria, Firmicutes, and Fusobacteria. Furthermore, nine phyla were recorded for carp fed SC, with the top three phyla being Fusobacteria, Firmicutes, and Proteobacteria. However, carp fed a basal diet exhibited 14 phyla, with the most abundant phyla being Fusobacteriota, Bacteroidota, and Proteobacteria. This study concluded that the tested feed supplements could cause considerable alterations in the composition of the gut microbiome of carps reared in recirculating systems.

Keywords: *Pediococcus acidilactici*; *Saccharomyces cerevisiae*; yeast prebiotic; common carp; 16S rRNA gene; gut microbiota; gut health



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1. Introduction

In aquaculture, the common carp (*Cyprinus carpio*) is considered a prominent freshwater fish species, with global production exceeding 4 million tons, constituting over 7.7% of total finfish production [1]. The common carp is able to consume diverse foodstuff and is a flexible feeder, switching from its preferred diet to alternative foods depending on food availability. However, a poor feed conversion ratio (%) and high mortality rates affect fish and result in reduced fish production. Several bacterial pathogens such as *Aeromonas*

salmonicida, *Staphylococcus* spp., *Mycobacterium* spp., *Vibrio harveyi*, and *Flavobacterium psychrophilum* have routinely caused poor fish production [2]. Antibiotics are commonly implemented in aquaculture to overcome pathogens and disease development; however, antibiotics are not environmentally friendly and are known for their negative impact on beneficial microbes because of the potential of bacteria to produce antibiotic-resistant strains [3]. The problem of antibiotic resistance development affects not only the aquaculture industry but also consumers and the environment [4,5]. Consequently, the use of antibiotics in aquaculture is directly related to the presence and spread of resistant bacterial pathogens in humans [6]. The rise in antibiotic resistance and consumer safety concerns are key factors that have motivated scientists to seek alternative control strategies, such as probiotics and prebiotics, in aquaculture.

One of the critical roles of the gut microbiota is to enhance immunity parameters and nutritive status through several means. The gut microbiota enhances the digestive process by providing digestive enzymes, which play an important role in polysaccharide degradation and vitamin synthesis [7]. In addition, the gut microbiome also plays a significant role in preventing the host from acting against pathogenic bacteria by competing for adhesion sites, absorbing nutrients, and producing antimicrobial compounds to reduce the intensity of the pathogens [7,8]. The use of probiotics and prebiotics in aquaculture has gained significant attention due to their potential to enhance the fish gut microbiota and improve overall health [9–12].

Powerful sequencing technologies such as Next-generation Sequencing (NGS) have shown that culture-based methods do not reflect the total microbial community [13]. Advances in high-throughput sequencing approaches have led to increased interest in the metagenome; however, there are few studies that have discussed the effect of prebiotics and probiotics on the microbial community of the intestine of freshwater fishes using NGS. Thus, the current study aimed to investigate the impact of two probiotics, namely, *Pediococcus acidilactici* and *Saccharomyces cerevisiae*, and the inactivated yeast prebiotic on the microbial communities of the intestine in common carp (*Cyprinus carpio* L.) using Illumina's NGS.

2. Materials and Methods

2.1. Ethical Statement

Common carp (*Cyprinus carpio*) experimental procedures in this investigation were carried out in accordance with technical and regulatory guidelines for animal welfare. The Department of Animal Husbandry, Institute of Animal Science, University of Debrecen obtained the required permit for these experiments from the Animal Protection Committee of the University of Debrecen, Hungary (15/2019/DE MÁB Kovács László). The permit, issued by the Hajdú–Bihar County Government Office on 5 March 2020, is valid for a period of five years and was signed by the Chief Veterinary Inspector for Animal Welfare.

2.2. Feed Composition

The dry matter composition of the basal diet that has been used comprises 33.5% crude protein, 6.9% crude fat, 1.32% crude fiber, 6.09% ash, and 15.04 MJ/kg digestible energy. The ingredients (g/kg) are 460 g wheat meal, 200 g poultry by-product meal, 150 g fish meal, 100 g JPC 56 soy protein concentrate, 20 g blood meal, 20 g vitamin and mineral premix, 20 g zeolite, 20 g fish oil, and 10 g glucose.

