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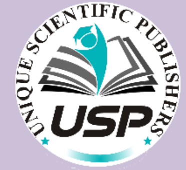

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Composition of the Microbiome of Rainbow Trout (*Oncorhynchus mykiss*) and Siberian Sturgeon (*Acipenser baerii*) and Options for its Correction with Probiotics

Zhumagul Kirkimbayeva [1], Nurzhan Sarsembayeva [2,*], Birzhan Biyashev [1], Ardak Akkozova [3], Arman Zhylkaidar [1] and Dinara Sarybaeva [1]

[1]Department of Microbiology, Virology and Immunology, Faculty of Veterinary Science, Kazakh National Agrarian Research University, Almaty, Republic of Kazakhstan
[2]Department of Veterinary Sanitary Examination and Hygiene, Faculty of Veterinary Science, Kazakh National Agrarian Research University, Almaty, Kazakhstan
[3]Department of Veterinary Sanitary Examination and Hygiene, Faculty of Veterinary Science, Kazakh National Agrarian Research University, Almaty, Kazakhstan
***Corresponding author:** nursarsembayeva@mymail.academy

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ABSTRACT

The microbiome of fish plays a major role in preserving the population's health and obtaining safe, high-quality products. Antibiotic treatment in aquaculture creates problems associated with the emergence of a resistant population of microflora, which can be transmitted to humans through food, direct contact, or the environment. Thus, it is a justified and promising research objective to search for methods to increase the fish population in commercial fish farming using effective probiotic and synbiotic preparations. The study analyzes the microbiome of rainbow trout (*Oncorhynchus mykiss*) and Siberian sturgeon (*Acipenser baerii*). The composition of fish intestinal microflora is studied using microbiological analysis with determination of resistance to antibiotics and biofilm-forming ability in the identified strains and molecular genetic identification of bacterial strains using Sanger sequencing of the 16S rRNA gene with phylogenetic analysis. The fish microbiome is found to include the representatives of the genus *Aeromonas, Acinetobacter, Citrobacter, Corynebacterium, Enterobacter, Escherichia, Hafnia, Pseudomonas,* and *Staphylococcus.* In fish with signs of pathological processes, the content of opportunistic microflora is significantly increased. The isolated pure cultures of microorganisms identified as *Aeromonas hydrophila, Aeromonas salmonicida, Aeromonas veronii,* and *Aeromonas sobria* are resistant to six antibiotics (out of the 19 tested) and can form biofilms. In laboratory conditions, the probiotic strain *Escherichia coli 39 SN* shows antagonistic properties to the *Aeromonas*. These results can be used in the development of probiotics and synbiotics for aquaculture.

Key words: Antibiotic resistance, Aquaculture, Biofilm formation, Microbiome, Prebiotics, Synbiotics

INTRODUCTION

Industrial fish farming plays an important role in the economy of the Republic of Kazakhstan (Mazina et al. 2022). It is one of the most promising industries as it provides income to many farms and enterprises, creates jobs for the population, increases the export potential of Kazakhstan's economy, and serves as a renewable source of biodiversity. In the last few years, Kazakhstan has witnessed an active growth of production indicators in fish farming, related to state support of the industry in the form of reduced tax burden and subsidies for fodder, breeding stock of valuable fish species, fry, and veterinary drugs. These measures are aimed at creating conditions for faster growth of the population of hydrobionts, the increase of fishery resources and the development of artificial population growth. There is a global trend of transition from commercial fishing to aquaculture development. Aquaculture is one of the most demanded and rapidly developing industries in the world that ensures food security, including protein-rich foods (Mukhambetov et al. 2023). The physiological norm of fish products is 20.7kg/person. In Kazakhstan, this indicator does not exceed 15kg; in some regions, it is even lower. Despite a

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positive dynamic in aquaculture production, several issues inhibit the growth and development of this industry. The fish population and production volumes are determined by the habitat, the quality of feed, and the absence of viral and bacterial infectious diseases and parasitoses.

When the necessary conditions are not met (increased fish population density, poor water and feed quality, etc.), the fish microbiome is the most vulnerable element. The composition of the microbiome of the fish organism, including intestinal microflora, changes under the influence of various endogenous and exogenous factors. The endogenous factors include the genetically formed qualities of fish: the structure of the digestive tract, enzymes produced in the intestine, the pH of the intestinal contents, osmotic pressure, the host's immunity, and others (Shivokene and Trepshene 1985). The exogenous factors include the environment, feed type and quality, temperature fluctuations, infection, etc. (Syvokiene et al. 1995; Syvokiene et al. 2003; Bykovskaia 2014).

Fish have a distinct intestinal microflora consisting of anaerobes, facultative anaerobes, and obligate anaerobes. The intestinal microflora of fish is simpler than that of warm-blooded animals. Some researchers believe that the composition of gastrointestinal microflora in fish is similar to that of the body surface, the gills, and the food lumps (Cahill 1990). Some evidence suggests the presence of obligate anaerobic bacteria in the intestines of fish, especially herbivorous ones (Clements 1997; Zainitdinova et al. 2022).

The quality of the composition of fish microbiocenoses is of great importance because the predominance of pathogens against the background of weakened defenses of the macroorganism can lead to an epizootic. Microorganisms that are pathogenic or conditionally pathogenic to physiologically healthy organisms can become pathogenic when the natural resistance of these organisms is weakened, especially under unfavorable conditions and stressful situations (Basankina 2020; Arinzhanov 2023).

The acquisition and transmission of genetic resistance by bacteria to the antimicrobial agents used against them is now a leading problem in aquaculture, medicine and the food industry (World Health Organization 2006; Zhang et al. 2020). The rise in bacterial resistance and the spread of such strains in nature pose a serious threat to human and animal health (Matyar et al. 2010; Lulijwa et al. 2019; Hossain et al. 2019). Due to the widespread and often uncontrolled use of antibiotics, the number of antibiotic-resistant bacteria has increased dramatically and serves as a major cause of morbidity and mortality (Shulgina et al. 2015; Preena et al. 2020). This phenomenon reduces the effectiveness of antimicrobial therapy and raises concerns about the safety of fish products (Bakiyev and Bissenbaev 2021).

Acquiring resistance to antimicrobial drugs, the *Aeromonas* and *Pseudomonas* genera cause diseases resulting in economic losses in aquaculture. Aeromonas is a genus of Gram-negative, facultative anaerobic bacteria that are ubiquitous in fresh, brackish, and brackish-marine water. Common pathogenic Aeromonas species include *Aeromonas hydrophila*, *Aeromonas caviae*, *Aeromonas veronii*, *Aeromonas sobria*, and *Aeromonas salmonicida*. *A. hydrophila, A. sobria* and *A. caviae* are the most dangerous to human health, as they cause diseases such as intestinal infections, gastroenteritis and septicemia (Da Silva et al. 2022). The Aeromonas genome has been shown to contain several antibiotic resistance genes, the most dominant of which are the class B, C and D β-lactamase genes, as well as several drug efflux pump protein genes, posing a threat to humans and aquatic animals (Dubey et al. 2022).

Pseudomonas is a genus of bacteria that can accelerate the decomposition of food products due to the production of non-cellular proteolytic, lipolytic and saccharolytic enzymes. Low number of *Pseudomonas* spp. do not pose a health risk to consumers. Instead, it can produce histamine and pose a health risk to humans if they multiply excessively in fish that are naturally high in free histidine (Sheng & Wang 2021)

The composition of fish microbiota is contingent on environmental conditions, i.e., fish habitat, which is why it is different in wild fish and aquaculture. In fish farms with a closed water supply system, the intestinal level shows a relative constancy, which depends on the colonization resistance of the host organism and the biological characteristics of microorganisms. Bacterial colonization of the digestive tract depends on the pH of the intestinal environment, the peristalsis, the content of bile acids and digestive enzymes, the host's immune response to bacterial invasion, and the presence of autochthonous bacteria and pronounced antagonists capable of biosynthesizing antibiotic-like compounds (Ringo et al. 2003; Sidorova and Kuchko 2023).

