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THE IMPACTS OF HEAVY METALS ON THE GUT MICROBIOME IN COMMON CARP (*CYPRINUS CARPIO*)

Pollution of freshwaters with heavy metals has become an acute problem in many countries including Kazakhstan, and industrial progress is the primary source of toxic heavy metals. Since gut microbe communities play a significant role in fishes' homeostasis, immune regulation, metabolism, and disease resistance, it is crucial to understand how heavy metals affect fish's gastrointestinal microbiome diversity. Applications of metagenomics using the 16S rDNA gene's hypervariable regions allow researchers to sequence the gastrointestinal microbiota's genome and identify the diversity of microorganisms, including those that cannot be cultured with traditional microbiological methods. Common carp (Cyprinus carpio) is resistant to highly polluted freshwaters with heavy metals and considered a bioindicator of freshwater pollution. Thus, this paper aims to overview heavy metals' influence on the gastrointestinal microbiome diversity in common carp. Future directions are also discussed to enhance our understanding about the relationships between different environmental factors and gut microbiome diversity of wild fish. Further, it is crucial to understand how each bacterium would help common carp resist heavy metal toxicity.

Key words: common carp, heavy metal, resistance, gut microbiome, metagenomics.

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Сазан балығының (*Cyprinus carpio*) ішек микрофлорасына ауыр металдардың әсері

Тұщы сулардың ауыр металдармен ластануы көптеген елдерде, соның ішінде Қазақстанда да өте өзекті мәселеге айналды. Өндірістің қарқынды дамуы ауыр металдармен ластанудың басты көзі болып саналады. Ішек микрофлорасы балықтардың гомеостазында, иммундық жүйесін реттеуде, зат алмасу үдерісінде және ауруларға төзімділігінде маңызды қызмет атқарғандықтан, ауыр металдардың балықтардың ішек микрофлорасының алуантүрлілігіне әсерін зерттеу өте қажет. 16S рДНҚ генінің гиперөзгермелі аймақтары негізінде жүзеге асырылатын метагеномика әдісі зерттеушілерге ішек микрофлорасы геномын секвенирлеуге мүмкіндік береді. Нәтижесінде дәстүрлі микробиологиялық әдістер арқылы анықталынбайтын микроағзалардың тізімін анықтауға болады. Қарапайым тұқы немесе сазан балығы (Cyprinus carpio) ауыр металдармен қатты ластанған тұщы суларда өте төзімді және тұщы сулардың ластануының биоиндикаторы болып саналады. Сондықтан осы мақаланың мақсаты сазан балығы ішек микрофлорасының алуантүрлілігіне ауыр металдардың әсеріне шолу жасау. Табиғи ортадағы сазан балығы микрофлорасының алуантүрлілігі мен әртүрлі қоршаған орта факторлары арасындағы қарым-қатынасы туралы біздің білімімізді толықтыруға алып келетін бағыт талқыланады. Сонымен қатар, ауыр металдардың улылығына сазанның төзімділігіне оң әсер ететін бактериялар түрлерін білу өте маңызды.

Түйін сөздер: сазан, ауыр металдар, резистенттілік, ішек микрофлорасы, метагеномика.

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Воздействие тяжелых металлов на микробиом кишечника сазана (*Cyprinus carpio*)

Загрязнение пресных вод тяжелыми металлами стало острой проблемой во многих странах, в том числе и в Казахстане, а промышленный прогресс является основным источником токсичных тяжелых металлов. Поскольку сообщества кишечных микроорганизмов играют важную роль в гомеостазе, иммунной регуляции, метаболизме и устойчивости к болезням рыб, крайне важно

понять, как тяжелые металлы влияют на разнообразие микробиома желудочно-кишечного тракта рыб. Применение метагеномики с использованием гипервариабельных областей гена 16S рДНК позволяет исследователям секвенировать геном микробиоты желудочно-кишечного тракта и идентифицировать разнообразие микроорганизмов, включая те, которые невозможно культивировать традиционными микробиологическими методами. Обыкновенный карп или сазан (Cyprinus carpio) устойчив к сильно загрязненным тяжелыми металлами пресным водам и считается биоиндикатором загрязнения пресных вод. Поэтому целью данной статьи является рассмотрение влияния тяжелых металлов на разнообразие кишечной микрофлоры карповых рыб. Обсуждается способ расширения наших знаний о разнообразии микрофлоры карпа в естественной среде и взаимосвязи между различными факторами среды.