2.3. Animal Housing

Common carp with an initial average body weight of 920 ± 161 g were reared in a recirculating system (RAS). The experiment was performed in the fish laboratory of the University of Debrecen, Department of Animal Husbandry. After the fish were ac-

climatized for 2 weeks, a total of 96 fish were distributed in 12 fish tanks (8 fish/tank, 24 fish per treatment). A commercial fish diet was used; its composition is mentioned above. Three different feed additives, each produced by Lallemand Animal Nutrition (Montreal, QC, Canada), were tested. Levucell contains the probiotic *Saccharomyces cerevisiae* var. *boulardii* CNCM 1-1079; Bactocell contains *Pediococcus acidilactici* CNCM I-4622 MA 18/5 M; and YANG is a product obtained from the inactivated yeast cell wall of *S. cerevisiae* and *Cyberlindnera jadinii*. The feeding experiment was designed to have three replications (3 tanks/treatment) and four treatments as follows: fish received the basal diet only (C) or the same basal diet supplemented with *P. acidilactici* (PA), *S. cerevisiae* (SC), and the yeast cell wall prebiotic (YANG), respectively, at 1 g/kg [14]. The water volume of each fish tank was 800 L. The feeding process was performed twice a day with feeding rates of 3% of the total biomass. Feces and uneaten feed were removed regularly when needed.

The dissolved oxygen level (mg/L) and water temperature (°C) were maintained at 6.78 ± 0.3 and 25.9 ± 0.9 , respectively. Meanwhile, the total dissolved solids (TDS; HI98130 Hanna Instruments, Woonsocket, RI, USA), NO_3^- , NO_2^- , and NH_4^+ concentrations (DR3900 Spectrophotometer, Hach, Loveland, CO, USA), and pH were monitored regularly, and they were at the optimum levels (Table 1). At the end of the trial, 1 to 2 g of fish digesta was collected from one fish per treatment in Eppendorf tubes and stored at -20°C until analysis.

Table 1. Water quality parameters of rearing system for common carp during experimental period (42 days).

Water Parameters	Mean $\pm$ Standard Deviation
Dissolved oxygen (mg/L)	6.78 ± 0.3
Temperature (°C)	25.9 ± 0.9
pH	7.4 ± 0.1
NO_3^- (mg/L)	5.78 ± 2.9
NO_2^- (mg/L)	0.24 ± 0.3
NH_4^+ (mg/L)	0.37 ± 0.18
TDS (ppm)	443 ± 6.72

2.4. DNA Extraction and Purification

Cell lysis and total DNA extraction were performed according to the manufacturer's protocol (Thermo Fisher Scientific, Waltham, MA, USA). After sample homogenization, the bacterial suspension was incubated for 3 min and then centrifuged at $16,000 \times g$ at 4°C for 10 min. The supernatant was used for DNA isolation. For DNA extraction from the cell suspension, a commercially available DNA kit, the QIAamp Fast DNA Stool Mini Kit (cat.n: 51604), was used according to the manufacturer's instructions (Qiagen GmbH, Hilden, Germany). Prior to the PCR reactions, the amount of isolated DNA was determined using a Qubit 2.0 fluorimeter with the Qubit™ dsDNA HS Quantitation Assay Kit (cat. n: Q32851) (Thermo Fisher Scientific, Waltham, MA, USA).

2.5. Library Preparation and 16S rRNA Sequencing

Library preparation was carried out according to the standard protocol (San Diego, CA, USA). The variable regions (V3 and V4) of the bacterial 16S rRNA were targeted and amplified to generate approximately 460 bp amplicons using universal primer sets 341F (5' CCTACGGGNGGCWGCAG 3') and 785R (5' GACTACHVG GGTATCTAATCC 3'), which were flanked by Illumina overhang adapter sequences (forward: 5' TCGTCG-GCAGCGTCAGATGTGTATAAGAGACAG 3'; reverse: 5' GTCTCGTGGCTCGGAGATGT-GTATAAGAGACAG 3') (Sigma Aldrich, St. Louis, MO, USA). The 16S amplicon libraries for each sample were amplified using PCR, with the following cycling conditions: initial

denaturation at 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 53 °C for 40 s, and extension at 72 °C for 1 min. Amplification success and the relative intensity of the bands were checked on a 2% agarose gel. Sequencing was then performed using a MiSeq Reagent Kit v3-618 cycle (MS-102-3003) according to the manufacturer's protocols (Illumina, Inc., San Diego, CA, USA). Illumina BaseSpace software (<https://basespace.illumina.com/>, Illumina Inc., San Diego, CA, USA) was used to demultiplex paired-end reads and generate FASTQ files. The data were analyzed using the Quantitative Insight Into Microbial Ecology pipeline (QIIME2, v. 2019.1) [15]. The abundance of each taxonomic group in each fecal sample was visualized using Krona v. 2.6.