Recent scientific, technical, and conceptual achievements in the study of the animal intestinal microbiome have provided a rationale for the use of medications that correct the qualitative composition of microflora and improve the organism's resistance, normalize digestive processes, reduce the use of antimicrobial drugs, and allow obtaining environmentally friendly and safe products (Aira et al. 2019; Pineiro and Cerniglia 2021). The microbiomes of all animal species have their specific features; they also conform to the common basic principles of the structural organization of bacterial communities as determined by evolutionary conditions, food habits, and habitats. Microbial autoflora associations carry out important activities, consisting in the breakdown of food substrates in the production of several substances vital to the host organism (Belkova et al. 2015). Thus, beneficial microorganisms or probiotics have emerged as new solutions that can help reduce the use of antibiotics in aquaculture. Probiotic microorganisms used in aquaculture include certain strains of yeast, algae and especially bacteria, including representatives of *Bacillus sp., Lactococcus sp., Micrococcus sp., Carnobacterium sp., Enterococcus sp., Lactobacillus sp., Streptococcus* and *Weissella sp*. Such bacteria are characterized as safe and can produce natural compounds with antimicrobial activity and also stimulate the immune system (Ben Mhenni et al. 2023).

Bacteria in the composition of intestinal microflora are responsible for the colonization resistance of fish gastrointestinal tract, have a direct antagonistic activity, and can suppress the possible presence of conditional pathogens and control the interaction of dominant species with other members of the associative symbiosis in the digestive tract (Sidorova and Kuchko 2023). Correction of

intestinal microflora allows for increasing the resistance of fish to pathogens.

The need to ensure the ecological safety of aquaculture, control the state of conditionally pathogenic microflora, inhibit pathogenic bacteria, boost the immune response, and reduce the consumption of feed and losses in breeding requires consistent control over the intestinal microbiome calls for the development of safe and effective probiotic preparations. The goal of this study is to research the intestinal microbiome of healthy and diseased individuals of rainbow trout (*Oncorhynchus mykiss*) and Siberian sturgeon (*Acipenser baerii*) and find opportunities to correct it using the probiotic strain *Escherichia coli 39 SN*.

To achieve this research goal, we set the following research objectives:

1. To collect samples in the form of intestinal fragments from healthy individuals of rainbow trout and Siberian sturgeon.
2. To collect samples in the form of intestinal fragments and ascitic fluid from diseased individuals of rainbow trout and Siberian sturgeon.
3. To conduct bacteriological studies and isolate pure cultures from the biomaterial of healthy and diseased fish.
4. To identify the isolated cultures by their morphological and culture-biochemical properties and by sequencing the 16S rRNA gene using the Sanger method (Guo & Pyle 2023).
5. To identify antibiotic-resistant representatives of the intestinal microflora and ascitic fluid of aquaculture.
6. To determine the antagonistic properties of the probiotic strain *E. coli 39 SN* against opportunistic bacteria of the genus *Aeromonas* isolated for the individuals of rainbow trout and Siberian sturgeon.

MATERIALS AND METHODS

The study was conducted in 2022-2023 at aquaculture fish farms in the Almaty region, Kazakhstan.

The Tengry Fish LLC fish farm is situated in the Almaty region, Uygur district, Kulzhat rural okrug, village of Kalzhat. A source of underground artesian water is located 300-500m away from the farm site. Fish are raised in pools and ponds at water temperatures of 27-28°C. The farm has a total of 35 pools and four ponds. The volume of each pool is 30m³ of water. The farm's capacity is sufficient to grow 300t of fish annually.

The MG LLC farm is located in the city of Esik, Almaty region. Water for the farm comes from the mountainous Issyk River. The water temperature reaches 13°C in summer and drops to 4-5°C in winter. Trout are raised on the farm from spawn to marketable weight (250-350g), up to more than 2-3kg. The farm has 150 iron barrels; their area totals 9m^3, of which 7m^3 is occupied by water. Fish are placed in barrels in the weight category of 30-45kg/1 m^3 of density.

This study on fish was reviewed and approved by the Ethics Committee of the Kazakh National Agrarian Research University (Minutes No. 77 of May 15, 2023). The subjects of the study were representatives of the microbiome of the intestine and ascitic fluid of healthy individuals of rainbow trout and Siberian sturgeon and diseased individuals with signs of pathological processes kept in cage conditions.

The intestinal microbiome of fish was analyzed using microbiological methods and molecular-genetic identification, specifically through 16S rRNA gene sequencing and phylogenetic analysis. The studies were conducted in the Antibacterial Biotechnology Laboratory of the Kazakh National Agrarian Research University following the techniques. We performed molecular genetic studies in an accredited laboratory of the Scientific and Practical Center of Microbiology and Virology.

Before sampling intestinal sections, the fish were cleaned with alcohol. The dissection was performed with sterile instruments. Samples were placed in pre-prepared sterile containers. Ascitic fluid was sampled using a disposable medical syringe.

For the non-selective accumulation of bacteria, samples were seeded in the primary enrichment medium and TSB medium. The cultures were then transferred into petri dishes with MPA and TSA. The resulting isolated colonies were studied and described according to the rules given in Methods of General Bacteriology (Vasilev et al. 2003). To obtain pure cultures of the isolated microorganisms, a portion of the isolated colony of each morphotype was transferred to an agar bevel. The obtained pure cultures were then used for all subsequent experiments.

Microscopic analysis of the main morphological features of the isolated cultures was conducted using the Gram stain method, the Gins-Burry method, and the Peshkov method. The culture, biochemical, and physiological properties of the isolated microorganisms were studied with the application of nutrient media for general, elective, and differential-diagnostic purposes (Gissa's medium, Clark broth, Kessler's nutrient medium, blood agar, bismuth sulphite agar, Ploskirev baktoagar, Levin's medium, etc.) following the procedures outlined by Sidorova and Kuchko (2023).

We determined catalase following the method described by Vasilev et al. (2003). The sensitivity of bacteria isolates to antibiotics was determined by the disk diffusion method (MU 4.2.1890- 04) using antibacterial drugs. The antagonistic activity of the probiotic strain *E. coli 39 SN* was tested against representatives of the genus *Aeromonas* isolated from healthy and diseased aquaculture specimens. The study focused on 16 strains: *A. hydrophila* (4), *A. salmonicida* (3), *A. veronii* (3), *A. sobria* (3), *Aeromonas media* (2), *Pseudomonas protegens* (1). The antagonistic activity index was measured by two *in vitro* methods: agar blocks (block modification) (Mashentseva and Khorolskii 2008; Blinnikova 2003) and delayed antagonism (MUK 4.2.2602-10, 2010). The antagonistic activity index was estimated by the diameter of inhibition of the growth of tested cultures by the *E. coli* 39 SN strain.

The ability of each isolate to form biofilms on the plastic surface was determined using 24-well polystyrene microtiter plates following modified protocols (Chief State Sanitary Doctor of the Russian Federation 2010). The daily culture of each isolate was grown in 10mL TSB and diluted to the final inoculum amount of 10^7 CFU/mL. 2mL of inoculum was transferred into a total of six wells, and wells containing only TSB (not inoculated broth) served as a negative control for each isolate. The 24-well plate was incubated at 25°C for 48h to allow biofilm formation. After

48h of incubation, the inoculum was removed from each well of the plate, and the wells were washed three times with sterile distilled water to remove loose cells. The plate was then air-dried for 15min to allow the cells to attach, and 2mL of 1% crystal violet solution was added to the treated and control wells to stain the attached cells. The plate was incubated at room temperature for 20min, after which the stain was removed from the wells, and the wells were washed five times with sterile distilled water to remove all traces of the stain. The plate was then air-dried for 15min, and then 2mL of 95% ethanol was added to each well to dissolve the stained cells. The biofilm formation by each strain in the wells was quantified by measuring optical density using a micro quant microplate spectrophotometer (model ELx800, BioTek Instruments, Winooski, VT).

The experiment was repeated three times for each isolate on separate days. The final biofilm formation capacity of each isolate was analyzed and presented as OD values minus the OD values of the negative control (final OD600 = mean isolate OD – mean negative control OD). The biofilm formation ability of each isolate was further analyzed by grouping them into three categories: 1) weak biofilm producers had the final values of OD600<0.3, 2) moderate biofilm producers had final OD600 values between 0.3 and 0.6, and 3) strong biofilm producers had final OD600>0.6 (Fig. 1).

The molecular genetic identification was carried out by sequencing the 16S rRNA gene using the Sanger method (Guo and Pyle 2023) in the accredited laboratory of the Scientific and Practical Center of Microbiology and Virology. Preliminary identification was performed by classical microbiological methods.