Кроме того, очень важно знать виды бактерий, положительно влияющие на устойчивость карпа к токсичности тяжелых металлов.

Ключевые слова: сазан, тяжелые металлы, резистентность, микробиом кишечника, метагеномика.

Introduction

Heavy metals are naturally occurring elements with high atomic weights. Many of them are essential for living organisms, mostly at lower concentrations [1]. Nevertheless, high concentrations of heavy metals can be toxic to living organisms, including freshwater fish; even traces of some heavy metals can have toxic effects. Since fish are the top consumers in freshwaters, heavy metals accumulate in different fish tissues through the food chain and directly from the contaminated aquatic environment. Human activities are the primary sources of heavy metal pollution [2, 3]; their toxic effects on the fish and other vertebrates are well-studied [4-6]. High concentrations of heavy metals caused histopathological damages in the gill structure of common carp (*Cyprinus carpio*) compared to control groups; damages included fusion of primary lamella and thinning and shortening secondary lamella [7]. Moreover, heavy metals exposure resulted in glomerular necrosis, congestion, and degeneration of tubules in the kidney of common carp [7]. However, little is known to scientists about the impacts of different heavy metals on fishes' gut microbiome composition.

Fish gastrointestinal microbiome, the collection of all microbes living on the gut's surface, has enormous impacts on the host metabolism, nutrient absorption, immune system regulations, and pathogen resistance. Gut microbiome diversity of fish considerably varies depending on their phylogenetics, surrounding environment, and diet [8-10]. Understanding the dynamics of the gut microbiome diversity of fishes caused by heavy metals is vital to maintain the health and disease resistance of economically important fish species. It is practically impossible to manipulate wild fish's gastrointestinal microbiome composition in rivers and lakes where pollution usually occurs. Thus, preventing freshwaters from heavy metal pollution could be a better solution to protect fish and other aquatic organisms from heavy metals' toxicity. However, growing human populations in the world can make it challenging to regulate heavy metals concentrations in freshwaters because it can increase human pressures on wildlife habitats. Due to these limitations, it is crucial to estimate the concentrations of heavy metals that can alter the gut microbiome composition of fish, and especially, identify the gut microbiome's compositions associated with the toxicity of heavy metals. Further, specific gut microbiome communities associated with the heavy metal pollution might be better indicators of heavy metal exposure in fish. However, traditional microbiological methods cannot identify the entire microbiome diversity of environmental samples collected directly from different environments [11].

Thanks to achievements in genome sequencing techniques, scientists can study the biodiversity of microorganisms, including those which cannot be cultured using microbiological techniques, by sequencing the entire genome or desired regions of the genome using single-stranded nucleotide primers. Metagenomics, as a young branch of genomics, allow researchers to sequence the whole genome of microorganisms sampled from the environment and understand how their diversity in the gut varies depending on endogenous and exogenous factors [12-14]. Furthermore, PCR-based studies can amplify targeted regions of 16S rRNA of unculturable microorganisms using DNA primers [6], including vertebrates' gut microbiome. The bacterial rRNA gene has conserved and variable sequence regions, and each region can have its importance in genomic studies depending

on the aim of the research. Variable regions of the 16S rRNA gene can help identify microorganisms down to genera level [15, 16] because the sequences of these regions differ across microorganism species. Thus, primers designed based on highly variable 16S rDNA regions are applied to identify operational taxonomic units (OTU). OTUs are a group of microorganisms that share at least 97-99% identical sequences of the 16S rDNA gene [17-19]. These species can be considered as phylogenetically close relatives if a 16S rRNA (rDNA) sequence of OTUs differ by less than 1% [18] or 3% [19] across identified species. Fish that consume relatively similar food resources might share the same OTUs. However, OUT composition can vary considerably among fish species depending on their guilds and digestive system morphology, physiological behavior [20]. However, the existence of the core gut microbiome across fish species is required further investigation.

Since the gut microbiome communities of fish affect physiological and biochemical homeostasis and disease resistance of fish, it is essential to understand how their diversity will change after exposure to heavy metals. Obtained results would be beneficial for future studies on each operational taxonomic unit's roles in common carp's ability to live in heavily polluted waters with heavy metals. Thus, this paper aims to overview heavy metals' influence on the gastrointestinal microbiome diversity in common carp. Because gut metagenomics is a recent field, there are relatively few studies available. Therefore, I will describe each in detail and follow with recommendations for future studies.

