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Research Article

Metagenomic analysis of the intestinal flora and antibiotic resistance genes of yellow catfish (*Pelteobagrus fulvidraco*)

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Abstract

High-throughput sequencing of intestinal microbial DNA using the Illumina platform was implemented to clarify the structure and function of intestinal flora and antibiotic resistance genes (ARGs) abundances in yellow catfish (*Pelteobagrus fulvidraco*). Species annotation and gene function analysis were performed on the metagenomic sequencing data. Intestinal bacteria were isolated and identified by 16sRNA. The results showed that intestinal flora was highly similar in the three *P. fulvidraco*. A total of 37 phyla and 788 genera of intestinal bacteria were identified. Proteobacteria, *Streptomyces*, and *Clostridium* are the dominant flora with the average relative abundance of 25.71%, 14.75%, and 5.15%, respectively. Six strains were successfully isolated and identified in our experiment. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis showed that the primary metabolic pathway is dominated by metabolism and organic systems, while the secondary metabolic pathway is dominated by sensory system, carbohydrate metabolism, replication, and repair. In addition, 499 ARGs of 37 resistance types were identified based on Antibiotic Resistance Genes Database. Tetracycline, polypeptide, macrolide, glycopeptide, and multiple drug resistance were highly abundant. The intestinal ARGs of *P. fulvidraco* were *macB, bcrA,* and *evgS*. In general, rich bacterial diversity and many types of ARG were detected in the intestine of P. fulvidraco. Moreover, probiotics are potentially a good alternative to antibiotic abuse in aquaculture industry. Therefore, analysis of intestinal flora, intestinal flora ARGs and gene functions is beneficial for the artificial farming of *P. fulvidraco*.

Keywords: *Pelteobagrus fulvidraco*, Metagenome, Intestinal flora, Antibiotic resistance genes

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Introduction

Pelteobagrus fulvidraco (Siluriformes: Bagridae), commonly known as the yellow catfish, is an important economic fish with a unique flavor of its meat, high nutrition, and fewer fishbones in China (Jee *et al*., 2009). The composition and structure of the intestinal microbial community of *P. fulvidraco* are affected by farming scale, feeding conditions, and water quality. Intestinal microorganisms play a key role in healthy fish growth. Intestinal microorganisms promoted their hosts' growth, development, and metabolism at appropriate concentrations. However, intestinal microorganisms caused disease when their concentrations are not held in check (Ringo *et al*., 2016).

Probiotics promoted the growth, metabolism, and maintenance of a balanced intestinal flora of their hosts. For example, *Bacillus pumilus* and *B. licheniformis* enhanced the immunity and disease resistance in tilapia (Aly *et al*., 2008), and *B. subtilis* inhibited growth of harmful microorganisms such as *Vibrio*, *Pseudomonas*, and *Aeromonas* (Olmos *et al*., 2020). Bacterial diseases have deleterious effects on the artificial breeding of *P. fulvidraco*, and these negative effects are exacerbated by environmental deterioration, intensive cultivation, and improper management. These pathogens caused hyperemia, bleeding, festering, abdominal dropsy, and even caused death in *P. fulvidraco* (Zhang *et al*., 2014). Many pathogens such as *A. hydrophila* also caused zoonotic diseases. Antibiotics are widely used in aquaculture to control the spread of disease and protect animal health (Muziasari *et al*., 2016). Approximately 75% of antibiotics cannot be absorbed in humans and animals (Chee-Sanford *et al*., 2009). Antibiotic residues such as norfloxacin, ciprofloxacin and enrofloxacin were detected in various fish and other aquatic products (He *et al*., 2012; Chen *et al*., 2015). The banned antibiotic chloramphenicol was detected in carp and silver carp muscles (Lu *et al*., 2009). Excessive exposure to antibiotics resulted in increases in the abundance of various antibiotic resistance genes (ARGs) in bacteria, and high antibiotic exposure caused multiple drug resistance in fish and increase the difficulty of disease control and prevention. Several experiments showed that the main pathogens were resistant to multiple drugs in *P. fulvidraco* (Jee *et al*., 2009; Quinn and Stevenson, 2012). Diverse bacterial pathogens and multiple drug resistance of bacteria negatively affected artificial farming of *P. fulvidraco* and had deleterious ecological effects. Probiotics are a promising alternative to antibiotics that promote intestinal flora balance and eliminate harmful pathogenic microorganisms. Research showed they colonized in intestines of fish *in-vivo* (Kuebutornye *et al*., 2020). Thus, non-toxic probiotics are safe and pollution-free for enhancing fish intestinal microflora. Additional research is needed to improve the efficiency of probiotics to promote growth, metabolism, and intestinal health in fish.

Traditional culture and 16S rDNA technology are used to study the intestinal microbes of *P. fulvidraco* (Wu *et al*., 2010). However, only cultivable microorganisms identified in vitro, and intestinal ARGs remain unexplored in *P. fulvidraco*. Here, metagenomic analysis was applied to clarify the composition and function of the intestinal flora of *P. fulvidraco*. We aimed to identify uncultured microorganisms and determine the abundances and types of ARGs in *P. fulvidraco*. We identified several novel ARGs in the microbial communities of *P. fulvidraco*. Macrogenomics provided a theoretical basis for the rational use of antibiotics in artificially farming of *P. fulvidraco* (Blair *et al*., 2015).