DNA isolation

In this work, we used daily bacterial cultures. Genomic DNA was isolated from bacteria using PureLink® Genomic DNA Kits (Invitrogene, USA). DNA concentration was determined using a Qubit fluorimeter (Invitrogen, USA) on a scale for dsDNA HS. The concentration of the obtained DNA ranged from 5.6 to 80.2ɳg/µL.

Gram-negative bacteria

Bacterial suspension at a concentration of $2x10^9$ was transferred to a 1.5mL tube and centrifuged at 10,000rpm for 5min. The supernatant was removed. The precipitate was resuspended in 180µL of dissolution buffer. 20µL Proteinase K was added, vortexed until homogeneous, and incubated at 56°C for 30min, stirring the sample periodically. After adding 20µL of RNase A solution, the sample was stirred by vortexing and incubated at room temperature for 10min. 200µL of lysis buffer was added to each sample. The sample was then vortexed for 15 seconds. 400µL of 50% ethanol was added and stirred by vortexing. The prepared lysate was then transferred to a GeneJET Genomic DNA Purification Column and centrifuged for 1min at 6,000rpm. The flowing liquid was removed, and the column was transferred to a new tube. Following this, 500µL of Wash Buffer I was added. The column was centrifuged for 1 min at 8,000rpm. The flowing liquid was removed, and the column was transferred to a new tube. 500µL Wash Buffer II was added. The column was centrifuged at 13,400rpm for 3 minutes. The column was then placed in a new tube. 50µL Elution Buffer was added to the center of the column. The column was centrifuged for 2min at maximum rpm. Finally, the column was removed, and DNA was stored at -20°C.

Gram-positive bacteria

Bacterial suspension at a concentration of $2x10^9$ was transferred to a 1.5mL tube and centrifuged at 10,000rpm for 5min. The supernatant was removed. The precipitate was resuspended in 180µL of lysing buffer for G+ bacteria, followed by incubation at 37°C for 30min. 200µL Lysis Solution and 20µL Proteinase K were added and vortexed until homogeneous, then incubated at 56°C for 30min, stirring the sample periodically. 20µL of RNase A solution was added, stirred by vortexing, and incubated at room temperature for 10min. 200µL of lysis buffer was added to each sample. Vortexing was performed for 15 seconds. 400µL of 50% ethanol was added and mixed by vortexing. The prepared lysate was transferred to GeneJET Genomic DNA Purification Columns and centrifuged for 1min at 6,000rpm. The flowing liquid was removed, and the column was transferred to a new tube. 500µL of Wash Buffer I was added. The column was centrifuged for 1 minute at 8,000rpm. The flowing liquid was removed, and the column was transferred to a new tube. 500µL Wash Buffer II was added, followed by centrifugation at 13,400rpm for 3min. The column was placed in a new tube. 50µL Elution Buffer was added to the center of the column. The column was centrifuged for 2min at maximum rpm. The column was then removed. DNA was stored at -20°C. The bacteria were identified by studying the sequence of the 16S rRNA gene region using universal primers (Table 1).

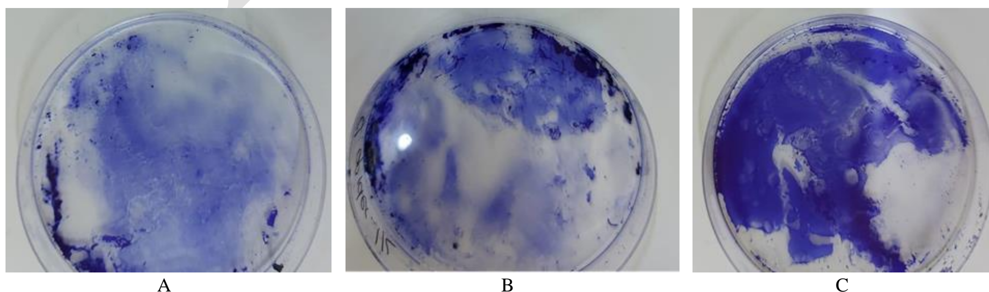

Fig. 1: Examples of varying biofilm-production ability in the isolated strains: a – weak biofilm producers; b – moderate biofilm producers; c – strong biofilm producers.

Table 1: Description of the universal primers of 16S rRNA.

Primer name and direction	Nucleotide sequence (5" to 3")	Amplicon (bp)
Forward 8F	5- AGAGTTTGATCCTGGCTCAG -3	
Reverse 806R	5- GGACTACCAGGGTATCTAAT -3	630

In Press

Reaction mixture preparation

The reaction mixture (30µL) contained 3µL 10x of reaction buffer (Fermentas), 2.5mM $MgCl_2$, 0.2mM of each deoxyribonucleoside triphosphate (dNTP), 10pmol of each primer, 1 unit of Taq–polymerase Maxima Hot Start Taq DNA Polymerase (Fermentas).

Amplification regime

Polymerase chain reaction (PCR) was run in a Mastercycler nexus gradient thermocycler (Eppendorf). The reaction was started by incubating the mixture at 95°C for 7min, followed by 30 cycles consisting of: 95°C for 30 seconds, 55°C for 40 seconds, and 72°C for 1min. The final elongation was performed at 72°C for 10min. The amplified product was separated in a 2% agarose gel, bands were stained with ethidium bromide and visualized in a UV transilluminator. The electrode buffer utilized was the 1xTAE buffer. PCR products were purified using the PureLink® PCR Purification Kit (Invitrogen, USA).

Sequencing reaction

Sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) according to the BigDye® Terminator v3.1 Cycle Sequencing Kit Protocol (Applied Biosystems USA) (Syvokiene et al. 1995). The purification of the sequencing product was performed using the BigDye XTerminator Purification kit (Applied Biosystems, USA) (Syvokiene et al. 2003). Capillary phoresis of the bacterial 16S rRNA gene fragment was performed on a 3500 DNA Analyzer automated sequencer (Applied Biosystems, USA).

Data processing and phylogenetic analysis

The sequencing results were processed using the SeqA software (Applied Biosystems). Homologous nucleotide sequences of 16S rRNA genes were found using the BLAST (Basic Local Alignment Search Tool) program in the International Gene Bank database of the USA National Center for Biotechnology Information (NCBI) (htpp://www.ncbi.nlm.nih.gov). Nucleotide sequence alignment was performed using the ClustalW algorithm. The phylogenetic tree was constructed in MEGA 11 using the Maximum Likelihood method.

RESULTS AND DISCUSSION

To study the intestinal microbiome of healthy fish and fish with signs of gastrointestinal tract infection, we obtained 78 samples of biomaterial: gill washes, ascitic fluid samples, and intestinal contents. Of these, 40 samples were obtained from rainbow trout and 38 from Siberian sturgeon. According to the obtained counts of the dominant intestinal microflora of rainbow trout, the total bacterial counts in gut contents and gill washes range from 5.7×10^4 to 2.3×10^7 CFU. Quantitative indicators vary depending on the time of year (Table 2). Similar results are obtained for the intestinal and gill microbiome of Siberian sturgeon. The bacteriological method of investigation based on the homogenization of intestines with their contents and subsequent seeding of the homogenate on selective media allows one to detect only those bacteria that grow on the used media. Therefore, the obtained quantitative results are approximate.

Table 2: Seasonal quantity of microflora

Species	Sample collection timing	Total bacterial count	
		In intestinal contents	In the gills
Rainbow trout	June	$2.3\cdot10^7$	$8\cdot10^5$
Rainbow trout	November	$5.7\cdot10^4$	$3\cdot10^2$
Siberian sturgeon	June	$2\cdot10^8$	$3\cdot10^6$
Siberian sturgeon	November	$6\cdot10^4$	$2\cdot10^3$

In our study of the composition of the microflora of the intestines, internal organs, and gills of rainbow trout and Siberian sturgeon, we isolated a total of 103 cultures. In studying the morphology and culture properties of the isolates, Gram-negative bacteria were found to be dominant, accounting for 92.2 % of the identified microorganisms. 79.6% of the identified microorganisms possess catalase activity. In microbiological studies on motility, 73.8% of microbial cultures show the ability to move.