Common carp is an economically significant cyprinid (*Cyprinidae*) species, and it generated 7.7 % of total aquaculture production in the world in 2018 (SOFIA, 2020). Moreover, common carp is highly resistant to polluted waters with heavy metals [21], and it is an interest of this study how the diversity of the gut microbiome changes because of heavy metal pollution. The gut microbiome diversity of common carp was thoroughly described in one study [8]. This paper compared the fecal microbiome diversity of three carp species captured from wild and laboratory-housed using the V6 hypervariable region of the 16S ribosomal RNA (rRNA). They found that *Proteobacteria, Firmicutes, and Fusobacteria* were the most abundant phyla among studied species and their habitats. However, there were significant differences between wild and laboratory-housed groups regarding the beta diversity within the species. Wild captured common carp had significantly higher amounts of *Clostridiales* than laboratory-housed common carp, whereas the abundance of *Fucobacteriales* was higher among laboratory-housed samples of the same species. Bacterial communities clustered by the environment of fish underlying the importance of the environment in shaping the gut microbiome composition of fishes. However, these authors did not find the significant effects of diet on the fecal microbiome diversity in common carp.

Applications of metagenomics in studying gastrointestinal microbiome diversity

In one experimental study [22], the authors randomly assigned juvenile common carp to different cadmium concentrations, 0 $\mu g\ L^{-1}$, 50 $\mu g\ L^{-1}$, and 500 $\mu g\ L^{-1}$. This study used the V3-V4 variable regions of the 16S rRNA to compare the gut microbiome's diversity among control and treatment groups. The results showed that cadmium (Cd) exposure considerably decreased the gut microbiome diversity of common carp and revealed an increased abundance of Cd-resistant microorganisms (*Methylobacterium* and *Methylophilus*). Increasing the abundance of Cd-resistant bacteria species in response to high cadmium concentrations might explain the resistance of the common carp in highly polluted waters, but it needs to be investigated. Furthermore, a hierarchical clustering tree demonstrated that microbial communities clustered into two groups: the microbiome compositions of the control and 50 $\mu g\ L^{-1}$ group clustered, while 100 $\mu g\ L^{-1}$ clustered separately. Despite the hierarchical clustering, the control and 50 $\mu g\ L^{-1}$ groups had relatively different operational taxonomic units. Metagenomics using 16S rRNA may not be sufficient in studying the role of each OTUs in the host immune system and heavy metal tolerance. However, this study stated that metagenomics allows researchers to identify the community of the gut microbiome associated with cadmium's toxicity.

Chronic copper exposure also negatively affected the gut microbiome composition and lipid metabolism in common carp in the treatment groups (0.07 mg/L, 0.14 mg/L, 0.28 mg/L) compare to control [23]. The decreased abundance of *Lactobacillus, Bacillus, and Akkermansia* in the

common carp's gut was observed from treatment groups (table 1). For example, the widely applied probiotic *Lactobacillus* prevents pathogen invasions and activates the nutrition intake of fishes. The abundance of *Lactobacillus* lowered to 0.82 % in the 0.07 mg/L Cu treatment group from 1.04 in control group. However, it decreased to 0.25 in the 0.28 mg/L Cu treatment group. Consequently, the risk of pathogen invasion increased in the gut of copper-exposed common carp following reduced abundance of beneficial bacteria. On the other hand, pathogen-related bacteria (*Pseudomonas and Acinetobacter*) were abundant from the gut samples of treatment groups (table 1). The presence of these genera in the gut of copper-exposed common carp might suggest that the gut's functional barrier was disrupted, and those fish are vulnerable to pathogen invasion.

Furthermore, Meng and others study found out that the gut microbiome communities associated with lipid metabolism and immunity were disturbed due to waterborne chronic copper exposure [24]. The expression of five genes related to lipid metabolism in common carp's liver was investigated using 18s mRNA primers and the results revealed suppressed expression of lipogenic enzymes. Lipogenic enzymes involve in energy storage through synthesis of triglycerides and fatty acids and their suppressed expression can indicate organism's reduced growth. These findings positively correlated with taxonomic composition analysis. *Allobaculum, Blautia, Faecalibacterium, Roseburia* and *Ruminococcus* are well-known as short chain fatty acid (SCFA) producers and the abundance of these microorganisms decreased in copper-exposed common carp's gut. Since SCFAs protect intestinal epithelial cells from pathogen invasion and the reduction of SCFA synthesis can indicate the energy disbalance and metabolic dysfunction in analysis of taxonomic composition supported this conclusion.