Materials and methods

Fish samples

Three healthy *P. fulvidraco* individuals were purchased from the Huimin Supermarket in Old Chaoyang Village, Huaxi District, Guiyang City, Guizhou, China. *P. fulvidraco* individuals were stored on ice. After 30 min, individuals were washed with 75% alcohol for 30 s, followed by sterilized ultrapure water, and then were dissected. The intestinal contents were removed and placed in sterilized centrifuge tubes. The intestinal contents were sampled using Omega Mag-Bind Soil DNA Kit (Shanghai Yuanmu Biological Co., Ltd.), and total DNA was extracted according to the kit instructions and stored in a refrigerator at -80°C for subsequent use. Illumina sequencing and library construction were performed by Shanghai Parsono Biological Company.

Metagenomic sequencing and analysis

Evaluation of sample quality

Paired-end libraries were constructed and sequenced using the Illumina NovaSeq/HiSeq high-throughput sequencing platform. Quality of raw data generated by sequencing was evaluated by FastQC (Gaud *et al*., 2021). A sequencing machine was used to screen and filter raw data.

Species annotation

The Kraken2 (Wood and Salzberg, 2014) tool was used to annotate non-spliced sequences to species. The BLASTN tool was used to annotate spliced sequences to species. Specifically, BLASTN searches of contig sequences against the nucleotide (nt) database of the National Center for Biotechnology Information (NCBI) were conducted. Species annotation information of target sequences was analyzed using Blast2lca software (Huson *et al*., 2007). The species abundance table was generated by integrating the species abundance information from each sample.

Gene prediction and functional annotation

The gene sequence files, protein sequence files, gene transfer format files, and general feature format files for the contigs were obtained using MetaGeneMark (Zhu *et al*., 2010) web tools (http://exon.gatech.edu/GeneMark/).

Redundant sets of data were removed using MMseqs2 (Steinegger and Söding, 2017) software. The non-redundant set of protein sequences was compared against the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa *et al.*, 2022) database (http://www.genome.jp/kegg/) and the EggNOG (Buchfink *et al.*, 2015) database (http://eggnog5.embl.de/), and the functions of genes were analyzed.

Annotation of ARGs

The annotation information of ARGs was obtained via comparison of the set of sequences against the Antibiotic Resistance Genes Database (ARDB).

Verification of the sequencing results

To verify the accuracy of the metagenomic sequencing data, we obtained random samples of *P. fulvidraco* from the same growing environment to isolate, culture, and purify intestinal bacteria. DNA was extracted for 16S rRNA amplification and sequencing. The primers of the 16S rRNA: 27F, 5'AGAGTTTGATCATGGCTCAG3'; 1492R, 5'GGTT ACCTTGTTACGACTT3'. BLAST searches were then conducted against the NCBI database.

Results

Assessment of sample quality

The total number of reads of the three *P. fulvidraco* samples was 74,683,642, 73,349,040, and 71,207,190. The total number of bases in these three samples (1, 2, and 3) was 11,218,648,324 bp, 11,018,392,684 bp, and 10,701,244,722 bp, respectively. The proportion of bases with an accuracy of 99% and 99.9% out of total bases was greater than 95% and 90%, respectively (Table 1).

Table 1: Data on the raw sequences.

Sample	Total number of reads	Total number of bases (bp)	N (%)	GC (%)	Q20 (%)	Q30 (%)
Sample-1	74,683,642	11,218,648,324	0.00033	39.90	95.79	90.63
Sample-2	73,349,040	11,018,392,684	0.00032	39.86	95.92	90.92
Sample-3	71,207,190	10,701,244,722	0.0004	39.84	95.85	90.65

Notes: In the table, N (%) is the percentage of ambiguous bases in total bases; GC (%) is the percentage GC content (i.e., the sum of G bases and C bases as a percentage of the total bases); Q20 (%) and Q30 (%) are the percentages of bases with 99% and 99.9% accuracy, respectively.

The number of effective sequences was greater than 97% of all sequences; the total number of effective bases in the three samples (1, 2, and 3) was 10,940,801,780 bp, 10,750,309,933 bp, and 10,441,115,363 bp, respectively (Table 2). The total number of species in the intestinal flora of the three samples (1, 2, and 3) according to the Chao1 (a nonparametric method for estimating the number of species in a community) and ACE indices were 22, 6, and 44, respectively. The Simpson index values were 0.852439364, 0.740813149, and 0.799409563, and the Shannon index values were 3.467284216, 2.236238705,

and 3.0967324, respectively (Table 3). This indicates that our sampling was sufficient and our data are robust. The species accumulation curve plateaued at higher numbers of samples, indicating that a total number of species will no longer increase significantly with the addition of new samples (Fig. 1).

Table 2: Statistics of the valid sequence data.

Sample	Library name	Proportion of effective sequences (%)	Total number of effective bases (bp)	Proportion of effective bases (%)
Sample-1	SPE	97.67	10,940,801,780	97.52
Sample-2	SPE	97.73	10,750,309,933	97.57
Sample-3	SPE	97.74	10,441,115,363	97.57

Table 3: Alpha diversity indices.