3. Results

The results obtained from the metagenomic analysis of the microbiome of common carp that were fed different feed additives (*P. acidilactici*, *S. cerevisiae*, yeast prebiotic, and the control diet) are reported in Figure 1 and Table 2. The results for carp fed a diet supplemented with 0.1% (1 g/kg of feed) of the yeast prebiotic include 11 different bacterial phyla, and Fusobacteria with an abundance of 55%, Firmicutes (36%), and Proteobacteria (6%) were the top phyla. The phylum Fusobacteria was dominated by *Fusobacterium*, while Firmicutes was dominated by the *Pedococcus*, *Streptococcus*, *Clostridium*, *Epulopiscium*, and *Lactococcus* genera. The phylum Proteobacteria was also dominated by several genera such as *Aeromonas*, *Citrobacter*, *Enterobacter*, *Dechloromonas*, and *Crenobacter*. Furthermore, carp fed 0.1% *P. acidilactici* showed 10 phyla. Proteobacteria, Fusobacterium, and Firmicutes were the top phyla. In Proteobacteria, the most common species were *Aeromonas*, *Shewanella*, and *Plesiomonas*, respectively, whereas *Cetobacterium* was the most common genus in Fusobacterium. In the sample from carp fed 0.1% *S. cerevisiae*, nine phyla were found, and the top three phyla were Fusobacterium (50%), Proteobacteria (35%), and Firmicutes (14%). In Fusobacterium, *Cetobacterium* was the most dominant genus, whereas *Aeromonas*, *Vibrio*, *Plesiomonas*, *Citrobacter*, and *Shewanella* were the most dominant genera in the phylum Proteobacteria. Lastly, in carp fed the basal diet (the control diet), 14 different phyla were present. The most dominant phyla were Fusobacteriota (68%), Proteobacteria (27%), and Bacteroidota (3%). In Fusobacteriota, *Fusobacterium* dominated, whereas *Aeromonas*, *Citrobacter*, *Dechloromonas*, *Plesiomonas*, and *Providencia* dominated the phylum Proteobacteria.

Table 2. Bacterial abundance in intestine of carp fed probiotics and yeast cell wall prebiotic.

Feed Supplements	Genus	Reads	Read %
Control	<i>Cetobacterium</i>	67.802	77.46
	<i>Streptococcus</i>	08.341	9.53
	<i>Citrobacter</i>	03.587	4.10
	<i>Aeromonas</i>	02.827	3.23
	<i>Chryseobacterium</i>	01.964	2.24
	<i>Uruburuella</i>	01.955	2.23
	<i>Plesiomonas</i>	01.154	1.23
	<i>Lactococcus</i>	00.382	0.44
	<i>Vibrio</i>	00.287	0.33
	<i>Shewanella</i>	00.239	0.27
<i>P. acidilactici</i>	<i>Vibrio</i>	26.318	53.78
	<i>Cetobacterium</i>	10.920	22.31
	<i>Aeromonas</i>	9.695	19.81
	<i>Lefsonia</i>	1.685	3.44
	<i>Mycobacterium</i>	0.194	0.40
	<i>Streptococcus</i>	0.127	0.26
	<i>Plesiomonas</i>	0.040	0.08
	<i>Shewanella</i>	0.026	0.05
	<i>Uruburuella</i>	0.026	0.05
	<i>Citrobacter</i>	0.011	0.02

Table 2. Cont.

Feed Supplements	Genus	Reads	Read %
<i>S. cerevisiae</i>	<i>Cetobacterium</i>	79.053	58.36
	<i>Aeromonas</i>	47.169	34.82
	<i>Vibrio</i>	3.478	2.57
	<i>Citrobacter</i>	2.866	2.11
	<i>Plesiomonas</i>	0.993	0.73
	<i>Chryseobacterium</i>	0.749	0.55
	<i>Shewanella</i>	0.487	0.36
	<i>Uruburuella</i>	0.317	0.23
	<i>Streptococcus</i>	0.279	0.21
	<i>Lefsonia</i>	0.082	0.06
Yeast cell wall	<i>Cetobacterium</i>	101.599	91.54
	<i>Aeromonas</i>	3.276	2.95
	<i>Plesiomonas</i>	2.748	2.48
	<i>Vibrio</i>	0.806	0.73
	<i>Citrobacter</i>	0.732	0.66
	<i>Colostridium</i>	0.605	0.55
	<i>Chryseobacterium</i>	0.511	0.46
	<i>Streptococcus</i>	0.393	0.35
	<i>Shewanella</i>	0.147	0.13
	<i>Uruburuella</i>	0.121	0.11