Aeromonas are small gram-negative bacilli. Mobile and immobile species have been identified. When the growth temperature regime was monitored, all isolates were found to grow at 20-25°C. After 24h of cultivation in meat peptone broth, the isolates grow, forming a tender film and flakes, with some tubes showing uniform turbidity and the formation of a whitish precipitate and a grayish film on the surface of the medium. In meat peptone agar (MPA), the isolates grew in the form of translucent, slightly bluish colonies. Some cultures in MPA formed smooth, rounded, translucent colonies with a whitish-yellow tint, no more than 1-3mm in diameter. In the test tubes with isolates left at room temperature (22-25°C), greenish and sometimes brown staining appeared after 48 hours of incubation (n=5). On MacConkey agar, growth was observed after 24 hours in the form of small, gray with a pink tint, round with smooth edges, protruding and glossy colonies with a diameter of less than 1mm. On TSA medium, the tested microorganisms formed round, grayish-yellow, translucent colonies. Cultivation of the studied strain on semi-liquid GRM agar at 20 and 30°C for 72h resulted in diffuse culture growth at the inoculation site, indicative of the mobility of 47 isolates of *Aeromonas*, while 12 isolates remained immobile.

To determine hemolytic activity, all cultures were sown on blood agar. The cultures were sown in pairs and incubated for 72hours at 37 and 30°C. In this case, the growth of gray, glossy, convex, round colonies with smooth edges and up to 2mm in diameter was detected on blood agar. After 48 hours, a thin zone of greenish color was observed around the colonies, indicating the ability of the studied strain to exhibit incomplete hemolysis (α-hemolysis).

Of the studied 59 isolates belonging to the genus *Aeromonas*, all produced the enzyme cytochrome oxidase, which was confirmed by the appearance of pink staining when a drop of 1% tetramethyl-p-p-phenylenediamine dihydrochloride solution was applied to the colony.

Catalase activity was also found in most of the tested strains. The presence of the DNAse enzyme was determined by incubating the tested strains for 72h at 37°C on DNAse test agar. The tested cultures formed large, transparent, round, even-edged, convex, glossy colonies. When 1 N HCl solution was added, 36 isolates out of 59 had transparent zones around the colonies (the diameter of

the zone ranging from 2 to 8mm). From the results obtained, 36 cultures display DNAse activity.

To detect gelatinase activity, the tested cultures were sown on media with 12% gelatin content. All isolates were found to have the ability to liquefy gelatin, i.e., to produce the gelatinase enzyme.

The study of biochemical properties on Gissa's medium with various carbohydrates has shown that the Aeromonas representatives have different enzymatic activities and decompose glucose (n=48), lactose (n=12), sucrose (n=50), mannitol (n=51), mannose (n=47), maltose (n=52), galactose (n=57), and arabinose (n=28) to form acid, with some strains also forming gas, form indole (n=47) and hydrogen sulfide (n=39), and do not decompose urea.

Representatives of the genus *Pseudomonas* showed the following properties: Gram-negative, non-spore-forming, motile bacilli. They grow under aerobic conditions with a wide temperature range (from 4 to 36°C), test positive for oxidase, and are catalase positive. They ferment glucose without releasing gas, form beta-hemolysis on blood agar, do not form indole, do not recover methyl red, and test negative on the Voges-Proskauer test.

Among the isolated cultures, some were not identified based on their biological properties. For this reason, Sanger sequencing of the 16S rRNA gene of the isolates was performed to confirm the results obtained and to identify the remaining unidentified cultures. The PCR product with universal primers for the 16S ribosomal RNA is shown in Fig. 2-15.

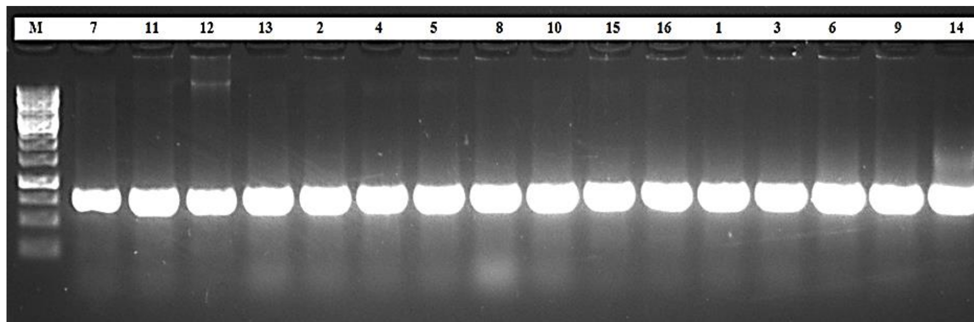

Fig. 2: Electrophoregram of the PCR product of the 16S rRNA gene.
M – DNA length marker 1 Kb

№ 15v – *Aeromonas hydrophila*
(№ 15, 29, 34, 35, 36, 49, 50, 51, 52, 53 analogous results)

Nucleotide sequence obtained by sequencing the 16S rRNA gene:

GTCGAGCGGCAGCGGGAAAGTAGCTTGCTACTTTTGCCGGCGAGCGGCGGACGGGTGAGTAATGCCTGGGAA
TTTGCCCAGTCGAGGGGGATAACAGTTGGAAACGACTGCTAATACCGCATACGCCCTACGGGGGAAAGGAGGG
GACCTTCGGGCCTTGCGCGATTGGATGTGCCCAGGTGGGATTAGCTTGTTGGTGGGGTAATGGCTCACCAAGGC
GACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAG
GCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCG
GGTTGTAAAGCACTTTCAGCGAGGAGGAAAGGTTGGTACCTAATACGTGTCAACTGTGACGTTACTCGCAGAA
GAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGC
GTAAAGCGCACGCAGGCGGTTGGATAAGTTAGATGTGAAAGCCCCGGGCTCAACCTGGGAATTGCATTTAAAA
CTGTCCAGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGA
ATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCATGTGCGAAAGCGTGGGGAG

Fig. 3: Electrophoresis of PCR products for microbiome bacterial isolates

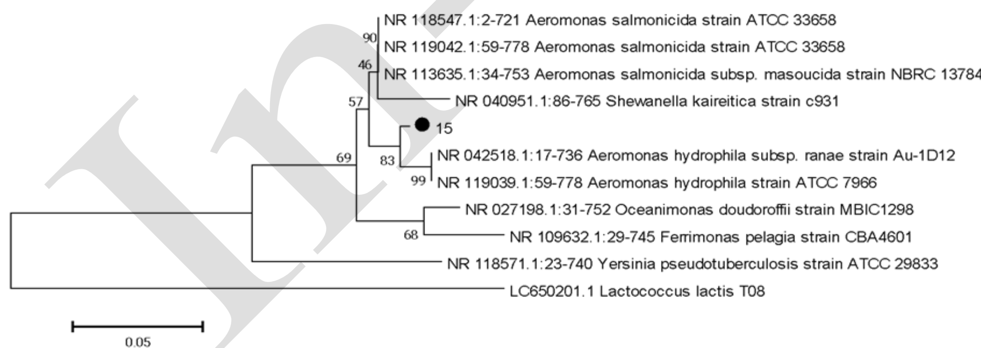

Fig. 4: Phylogenetic tree of *Aeromonas hydrophila* subsp. ranae
The level of homology with the closest strain NR 042518.1:17-736 *A. hydrophila* subsp. *ranae* strain Au-1D12 is 98.89%.