Sample	Chao1 index	ACE index	Shannon index	Simpson index
Sample-1	22	22	3.467284216	0.852439364
Sample-2	6	6	2.236238705	0.740813149
Sample-3	44	44	3.0967324	0.799409563

Notes: Chao1 richness estimator: estimates the number of species actually present in the flora by counting rare species detected only once and twice in the flora. The ACE richness estimator: defaults to include species with a sequence size below 10 to estimate the number of species actually present in the flora. Shannon diversity index: comprehensively considers the richness and evenness of the flora, while Simpson index is more sensitive to evenness and dominant species in the flora.

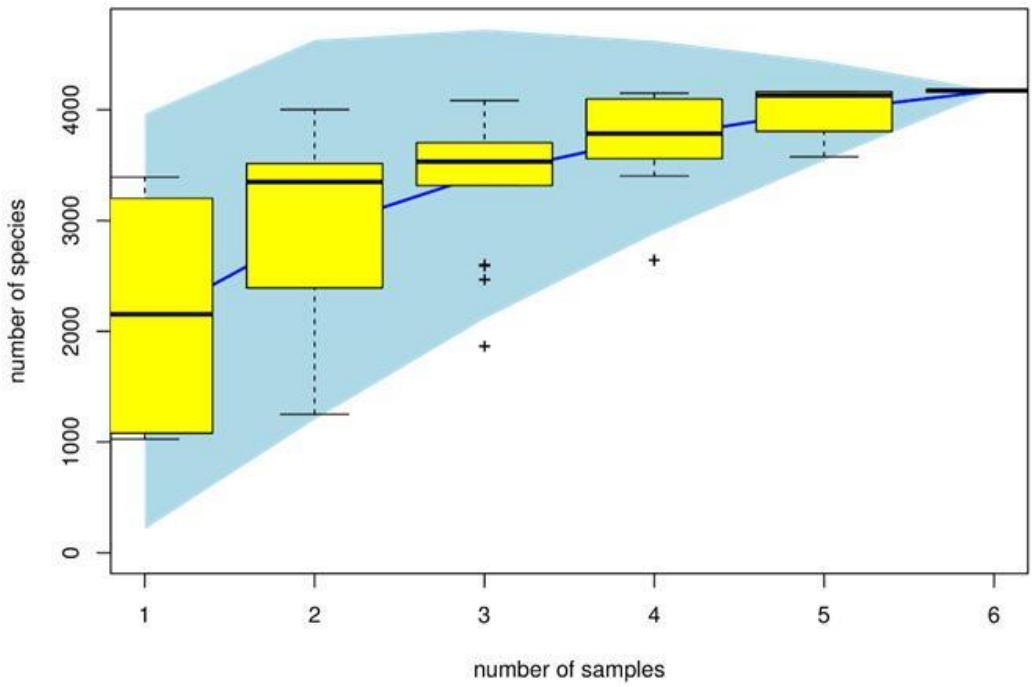

Figure 1: Species accumulation curve.

Species composition of the intestinal flora

We analyzed the relative abundances of bacteria with an average relative abundance greater than 1% in the samples. The four most common phyla detected were Proteobacteria (25.71%), Actinomycetes (24.62%), thick-walled fungi (21.78%), and Bacteroidetes (10.09%) (Fig. 2).

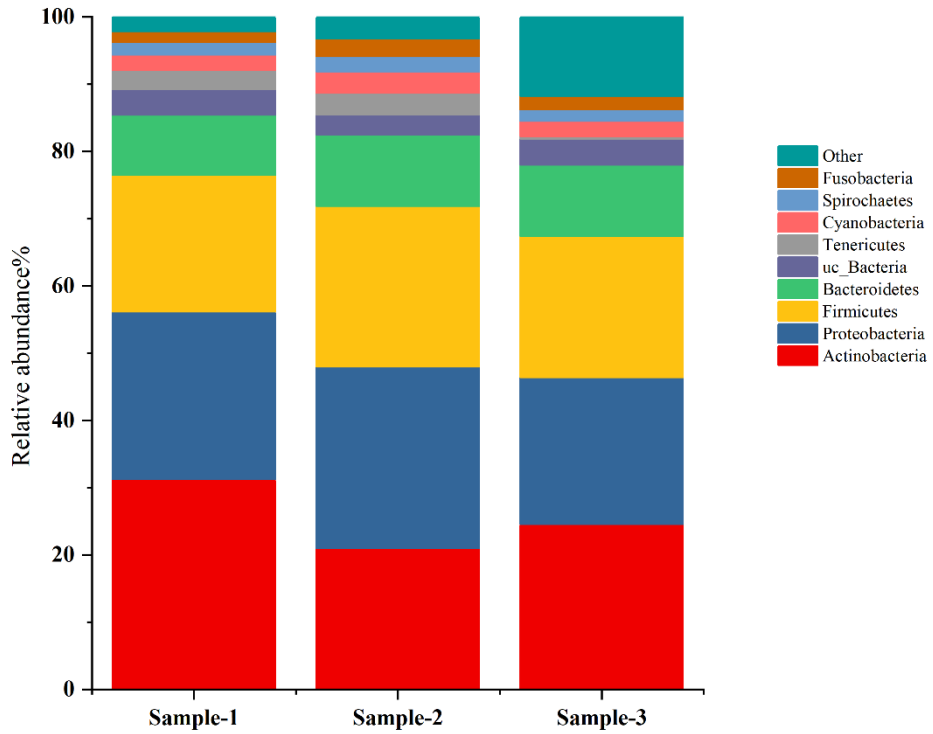

Figure 2: The relative abundances of the dominant phyla in each sample.