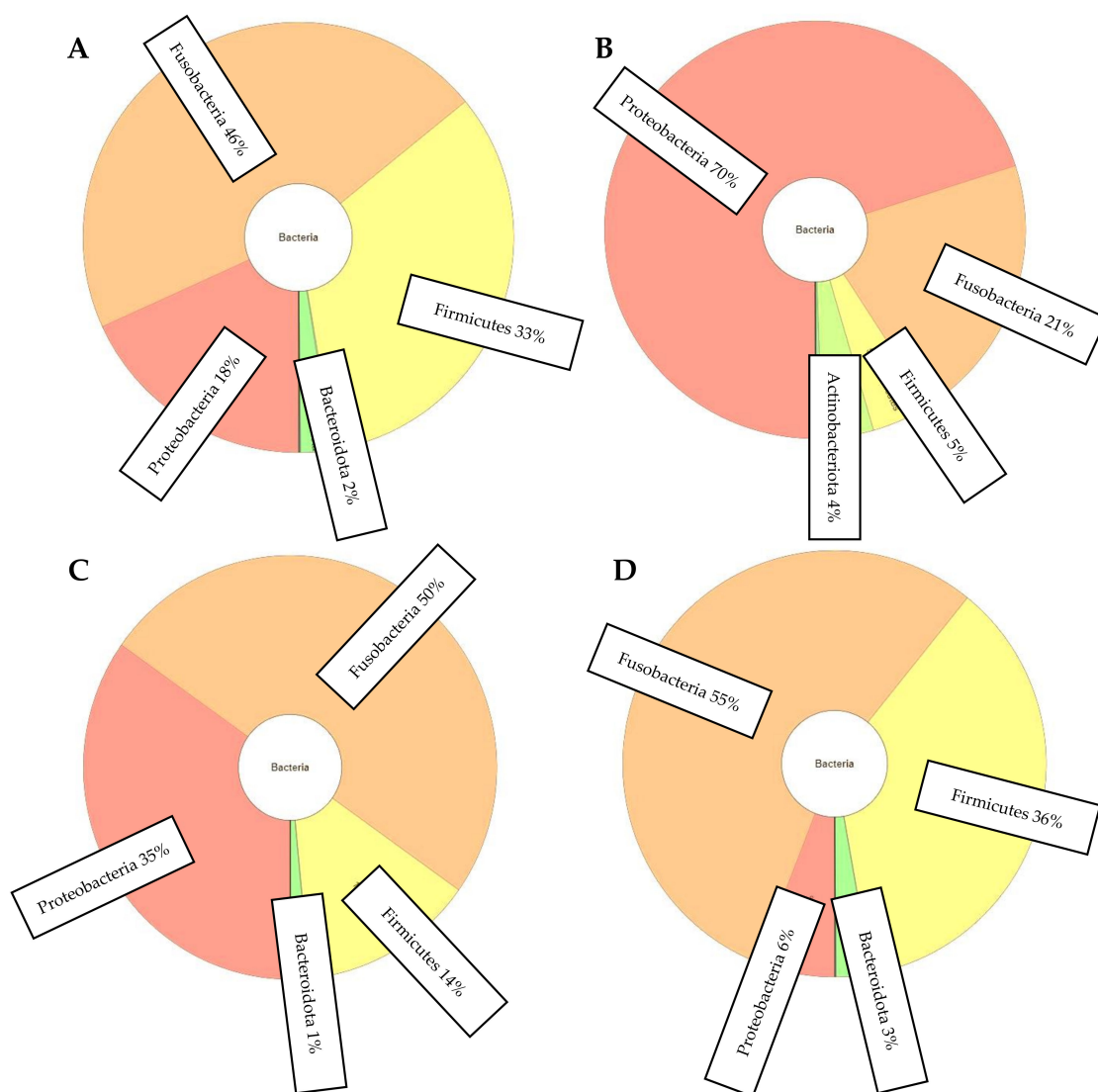


Figure 1. Krona pie charts adjusted at phylum level. The charts show the abundance in the intestinal bacterial community of carps fed (A) the control diet, (B) *P. acidilactici*, (C) *S. cerevisiae*, or (D) the yeast cell wall prebiotic for 42 days.