№ 13– *Aeromonas salmonicida*
(№ 25, 26, 27, 28, 32, 33, 37, 39, 40, 42, 43 -analogous results)

Nucleotide sequence obtained by sequencing the 16S rRNA gene:

GGAAAGTAGCTTGCTACTTTTGCCGGCGAGCGGCGGACGGGTGAGTAATGCCTGGGGATCTGCCCAGTCGAG
GGGGATAACAGTTGGAAACGACTGCTAATACCGCATACGCCCTACGGGGGAAAGGAGGGGACCTTCGGGCCT
TTCGCGATTGGATGAACCCAGGTGGGATTAGCTAGTTGGTGGGGTAATGGCTCACCAAGGCGACGATCCCTAG
CTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGG
GGAATATTGCACAATGGGGGAAACCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAA
GCACTTTCAGCGAGGAGGAAAGGTTGGCGCCTAATACGTGTCAACTGTGACGTTACTCGCAGAAGAAGCACC
GGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGC
GCACGCAGGCGGTTGGATAAGTTAGATGTGAAAGCCCCGGGCTCAACCTGGGAATTGCATTTAAAACTGTCCA
GCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCG
GTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGA
TACC

Fig. 5: Phylogenetic analysis showing homology with *A. hydrophila* subsp. Ranae

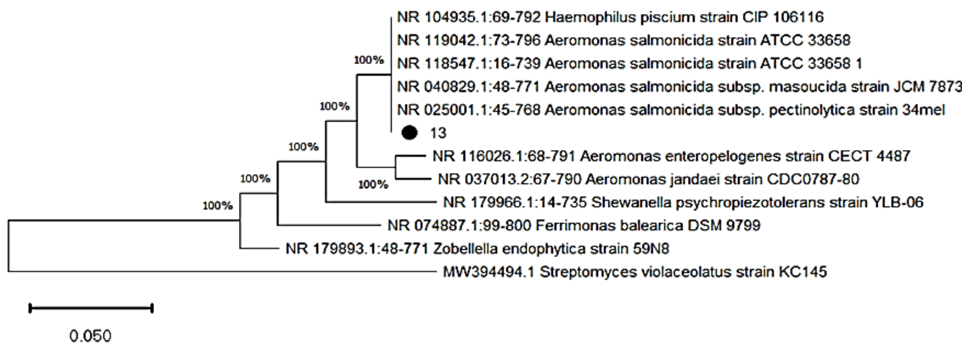

Fig. 6: Phylogenetic tree of *Aeromonas salmonicida* subsp. pectinolytica
The level of homology with the closest strain NR 025001.1:45-768 *A. salmonicida* subsp. *pectinolytica* strain 34mel is 100.00%.

4 – *Aeromonas veronii*
(№ 44, 45, 46, 47, 48, 54, 55, 56 - analogous results)

Nucleotide sequence obtained by sequencing the 16S rRNA gene:

**TGCAAGTCGAGCGGCAGCGGGAAAGTAGCTTGCTACTTTTGCCGGCGAGCGGCGGACGGGTGAGTAATGCC
TGGGGATCTGCCCAGTCGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATACGCCCTACGGGGGAA
AGCAGGGGACCTTCGGGCCTTGCGCGATTGGATGAACCCAGGTGGGATTAGCTAGTTGGTGAGGTAATGGCT
CACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGAC
TCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCCATGCCGCGTGTGTG
AAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAGGAAAGGTTGGTAGCTAATAACTGCCAGCTGTG
ACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTT
AATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTGGATAAGTTAGATGTGAAAGCCCCGGGCTCAAC
CTGGGAATTGCATTTAAAACTGTCCAGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAA
ATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGA
AAGCGTGGGGAGCAAACAGGATTAGATACC**

Fig. 7: Phylogenetic tree of Aeromonas species isolates

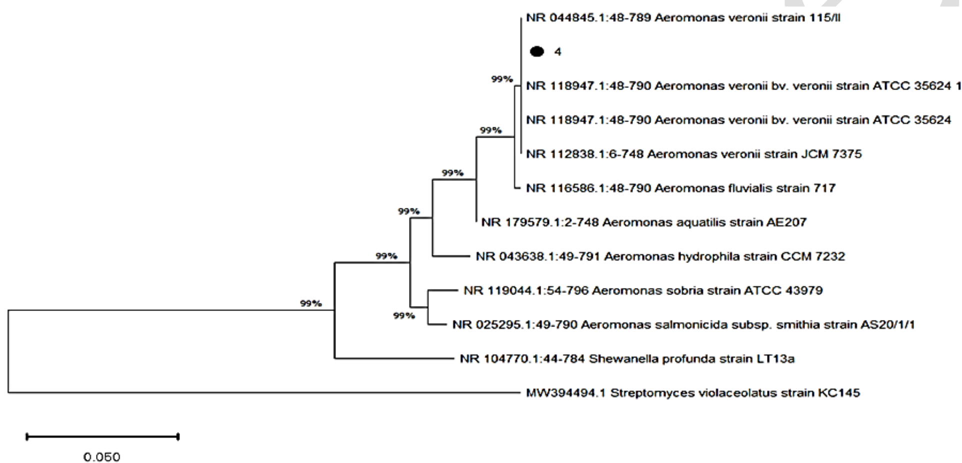

Fig. 8: Phylogenetic tree of *Aeromonas veronii* bv. veronii.
The level of homology with the closest strain NR 118947.1:48-790 *A. veronii bv. veronii* strain ATCC 35624 1 amounts to 100.00%.

№ 21v – *Aeromonas sobria*
(№ 57, 58, 59, 65, 66, 67, 72, 73, 80- ;analogous results)

Nucleotide sequence obtained by sequencing the 16S rRNA gene:

**CGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGT
TAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTGGATAAGTTAGATGTGAAATCCCCGAGCTCA
ACCTGGGAATTGCATTTAAAACTGGTCAGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCG
GTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACG**

Fig. 9: Phylogenetic tree for selected bacterial strains

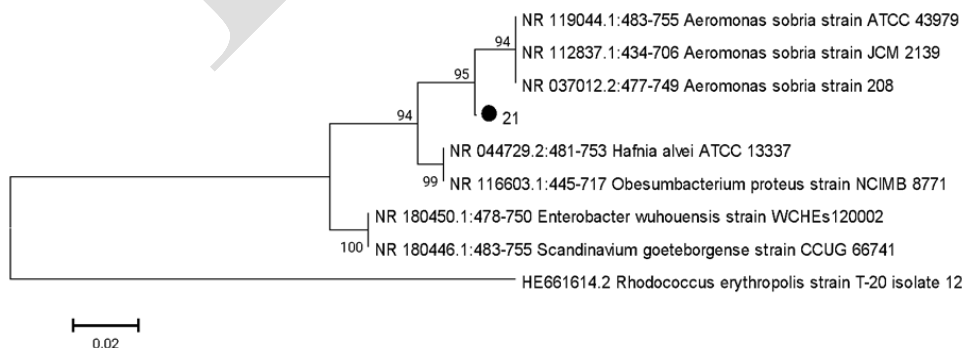

Fig. 10: Phylogenetic tree of *Aeromonas sobria*
The level of homology with the closest strains of *A. sobria* reaches 98.90%.

The microbiological analysis and genotyping of rainbow trout microflora led to the isolation and identification of 103 microbial cultures. Molecular genetic identification of bacterial strains of the microbiomes of the gut, internal organs and gills of fish, also performed by Sanger sequencing of the 16S rRNA gene, revealed 103 cultures,

№ 8 – *Aeromonas rivipollensis*
(№ 60, 63, 69, 70 - analogous results)

Nucleotide sequence obtained by sequencing the 16S rRNA gene

AGTCGAGCGGCAGCGGGAAAGTAGCTTGCTACTTTTGCCGGCGAGCGGCGGACGGGTGAGTAATGCCTGG
GAAATTGCCCAGTCGAGGGGGATAACAGTTGGAAACGACTGCTAATACCGCATACGCCCTACGGGGGAAAG
CAGGGGACCTTCGGGCCTTGCGCGATTGGATATGCCCAGGTGGGATTAGCTTGTTGGTGAGGTAATGGCTCA
CCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACT
CCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCCATGCCGCGTGTGTGA
AGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAGGAAAGGTTGGCAGCTAATATCTGTCAACTGTGA
CGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTA
ATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTGGATAAGTTAGATGTGAAAGCCCCGGGCTCAACC
TGGGAATTGCATTTAAAACTGTCCAGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAA
ATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCG
AAAGCGTGGGGAGCAAACAGGATTAGATACCT

Fig. 11: PCR amplification results of bacterial isolates

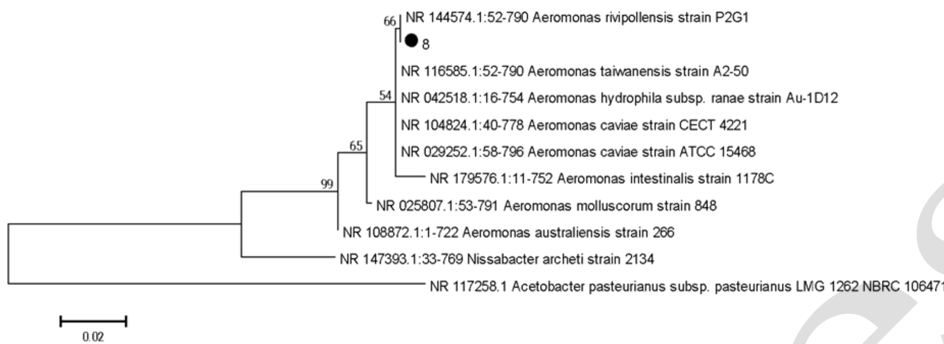

Fig. 12: Phylogenetic tree of *Aeromonas rivipollensis*
The level of homology with the closest strain NR 144574.1:52-790 *Aeromonas rivipollensis* strain P2G1 is 99.59%.