Twelve of the most common genera were *Streptomyces* (14.75%), *Clostridium* (5.15%), *uncultured bacteria* (3.55%), *Gordonella* (2.24%), *Bacillus* (2.70%), *Pseudomonas* (2.35%), *Sinomonas* (2.27%), *Yersinia* (23.9%), *Acinetobacter* (2.04%), *Streptococcus* (1.51%), *Clostridium* (1.36%), and *Aeromonas* (1.02%). *Mycoplasma* average relative abundance was 2.12% (Fig. 3). The most common species were *Streptomyces* (1.53%), *Yersinia* (0.87%), *Pseudomonas* (0.87%), *Bacillus cereus* (0.74%), *Marseilla* (0.39%), and *Gordonia* HY186 (0.34%). Probiotics comprised approximately 30% of the most common bacteria, and pathogenic bacteria comprised approximately 5% of the most common bacteria (Fig. 4). Some variation was observed in the abundances of phyla, genera, and species among samples. Analysis of the association network diagram revealed that Bacteroides, Proteobacteria, and Fusobacterium were dominant bacteria. These three bacteria were most closely related to other bacteria. Fusobacterium had a positive effect on the abundance of other bacteria (Fig. 5).

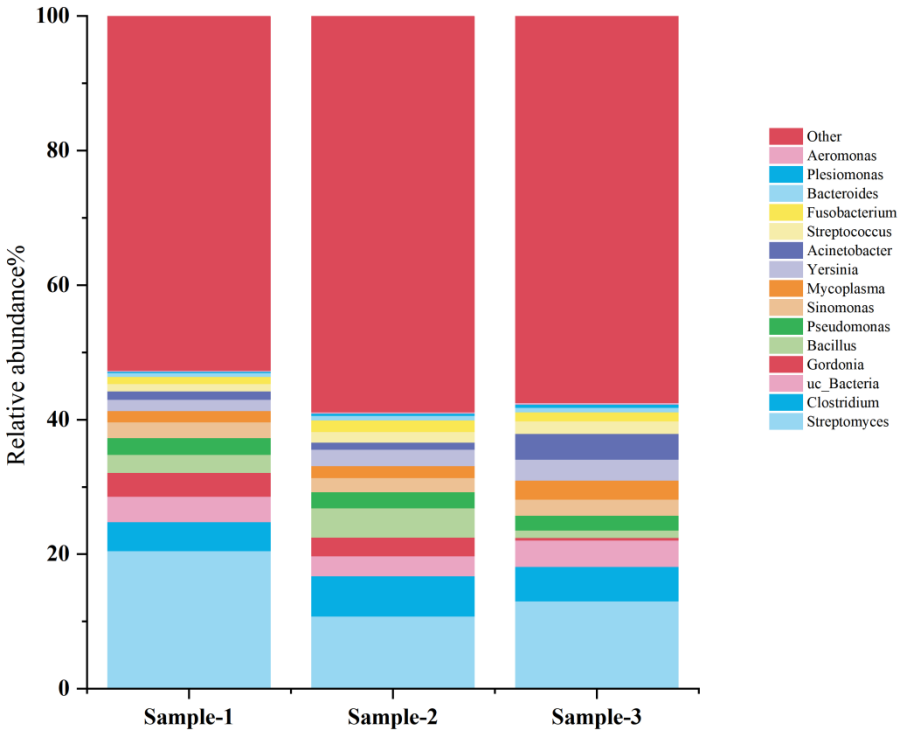

Figure 3: The relative abundances of the most common genera in each sample.