4. Discussion

Although the mechanisms of action of probiotics are not fully understood, Butt and Volkoff [16] suggested that probiotics could compete with pathogenic bacteria for niches by secreting antimicrobial peptides. As an alternative technique to antibiotic use, the implementation of natural alternatives in aquaculture via feed supplementation is becoming a major focus of current research [17]. Previous results have shown that a probiotic-supplemented diet could be promising in aquaculture industries [18,19]. The intestines of aquatic animals provide a favorable environment for beneficial microbes to colonize and thrive [20]. The composition of the gastrointestinal tract (GIT) microbiome can shape host growth, physiology, and health performance. High diversity in the intestinal microbiota is considered beneficial for host health. Thus, we investigated the effects of the probiotics *P. acidilactici* and *S. cerevisiae* and yeast prebiotics on the GIT microbiome of the common carp.

In the control group, the gut microbiota was mainly dominated by *Clostridium* (77.44%), followed by *Streptococcus* (9.15%) and *Citrobacter* (4.1%). *Clostridium*, a genus within the phylum *Firmicutes*, includes several species known for their fermentative metabolism, contributing to intestinal health. However, the presence of *Citrobacter* and *Aeromonas*, both members of *Proteobacteria* and potential opportunistic pathogens [21,22], could suggest a relatively less competitive microbial environment in the absence of probiotic and prebiotic supplementation.

In *P. acidilactici*, a major shift in microbial composition was observed, with *Vibrio* (53.78%) and *Cetobacterium* (22.31%) becoming the most dominant genera. The absence of potentially harmful genera such as *Citrobacter* or *Aeromonas* might suggest a suppressive effect of *P. acidilactici* on undesirable bacteria.

In *S. cerevisiae*, the gut microbiota was substantially dominated by genera such as *Cetobacterium* (58.36%) and *Aeromonas* (34.82%). *Aeromonas* spp. are commonly known as pathogens and are widespread in aquatic environments; however, they are also members of the freshwater fish microbiome that significantly contribute to the fermentation of organic substances, antibacterial activity, and cellulose degradation [23]. Additionally, *Aeromonas* spp. have been identified as causative agents of human diseases, including septicemia, which is characterized by the presence of *Aeromonas septicemia* [24]. *Aeromonas* spp. have also been confirmed to be important spoilage bacteria that reduce the quality of aquatic animals [25]. In addition, *Shewanella* species were recorded with high abundance compared to other groups. *Shewanella* species, a prominent group of omega-3 fatty acid-producing bacteria, are frequently isolated from the gastrointestinal tract (GIT) of fish and other vertebrate animals [26]. However, the genus *Shewanella* is also regarded as a critical spoilage bacterium that can produce hydrolytic and extracellular enzymes, which may adversely affect meat quality [27].

In the yeast cell wall prebiotic, *Cetobacterium* spp. (91.54%) were the most dominant. The yeast prebiotic showed a remarkable abundance of the phylum *Bacteroidota* compared to the other groups of feed supplements. This result is in line with Feher et al. [28], who noticed that supplementation with phytonutrients (fermented corn and pepper pulp) could increase the abundance of *Bacteroides* in the GIT of common carp. In fish, carbohydrate fermentation is mostly performed by bacteria which belong to the genus *Bacteroides*, known as good producers of short-chain fatty acids (SCFAs) that play a critical role against inflammation. Generally, due to the shorter retention times of SCFAs in omnivorous and herbivorous carps, probiotic and prebiotic feed supplementation could be a good technique for keeping SCFAs at their optimum levels [29].

In conclusion, dietary supplementation with probiotics (*P. acidilactici* and *S. cerevisiae*) and a yeast cell wall prebiotic (mixture of *S. cerevisiae* and *Cyberlindnera jandinii*) at 1 g/kg for

42 days could cause some alterations in the gut microbiome of carp reared in a recirculating system. However, this is a preliminary result based on a small sample size, and abundance estimation was based on Krona pie charts. Further studies with larger samples are required to validate these findings.

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