№ 1 – *Pseudomonas protegens*
(№ 30, 31, 38, 61, 62, 64, 71- analogous results)

Nucleotide sequence obtained by sequencing the 16S rRNA gene

CATGCAGTCGAGCGGCAGCACGGGTACTTGTACCTGGTGGCGAGCGGCGGACGGGTGAGTAATGCCTAGG
AATCTGCCTAGTAGTGGGGGATAACGTCCGGAAACGGGCGCTAATACCGCATACGTCCTACGGGAGAAAGT
GGGGGATCTTCGGACCTCACGCTATTAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGAGGTAATGGCTCAC
CAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTC
CTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAA
GAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTTACCTAATACGTGATTGTTTTGACG
TTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAAT
CGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGTTAAGTTGGATGTGAAAGCCCCGGGCTCAACCTG
GGAACTGCATCCAAAACTGGCAAGCTAGAGTATGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAAT
GCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGAA
AGCGTGGGGAGCAAACAGGATTAGATACCCT

Fig. 13: Electrophoresis of 16S rRNA gene amplification products

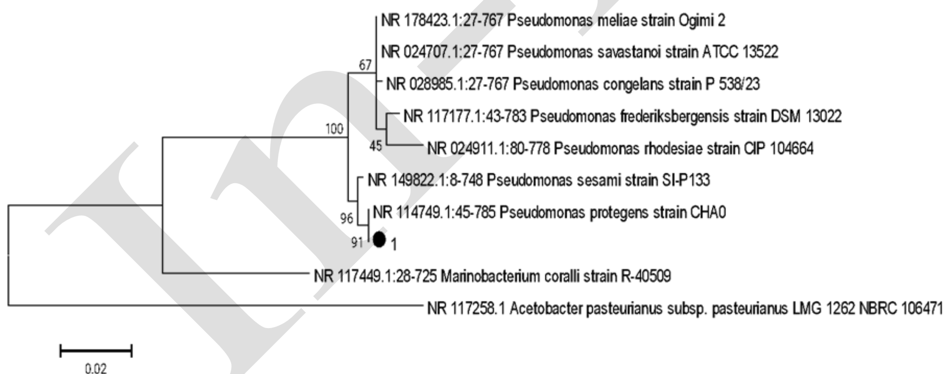

Fig. 14: Phylogenetic tree of Pseudomonas protegens
The level of homology with the closest strain NR 114749.1:45-785 *P. protegens* strain CHA0 is 99.87%.

including the strains of *A. media* (n=8), *Pseudomonas juntendi* (n=5), *Acinetobacter calcoaceticus* (n=3), *Staphylococcus aureus* (n=3), *Staphylococcus equorum* (n=4), *Obesumbacterium proteus* (n=2), *Enterobacter cloacae* (n=4), *Hafnia alvei* (n=4), *Yersinia entomophaga* (n=3), *E. coli* (n=5), *Arthrobacter gandavensis* (n=1), and *Acinetobacter sp.* (n=2) (Table 3).

The fish body has several defenses that prevent the penetration of pathogens, their reproduction, and the action of toxins released by them. These include skin and mucosal barriers and nonspecific cellular (phagocytosis) and humoral factors. These factors in their totality and interaction with each other and the external environment ensure homeostasis and protect against the introduction of pathogens (Kokou et al. 2019). The microbiota of fish, especially the intestinal microbiota, is important for fish health and influences nutritional metabolism, various physiological functions, productivity, and the formation of immunity. The microbiota composition depends on fish genetics, developmental stage (age), habitat, food habits, and diet. Several researchers have demonstrated the identity of microorganisms by studying the composition of the intestinal microflora of a single species of fish grown in laboratory conditions and caught in the wild and established that the host intestine provides a habitat for certain bacterial taxa (Vestrum et al. 2020).

Table 3: Number of bacteria isolated from healthy and diseased fish.

Isolated cultures	Total, *n/%*	Including	
		From healthy fish, *n/%*	From fish with signs of disease, *n/%*
Total across all isolates	103/100	46/44.6	57/55.4
Aeromonas hydrophila	14/13.6	5/27.3	8/72.7
Aeromonas salmonicida	12/11.65	3/25	9/75
Aeromonas veronii	9/8.7	3/33.3	6/66.7
Aeromonas rivipollensis	6/5.82	4/66.7	2/33.3
Aeromonas media	8/7.8	5/62.5	3/37.5
Aeromonas sobria	10/9.7	2/20	8/80
Pseudomonas protegens	8/7.8	5/62.5	3/37.5
Pseudomonas juntendi	5/3.9	3/60	2/40
Carnobacterium maltaromaticum	3/2.9	2/66.7	1/33.3
Staphylococcus aureus	3/2.9	2/66.7	1/33.3
Staphylococcus equorum	4/3.9	2/50	2/50
Obesumbacterium proteus	2/1.9	2/100	-
Enterobacter cloacae	4/3.9	3/75	1/25
Hafnia alvei	4/3.9	1/25	3/75
Acinetobacter calcoaceticus	1/0.97	-	1/100
Yersinia entomophaga	3/2.9	1/25	2/75
Escherichia coli	5/4.85	2/40	3/60
Arthrobacter gandavensis	1/0.97	-	1/100
Acinetobacter species	2/1.9	1/50	1/50

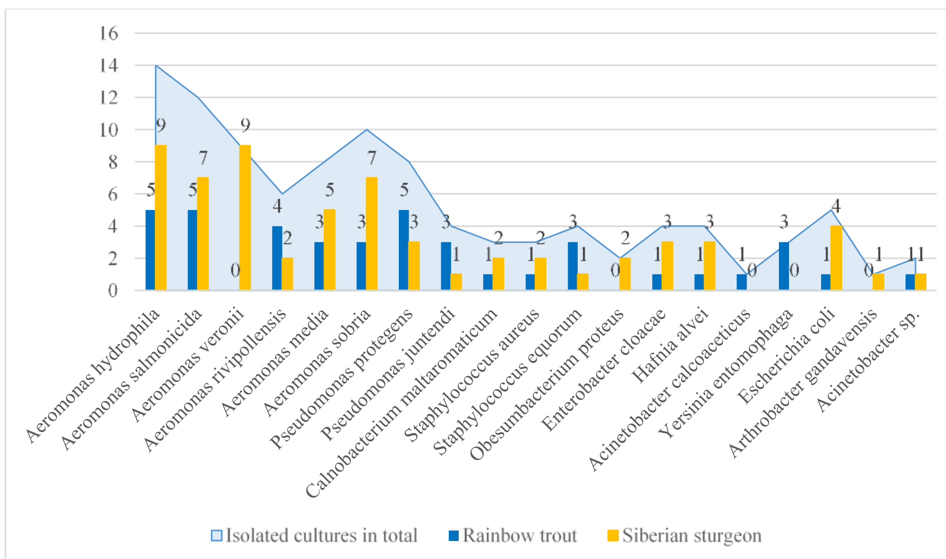

Fig. 15: Genus composition of the microbiome of the intestines, internal organs, and gills of rainbow trout and Siberian sturgeon.

The intestinal microbiota prevents the development of pathogens by directly inhibiting them, competing for nutrients, providing resistance to colonization, or interacting with host factors. The influence of various factors (endogenous and exogenous) on the condition of fish can disrupt homeostasis, leading to changes in the composition of the normoflora, which includes commensal microbes necessary to support the development and maturation of the immune system. Under certain conditions, some species of opportunistic microflora show aggressive properties towards the host, causing pathological processes and complicating the epizootic situation (Luan et al. 2023).

As a result of the microbiological analysis and genotyping of the microflora of rainbow trout and Siberian sturgeon individuals, a total of 103 microbial cultures were isolated and identified. In the studied fish species, the bacterial flora of the digestive tract is similar to that of the skin and gills.