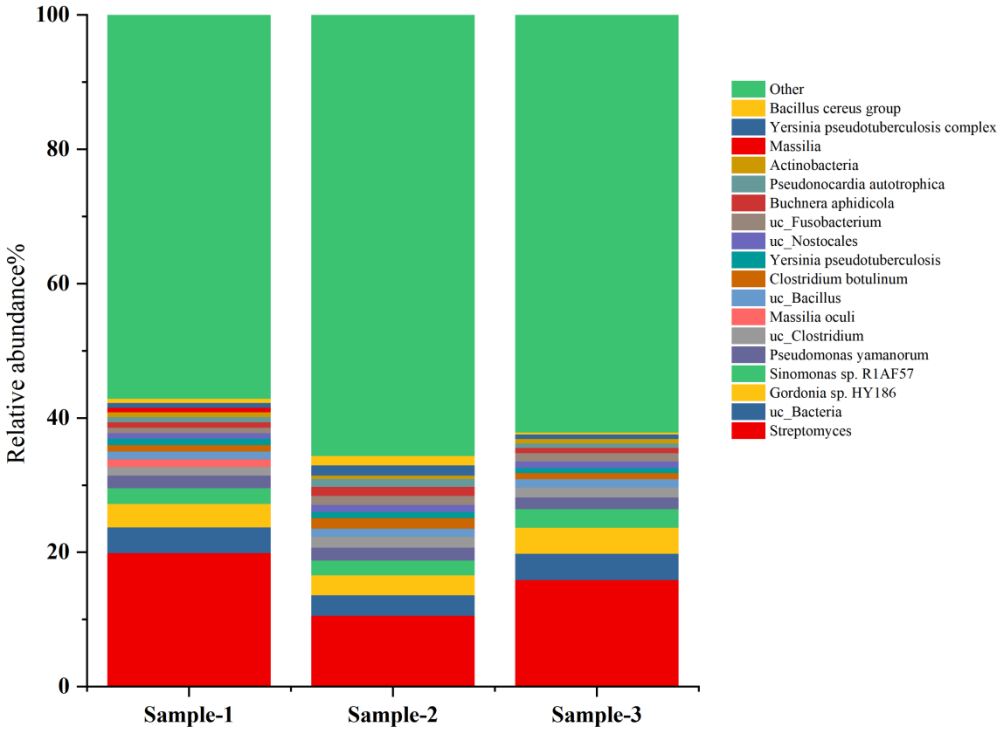

Figure 4: Relative abundances of the most common intestinal microbial species in each sample.

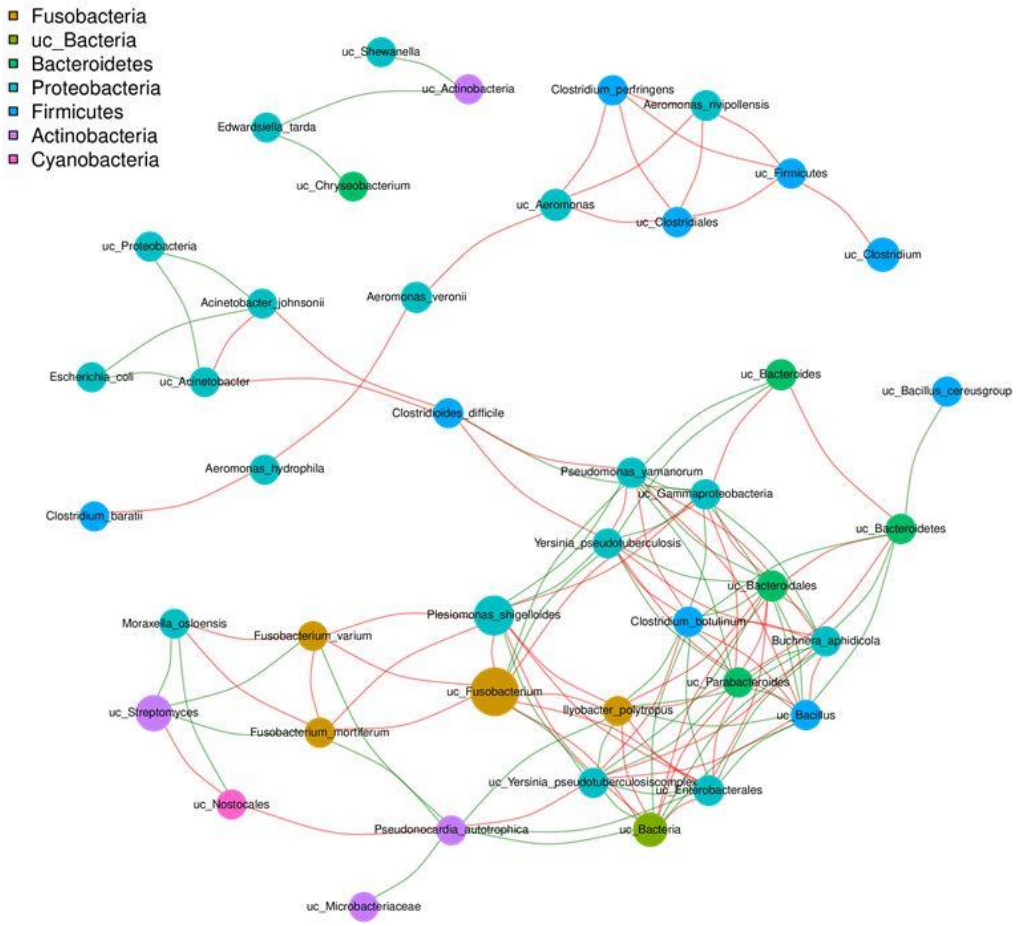

Figure 5: Association network of the most common intestinal microbial species. Each node corresponds to the dominant genus; red lines indicate positive correlations, and green lines indicate negative correlations. Node size is positively correlated with the average relative abundance of species.

Functional analysis of the intestinal flora

Primary metabolic pathways were detected according to the KEGG pathway: cellular processes, environmental information processes, gene information processes, human diseases, and metabolic and organic systems. The metabolic and organic systems were the most abundant. A total of 38 secondary metabolic pathways were identified according to KEGG pathway. The three most abundant secondary metabolic pathways were the sensory system, carbohydrate metabolism, and replication and repair, and their relative abundances were 27.16%, 8.56%, and 8.96%, respectively. The second most abundant secondary metabolic pathways were vitamin metabolism, amino acid metabolism, other amino acid metabolism, polysaccharide synthesis and metabolism, and nucleotide metabolism. Cluster analysis revealed high abundances of cell growth and death, nucleotide metabolism, terpenoids and polyketides metabolism,

drug inhibition, membrane transport, prokaryotic cell communication, secondary substance biodegradation and metabolism, and toxic substance biodegradation and metabolism, suggesting that the intestinal flora of *P. fulvidraco* was primarily involved in the nerve conduction of the neurosensory system.