Kakade and others first studied the impacts of a mixture of different heavy metals [chromium (Cr), cadmium (Cd), copper (Cu)] on the gut microbiome communities of common carp collected from the Yellow River, China [7]. Gut contents were sampled for DNA extraction and PCR amplification from the control, 0.8mg/L, and 3.2 mg/L treatment groups. For PCR amplification, they used the V3 and V4 variable regions of the 16S rRNA gene. The sequencing results demonstrated that high concentrations of heavy metals have led to significant changes at the phyla level in the treatment groups after 7 and 28 days of exposure. On day 14, *Firmicutes* (99.16%) were only phyla present in the 3.2 mg/L group, while on day 7, *Firmicutes* was 3.54%. On day 7, in the 3.2 mg/L group *Proteobacteria* was the most abundant (82.45%) phyla; however, this phylum disappeared from the gut content on day 14 (Figure 1). The authors think that *Firmicutes* could be highly resistant to high concentrations of heavy metals, especially Cr because its concentration was the highest among heavy metals in the mixture. It showed high accumulation compare to Cu and Cd at the end of the study. Also, the disappearance of other taxa in the 3.2 mg/L groups suggest that they might have a limited tolerance to exposure to extreme chromium concentrations. Another study found that Bacteroides abundance increased following cadmium exposure. [24]. However, this study indicated that supplementing probiotics such as *Bacillus coagulans* can alleviate cadmium toxicity and relieve intestinal barrier damage resulted cadmium exposure.

Chupani et al. (2019) studied the effect of food-borne ZnO nanoparticles on common carp's gastrointestinal microbiota [25]. For PCR amplification, they targeted the V4 variable region (515F-806R) of the 16S rRNA. They did not find significant differences in the gut microbiota compositions between the control (no added ZnO nanoparticles) and treatment groups (500 mg/kg feed ZnO nanoparticles). However, treatment group individuals had a relatively high abundance of *Flavobacterium*, *Chryseobacterium*, and *Aeromonas* compared to control group individuals. *Flavobacterium* and *Aeromonas* are associated with bacterial pathogens of fish in the wild, for instance. The presence of *Flavobacterium* and *Aeromonas* might indicate the disturbance of the balance of the gut microbiome of common carp after exposure to ZnO nanoparticles. It can lead to thriving opportunistic pathogens in the gastrointestinal tract of fish when exposed to toxic heavy metals in the wild and aquaculture. Moreover, this study showed that gastrointestinal microbial species diversity differs among individuals studied but did not significantly change in the gut microbiome diversity between the control and treatment groups.

Table 1 – Relative abundance (%) of sequences associated with lipid metabolism, probiotics and potential pathogens in the intestinal tissues of common carp following exposure to different concentrations of Cu (Meng et al. 2018)

Phyla/genera	Control	0.07 mg/L Cu	0.14 mg/L Cu	0.28 mg/L Cu
F/B	1.10 ± 0.21^{a}	0.88 ± 0.11^{a}	0.51 ± 0.03^{b}	1.34 ± 0.16^{a}
Blautia (%)	0.07 ± 0.01^{a}	0.03 ± 0.01^{a}	NF	NF
Phascolarctobacterium (%)	0.06 ± 0.01^{a}	0.07 ± 0.02^{a}	0.03 ± 0.01^{a}	0.02 ± 0.01^{a}
Roseburia (%)	0.14 ± 0.01^{a}	0.12 ± 0.01^{a}	0.02 ± 0.01^{b}	0.03 ± 0.01^{b}
Faecalibacterium (%)	0.12 ± 0.01^{a}	0.06 ± 0.03^{b}	0.02 ± 0.01^{c}	0.02 ± 0.01^{c}
Ruminococcus (%)	0.19 ± 0.02^{a}	0.08 ± 0.03^{b}	0.03 ± 0.01^{b}	0.06 ± 0.02^{b}
Coprococcus (%)	0.08 ± 0.02^{a}	0.07 ± 0.02^{a}	0.02 ± 0.01^{b}	0.02 ± 0.01^{b}
Bacteroides (%)	0.29 ± 0.01^{ab}	0.68 ± 0.02^{a}	0.23 ± 0.03^{ab}	0.12 ± 0.04^{b}
Allobaculum (%)	0.11 ± 0.02^{a}	0.07 ± 0.02^{ab}	0.03 ± 0.01^{b}	0.02 ± 0.01^{b}
Lactobacillus (%)	1.04 ± 0.29^{a}	0.82 ± 0.20^{a}	0.33 ± 0.04^{b}	0.25 ± 0.12^{b}
Bifidobacterium (%)	0.15 ± 0.01^{a}	0.09 ± 0.02^{b}	0.03 ± 0.02^{c}	0.03 ± 0.01^{c}
Akkermansia (%)	0.10 ± 0.02^{a}	0.03 ± 0.01^{b}	0.02 ± 0.01^{b}	0.01 ± 0.00^{b}
Acinetobacter (%)	0.17 ± 0.02^{b}	0.33 ± 0.06^{b}	0.63 ± 0.10^{a}	0.57 ± 0.07^{a}
Pseudomonas (%)	1.20 ± 0.22^{b}	1.03 ± 0.35^{b}	0.52 ± 0.05^{b}	6.11 ± 0.52^{a}