According to the results, the species composition of bacterioflora in the population of healthy and sick fish did not have reliable differences. When comparing the composition of the microbiome of healthy and diseased fish, there is a significant change in the quantitative composition of the isolated bacterioflora during pathological processes in the intestine and internal organs (Fig. 16).

The composition of associative symbiosis in diseased fish is dominated by opportunistic microflora. The content of *A. hydrophila* in the biomaterial from diseased fish is 45.4% higher than in healthy fish, the content of *A. salmonicida* is 50% higher, the content of *A. veronii* – 33.4% higher, and *A. sobria* – 60% higher.

Molecular genetic studies established that the strains of *A. rivipollensis* are the closest relatives of *A. media* ATCC 33907(T) (99.4%) and *A. hydrophila* ATCC 7966(T) (99.3%). The role of this member of the genus *Aeromonas* in aquaculture pathology is still under investigation (Marti and Balcazar 2015).

Representatives of the genus *Pseudomonas* were isolated from the samples of healthy and sick fish. Molecular genetic identification showed the presence of two species in the studied fish organisms – *P. protegens* and *P. juntendi.* In contrast, Ben Mhenni et al. (2023)

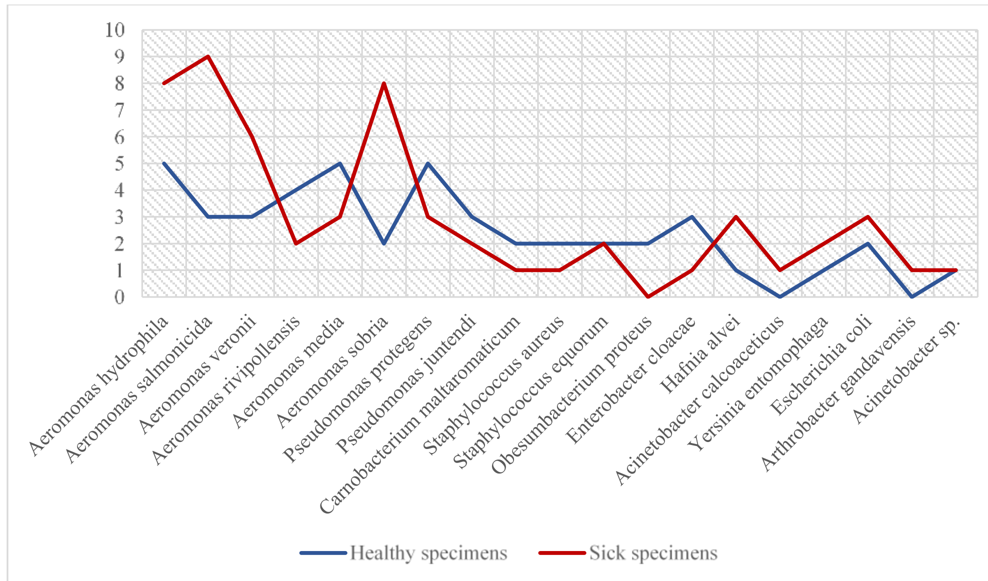

Fig. 16: Composition of the intestinal microbiome in healthy and sick fish.

when sequencing 16S RNA from salmon, flounder and cod fillets, found that 45.95% of the strains were *P. fluorescens*, followed by *P. fragi* (21.62%), *P. marginalis* (10.81%), *P. taetrolens* (8.11%), *P. lundensis*, *P. syringae*, *P. tolaasii*, *P. viridilivida* and *P. chlororaphis* (2.70% each one). (Ben Mhenni et al. 2023). It should be noted that *P. protegens,* and *P. juntendi* are typical soil microorganism that can be isolated from the roots of various plant species and in aquatic environments and has an extremely versatile metabolism, however, their pathogenicity has not been previously reported.

The population composition of various opportunistic microbe species inhabiting the bodies of animals, birds, and fish is marked by variability, which manifests in changes in the qualitative composition under the influence of endogenous and exogenous factors. Microorganisms that are pathogenic or conditionally pathogenic to physiologically healthy organisms may become pathogenic when the body's natural resistance is weakened, especially under unfavorable conditions and stressful situations. A frequently occurring fish disease caused by opportunistic microorganisms is bacterial hemorrhagic septicemia, characterized as a polyetiological fish disease (Basankina 2020).

In aquaculture farming, antibiotics are used extensively, as their introduction into fish feed in small amounts promotes the growth and survival of fish and regulates microbiological processes in the gastrointestinal tract of fish, ensuring microbial balance and preventing the emergence of mass diseases by reducing the number of pathogenic bacteria in the microbiome.

However, the large-scale use of antibiotics in aquaculture has led to a dramatic decrease in their effectiveness, the disruption of microbiocenoses, the emergence of resistant strains of bacteria, and the creation of favorable conditions for associative diseases (Dixon 1994; DePaola et al. 1995; Delcour 2009).

Many researchers report that antibiotic resistance is becoming increasingly common among the commensal bacteria in fish grown in confined aquaculture systems due to the excessive unregulated use of antibiotics for prevention and treatment (Cabello et al. 2013).

The following studies focused on determining the level of antibiotic resistance of isolated cultures. Antibiotic susceptibility was determined using the conventional disk-diffusion method (Kirby-Bauer disk diffusion susceptibility test) by sowing on Mueller-Hinton agar using standard paper disks (produced by Liofilchem S.r.l.), containing from 10 to 50μg of antibacterial preparations/disk. The diameter of the culture growth suppression zones was counted after 18-24 hours of cultivation in a thermostat at 25°C.

Our data on the prevalence of antibiotic-resistant microorganisms in aquaculture confirm the findings of several researchers concerned about the spread of antimicrobial resistance in fish normoflora. Studies on the antibiotic sensitivity of *Aeromonas* were carried out in two replications. All strains tested for antibiotic resistance were resistant to penicillin, ampicillin, amoxicillin, lincomycin, and tylosin. *A. hydrophila* had high resistance to nine antibiotics out of the 19 tested (Table 4). A medium degree of resistance was detected for three antibiotics. Finally, high susceptibility was detected to aminoglycosides, fluoroquinolones, and 3rd generation cephalosporins.

Next, we focused on identifying biofilm-forming strains among the microorganisms isolated from rainbow trout and Siberian sturgeon. Biofilms are a form of microbial communities fixed on abiotic and biotic surfaces and include microbial cells and associated extracellular matrix, which consists of polysaccharides, proteins, and DNA. Biofilms are ubiquitous. Bacteria living in biofilms differ significantly in their biological properties from planktonic forms. The interaction of biofilms with the macroorganism's immune system and the antibiotic resistance of bacteria in biofilms attract the most attention. Bacteria in biofilms become difficult to reach for immune system factors (Romanova and Gintsburg 2011).

The biofilm structure and the physiological features of film bacteria increase the community resistance to antimicrobial agents many times compared to planktonic cultures. Microorganisms included in biofilms were 100-1,000 times less susceptible to most antibiotics and other biocidal agents than planktonic cells. Some antibiotics in non-lethal concentrations can stimulate biofilm formation, which confirms the biological importance of biofilm formation by microorganisms for their defense against

Table 4: Interpretation of microorganism growth retardation zone diameters in determining the sensitivity of *Aeromonas* genus representatives to antimicrobials

Antibiotic, antibiotic content per disk (µg)	Classification	*A. hydrophila*	*A. salmonicida*	*A. veronii*	*A. sobria*
Erythromycin (E), 15	Macrolides	R	R	R	I
Tylosin (TY), 30		R	R	R	R
Tetracycline (TE), 30	Tetracyclines	S	R	I	I
Chloramphenicol (C), 10	Chloramphenicol	R	R	S	S
Vancomycin (VA), 5	Glycopeptides	R	R	I	R
Ampicillin (AMP), 10	β-lactam	R	R	R	R
Amoxicillin (AC), 10.1		R	R	R	R
Penicillin (GP), 10		R	R	R	R
Streptomycin (S), 10	1st generation Aminoglycosides	S	I	S	I
Kanamycin (K), 30		S	S	S	I
Gentamicin, 10 (Bioanalyse, Turkey)	2nd generation Aminoglycosides	I	S	I	I
Rifampicin (RD), 30	Ansamycins	R	R	I	R
Norfloxacin (NOR), 10	Fluoroquinolones	I	R	S	S
Enrofloxacin (ENR), 5		S	I	S	R
Flumequine (UB), 30		S	R	S	S
Cefoxitin FOX, 30	2nd generation Cephalosporins	R	R	R	I
Cefoperazone (CFP), 30	3rd generation Cephalosporins	S	S	S	S
Trimetroprim (TM), 5	Dihydrofolate reductase inhibitors	S	I	S	I
Lincomycin (MY), 15	Lincosamide group	R	R	R	R
Furazolidone (FR), 50	Nitrofuran derivatives	I	R	I	I

Note: Control values of *Aeromonas* growth suppression by antibiotics according to the recommendations provided by the Clinical & Laboratory Standards Institute, USA, 2014. (Source of data: http://www.clsi.org): R – resistant; S – susceptible; I – intermediate/weak susceptibility.