Sensory perception of the external environment (via, for example, hearing, vision, and tactile senses) aids host metabolism, energy acquisition, and nutrient absorption. It also has positive effects on carbohydrate digestion and amino acid metabolism (Fig. 6).

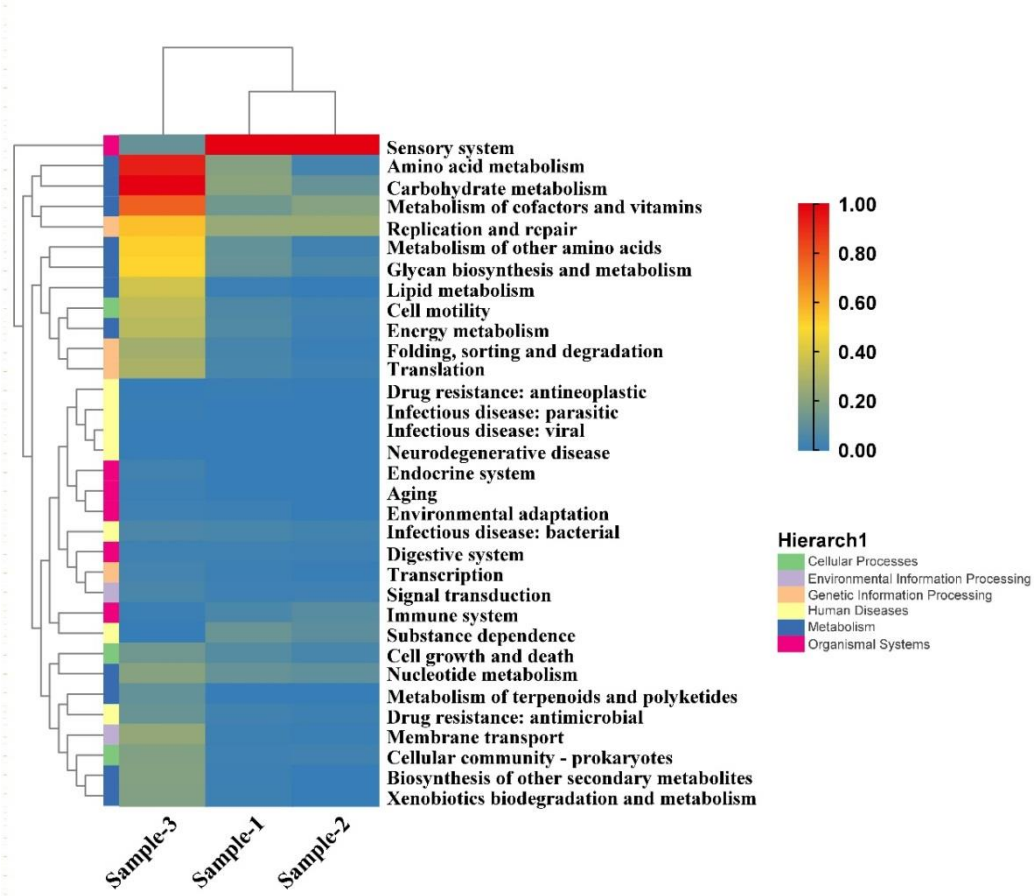

Figure 6: Relative abundances of the KEGG pathways in each sample

Annotation of intestinal ARGs

The drug resistance of the intestinal microorganisms in *P. fulvidraco* is mainly conferred by the ARGs of intestinal bacteria. A total of 21,482 resistance genes, including 888 ARGs and 35 types of ARGs, were annotated through comparison against the ARDB. ARGs with a relative abundance greater than 1% are shown in Table 4. The most common types of ARGs were multiple drug resistance, polypeptide, glycopeptide, tetracycline, and macrolide ARGs, and their relative abundances were 36.44%, 9.82%, 9.68%, 9.11%, and 8.00%, respectively (Fig. 7). The most common ARG was *macB*,that had a relative abundance of 6.28%. The second most common ARGs were *RanA*, *bcrA*, and *evgS*, and their

relative abundances were 3.14%, 3.05%, and 2.67%, respectively. Drug resistance was mainly mediated by target change and efflux pumps. Multiple drug resistance, glycopeptide, macrolide, polypeptide, tetracycline, and fluoroquinolone ARGs are common in *P. fulvidraco*.

Table 4: The most common ARGs detected in the three samples of *P. Fulvidraco*.

ARG	Quantity	Antibiotic resistance type	Resistance mechanism
adeL	238	Fluoroquinolones; tetracyclines	efflux
arlR	319	Fluoroquinolones; acridine pigments	efflux
arlS	367	Fluoroquinolones; acridine pigments	efflux
bcrA	656	Polypeptide	efflux
cdeA	424	Fluoroquinolones; acridine pigments	efflux
efrA	227	Macrocyclic esters; fluoroquinolones; welfare class	efflux
evgS	574	Macrocyclic esters; fluoroquinolones; penicillin; tetracyclines	efflux
macB	1348	Macrocyclic esters	efflux
TxR	335	Tetracyclines	efflux
msbA	311	Nitroimidazoles	efflux
tetA(58)	487	Tetracyclines	efflux
mtrA	213	Macrocyclic esters; penicillin	efflux
PmrF	228	Polypeptide	target alteration
RanA	675	-	efflux