Note: Values are mean ± SEM and values with different letters within the same row are significantly different at $P < 0.05$; NF means not found.

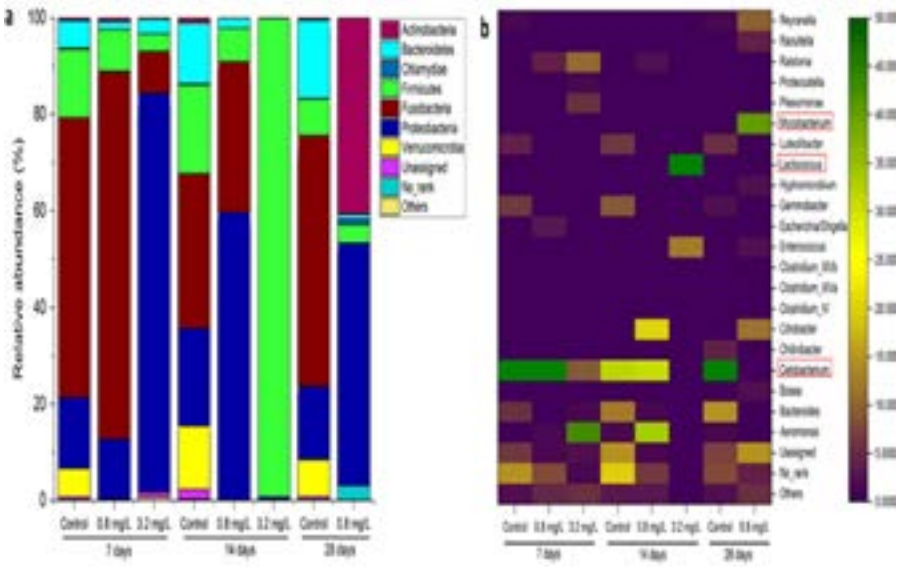

Figure 1 – Effects of heavy metal exposure on the gut microbiota at 7, 14, and 28 days in control, 0.8 mg/, and 3.2 mg/L groups; a) Relative abundance of identified gut microbiota at the phylum level (adapted from Kakade et al. 2020)

Conclusion and future directions

In the future, understanding the role of each operational taxonomic unit of the gut microbiome in the host physiology, homeostasis, metabolism, and disease resistance would be more beneficial for regulating the diet of fish in hatcheries and aquaculture. Changes in the gut microbiome composition caused heavy metal exposure helps to estimate threshold concentrations of heavy metals in freshwaters. Future studies should also use wild fish because other environmental factors such as temperature, habitat usage, and food availability are different from laboratory conditions. The effects of heavy metals interacting with other environmental factors might affect the gut microbiome composition of fish differently. With regard to heavy metals, it is understandable to conduct experiments in a laboratory where other variables are under control to distinguish heavy metals' real impacts on the gut microbiome composition. Moreover, using different DNA extraction methods [26] and different 16S rDNA primers might result in bias in microbiome diversity analysis. Thus, I recommend a comprehensive evaluation of methods for assessing fish gut microbiomes to develop standards that will allow direct comparisons across studies.

Heavy metal exposure alters the gut microbiome composition and decreases the diversity of the gut microbiota at phylum and genera level in common carp. The influence of heavy metals varies depending on a type of heavy metal exposed. Nevertheless, heavy metals resulted in gut dysbiosis and increased pathogen invasion. Metagenomics allow researchers to identify the gut microbiome diversity and composition, however it limits to understand how microorganisms help common carp to resist highly polluted waters.

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