Table 5: Comparative data on the biofilm-forming ability of different microorganism genera.

Isolated cultures	Total, n/%	Level of biofilm formation		
		*weak biofilm producers, n	**moderate biofilm producers, n/%	***strong biofilm producers, n/%
Total across all isolates	103	24/23.3	23/22.3	30/29.1
Aeromonas hydrophila	14	2	4	5
Aeromonas salmonicida	12	2	4	4
Aeromonas veronii	9	2	1	4
Aeromonas *rivipollensis*	6	1	3	1
Aeromonas *media*	8	2	3	2
Aeromonas sobria	10	3	1	5
Pseudomonas protegens	8	2	1	3
Pseudomonas juntendi	5	1	1	1
Carnobacterium maltaromaticum	3	2	1	-
Staphylococcus aureus	3	-	2	1
Staphylococcus equorum	4	1	3	-
Obesumbacterium proteus	2	1	-	1
Enterobacter cloacae	4	1	1	-
Hafnia alvei	4	1	2	1
Acinetobacter calcoaceticus	1	-	-	-
Yersinia entomophaga	3	1	-	-
Escherichia coli	5	1	-	2
Arthrobacter gandavensis	1	-	-	-
Acinetobacter species	2	1	-	-

Note:
When measured on a spectrophotometer, the colored wells provide the optical density:
*strains with optical density of OD600<0.3,
**strains with optical density OD600 from 0.3 to 0.6,
***strains with optical density OD600>0.6.

harmful factors (Fong and Yildiz 2015; Hobley et al. 2015; Mardanova et al. 2016). Thereby, the objective of the following studies was to determine the biofilm-forming ability of the isolated strains. The results are reflected in Table 5. 74.8% of tested cultures were biofilm-forming, with 39% of the strains (n=30) being strong biofilm producers. Of the opportunistic microflora, 33.3 to 50% are strong biofilm producers (*A. hydrophila – 35.7%, A. salmonicida – 33.3%, A. veronii – 44.4%, A. sobria – 50%).*

The predominant genus of bacteria in rainbow trout and Siberian sturgeon biomaterial samples was *Aeromonas*. These species have emerged as significant pathogens due to their increasing resistance to various antimicrobials. This resistance is primarily attributed to the indiscriminate use of antibiotics in fish farming, leading to multidrug-resistant strains that pose risks to both aquatic life and human health. Many studies indicated that *Aeromonas* spp. exhibit high levels of resistance to multiple antibiotic classes. For instance, Fauzi et al. (2021) found that resistance rates were highest for β-lactam antibiotic ampicillin (100%), followed by aminoglycoside antibiotics like streptomycin (59%), and kanamycin (41%). Another

study highlighted that *A. hydrophila* showed significant resistance to beta-lactam antibiotics, including ampicillin (>89%) and cephalothin (>84%) (Martins et al. 2023). Our data display that *Aeromonas* are indeed resistant to antibiotics that target bacterial cell wall synthesis - ampicillin, amoxicillin, penicillin and cefoxitin. Also, all four *Aeromonas* species tested were susceptible to third-generation cephalosporin. However, susceptibility to streptomycin and kanamycin was increased in our analysis, highlighting the fish species- and habitat-dependent nature of antibiotic resistance.

Nascimento et al. (2024) investigated the efficacy of reserve antibiotics of the cyclosporine and carbopene classes on isolates of *Aeromonas salmonicida*, *A. popoffii*, *A. media*, *A. hydrophil*, *A. hydrophila* and *A. veronii*. *Aeromonas salmonicida* was found to be the most resistant species, particularly to ceftazidime, cefoxitin, and imipene. 19 and 28% of isolates showed intermediate resistance to imipenem and meropenem, respectively, and resistance was associated with the ability to form biofilms. More than half of the isolates had this ability, with 90% of them having low optical density at 570nm (Nascimento et al. 2024). Our study included a broader panel of antibiotics and identified *A. hydrophila* as the most resistant. Moreover, all *Aeromonas* species were strong biofilm formers, so further research requires studying the characteristics and causes of biofilm formation by these bacteria.

Antimicrobial resistance in aquaculture is becoming an increasingly important issue as excessive and inappropriate use of antibiotics in fish farming and other forms of aquaculture is leading to the emergence of resistant strains of pathogens. This not only threatens animal health but may also pose a risk to human health and ecosystems as a whole. Probiotics, prebiotics and quorum sensing inhibitors have been investigated in many studies as alternative approaches to eliminate biofilms (Sam-On et al. 2023; Munni et al. 2023; Neil et al. 2024).

Preliminary studies to address the correction of fish intestinal microflora aimed at increasing the natural resistance of fish population and reducing the risk of intestinal infections consisted in determining the antagonistic activity of the probiotic strain *E. coli 39 SN* against opportunistic microflora. Preliminary studies demonstrated that the *E. coli* 39 SN strain has probiotic properties and performs well in experiments on farm animals (calves, lambs) (Biyashev et al. 2022).

The results show that the *E. coli* 39 SN strain has an inhibitory effect on the opportunistic microflora isolated from the diseased fish. In the study of the tested cultures of *Aeromonas* and *Pseudomonas* by the method of agar blocks and perpendicular streaks, we found that the antagonistic properties of the probiotic strain *E. coli* 39 SN enhances cultivation on the medium with the addition of 5% vermiculite. The stimulating effect of vermiculite likely relates to the prebiotic effect of the natural mineral. This provides an opportunity for further research to utilize it as a new probiotic product with multiple beneficial effects, including tolerance preservation, which is the most significant for sustainable microbiocenosis (Ursova 2015). However, only one probiotic strain of the genus Escherichia has been previously reported – *Escherichia coli* Nissle 1917, which is known for its immunomodulatory properties in the treatment of inflammatory bowel diseases (Behrouzi et al. 2020). The reason why *E. coli* strains are not widely used as antibacterial agents is the opportunistic status of these bacteria. Commensal strains acquire virulence through various mechanisms of horizontal gene transfer (Shoemaker et al. 2021). In addition, *E. coli* is becoming increasingly resistant to antibiotics and is now ranked third in the list of 12 antibiotic-resistant priority pathogens described by WHO. Thus, although *E. coli* 39 SN strain has demonstrated its antibacterial efficacy, further safety studies are required for its use in aquaculture.

Conclusion

There are several problems in modern commercial aquaculture production, one of the most important of which is infectious diseases caused by pathogenic or opportunistic microflora. The use of antibiotics in aquaculture for treatment, disease prevention, and growth stimulants leads to the development of a stable population of microflora that pose a threat of transmitting resistant bacteria to humans, whether through food, direct contact, or the environment. The study of the aquaculture microbiome allows one to objectively assess the condition of the fish organism and obtain a long-term forecast of pathological changes caused by endogenous and resident agents. It is promising to investigate the antagonistic activity of probiotic strains against pathogenic and opportunistic microflora with the prospect of developing probiotics and synbiotics that offer an alternative approach to maintaining veterinary welfare in aquaculture.

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Conflict of Interest: The authors declare no conflict of interest.

Authors contributions: Zhumagul Kirkimbaeva: data acquisition and data collection. Nurzhan Sarsembayeva: data analysis, statistical work. Birzhan Biyashev: data interpretation, critical revisions, interpretation of results. Ardak Akkozova: conceptualization, research design, technical support. Arman Zhylkaidar: funding acquisition, project administration, supervision, methodological development. Dinara Sarybaeva: literature review, manuscript writing, overall study supervision.

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