Note: Indicates that the antibiotic type was not annotated

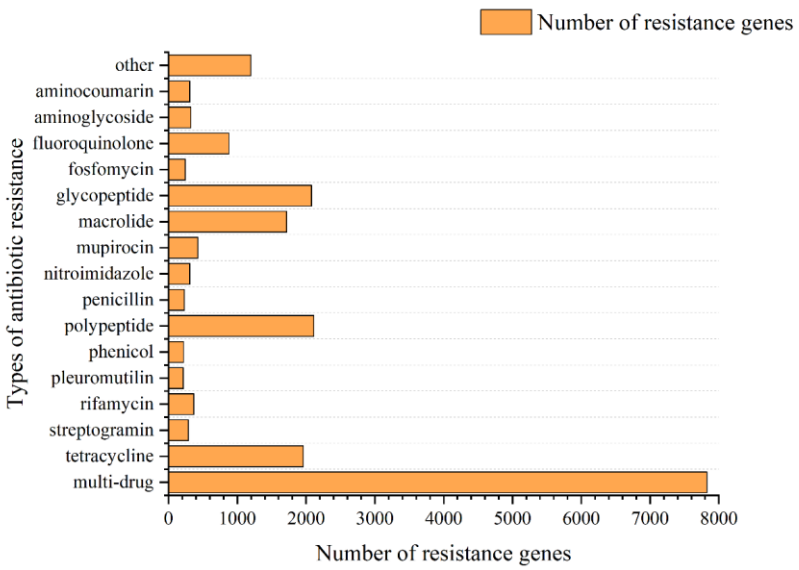

Figure 7: Total number of each type of ARG identified in the three samples of *P. Fulvidraco*.

Probiotics were collected from the microbial samples. Several probiotics are potentially a good alternative for six types of common antibiotics in the intestines of *P. fulvidraco* (Sup Table S1). The six most common antibiotics in the intestine had toxic side effects on the nervous system and digestive systems in *P. fulvidraco*. *Bacillus*, lactic acid bacteria, and *Clostridium* prevent bacterial diseases, promote a balanced intestinal flora, eliminate harmful

bacteria, enhance the growth of beneficial bacteria, and increase immunity and growth performance.

Supplementary Table S1: Probiotics that could be used as replacements for various types of antibiotics

Antibiotic	Toxic side effects	Probiotics	Probiotic effect
polypeptide	Neurotoxicity, kidney toxicity	*Bifidobacterium*, *Lactobacillus*,	Promoting the growth of probiotics that inhibit the growth of harmful bacteria
glycopeptide	Kidney and ear toxicity, prone to dependency	*Bacillus*, *Lactobacillus*	Promoting digestion and absorption and enhancing immunity
tetracycline	Digestive tract reactions, liver and kidney damage, abnormal bone development, allergies, microbial imbalance	*Bacillus*, *Streptomyces*, *Lactobacillus*, *Clostridium butyricum*	Promoting growth, controlling the growth of fungi and bacteria
macrolide	Digestive tract symptoms, hepatotoxicity, allergic reactions, cardiotoxicity	*Bacillus*, *Lactobacillus*	Promoting digestion improving intestinal structure, inhibiting the growth of pathogens
fluoroquinolone	Central nervous system toxicity, gastrointestinal reaction, photosensitive reaction	*Bacillus*, *Lactobacillus*, *Bifidobacterium Clostridium butyricum*	Inhibiting enteritis, preventing bacterial diseases
penicillin	Gastrointestinal, central nervous system, and allergic reactions	*Bifidobacterium*, compound bacteria *Bacillus*	Inhibiting enteritis and the growth of harmful pathogenic bacteria, improving immunity

Verification of the sequencing results

We isolated six bacteria, *Aeromonas versonii*, *Aeromonas caviae*, *Bacillus subtilis*, *Aeromonas hydrophila*, and *Streptococcus*, and the similarity was 100%, 99.79%, 99.93%, 99.87%, 99.65%, and 99.77%, respectively (Fig. 8).

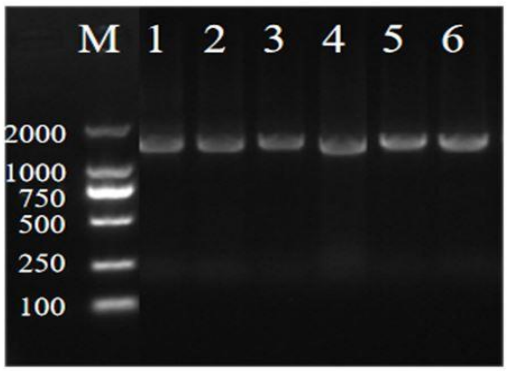

Figure 8: Results of the amplification of the 16S rRNA gene of intestinal bacteria in *P. fulvidraco*M: D2000, 1: Aeromonas versonii, 2: Aeromonas caviae, 3: Bacillus subtilis, 4: *Aeromonas hydrophila*, 5: *Streptococcus*, and 6: *Bacillus cereus*.

Discussion

In this study, the most common intestinal microbes were Proteobacteria, thick-walled fungi, *Clostridium*, and Bacteroidetes in *P. fulvidraco*, and these findings are consistent with the results of a previous study that used traditional sequencing and 16S rDNA data (Wu *et al*., 2010). Thus, our findings confirm the utility of metagenomic sequencing. Proteobacteria and Firmicutes were the most common phyla. Proteobacteria are sensitive to environmental factors (Faith *et al*., 2013) and contain a large number of pathogenic bacteria. Moreover, an increased abundance of Proteobacteria causes various diseases (Caporaso *et al*., 2011) and an imbalance in intestinal flora (Shin *et al*., 2015). Thick-walled bacteria can promote fat absorption and lipid droplet formation in the intestinal epithelium and liver (Semova *et al*., 2012). *Streptomyces*, *Clostridium*, *Bacillus*, *Aeromonas*, and *Mycoplasma* were the most common genera identified in our study. *Streptomyces* produced large numbers of antibiotics that prevent animal diseases and promote growth and development of animals (Li *et al*., 2017). *Clostridium* and *Bacillus*, non-pathogenic are often acted as antibiotics. For example, *Bacillus* promotes growth and enhances immunity. *Clostridium butyricum* inhibits the growth of *C. perfringens*, *E. coli*, and *S. aureus*. A high abundance of *Aeromonas* is probably due to intestinal dysfunction (Manna *et al*., 2013) or reflect fish adaptation to the environment (Perez-Sanchez *et al*., 2014). *Mycoplasma*, an important zoonotic bacterium, could cause pneumonia and respiratory inflammation (Volokhov *et al*., 2012). The most abundant bacteria in the intestine are probiotics at the species level. For example, *Gordonella* degraded alkanes (Martinkova *et al*., 2009). *Marseillae* tolerates heavy metals and promotes phosphorus solubilization and phenanthrene degradation (Lee *et al*., 2017). *Pseudonocardia*, an agriculturally important bacterial genus, improves water environment quality and promotes the healthy growth of aquatic products (Qin *et al*., 2008). *B. cereus* is a conditional pathogen, and non-pathogenic strains inhibit disease (Hong *et al*., 2016). *Yersinia* is a common pathogen in freshwater fish and it causes zoonoses (Tan *et al*., 2015).

We isolated and identified six strains, and three of them were dominant strains in P. fulvidraco by metagenomic analysis. These findings confirm the utility of metagenomic technology for obtaining large amounts of bioinformatics data. Analysis of phyla, genera, and species revealed that the most common non-pathogenic bacteria mediated nutrient absorption and metabolism, enhanced immunity, and promoted growth and development in *P. fulvidraco*. Moreover, many of these bacteria enhance the resistance of animals to disease. Pathogenic bacteria caused an imbalance of intestinal homeostasis, inflammation, and diseases in the intestine (Shanahan, 2010). Pathogenic bacteria were affected by various environmental factors and microbial interactions (Smith *et al*., 2007). This finding indicated that the

data is more comprehensive via metagenomic technology. In sum, the intestinal flora plays a key role in promoting growth and development in *P. fulvidraco*. Metagenomic technologies also identified important genes. However, more research is needed to elucidate environmental influences on these genes.

We found that *macB*, a macrolide resistance gene, was the most abundant ARG in the intestinal microbes of *P. fulvidraco*. This gene has been detected in river water (Stoll *et al*., 2012). The ARGs of intestinal microorganisms of carp revealed seven types of ARGs including quinolone, sulfonamide, aminoglycoside, macrolide, chloramphenicol, tetracycline, and β-lactam ARGs (Yuan *et al*., 2019). We also identified previously unreported ARGs with highly abundant such as *TxR*, *msbA*, and *PmrF* genes in *P. fulvidraco*. The difficulty of cultivating drug-resistant bacteria with these genes and expressing resistance by a single resistance gene, and the undetectability of conventional technique explains the lack of relevant studies. A large number of multiple drug resistance ARGs were detected in *P. fulvidraco*, and it indicated that many antibiotics have been used in the breeding of *P. fulvidraco*. Our study revealed that the resistance of most intestinal bacteria to antibiotics is achieved via efflux pumps in *P. fulvidraco*. Among tetracycline ARGs, 74% of the genes were related to the efflux pump mechanism. For example, *tetA*, *tetY*, and *tetZ* encode efflux pump proteins that promoted tetracycline excretion from cells as well as reduced intracellular tetracycline and toxicity to themselves, but they increase antibiotic resistance (Liu *et al*., 2018). The efflux pump proteins also pump other antibiotics outside the cells. Genes encoding efflux pump proteins are often located in transposons, integrons, or plasmids. And they are usually connected to other ARGs that enhances multi-drug resistance in microorganisms (Calero-Cáceres *et al*., 2019). Multiple drug resistance genes integrated into pathogenic bacteria are spread into man through the food chain. The persistence, migration, transport, and spread of bacteria is probably more harmful compared with antibiotic residues (Murray *et al*., 2018). Probiotics have been proved to be a good alternative for the antibiotic abuse in aquaculture industry. Probiotic additives provide an efficient and green ways compared to antibiotics to exert antimicrobial properties.

Intestinal microorganisms are closely associated with host health. In our study, intestinal microbes were investigated by metagenomic technology in *P. fulvidraco*. Identification of the ARG of intestinal flora pathogens provided insight into the pathogenic mechanisms of bacteria and clinical screening of drugs. The analysis of intestinal flora, intestinal microorganisms ARGs and gene function is beneficial for the artificial farming of *P. fulvidraco*.

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