Enzymatic potential of the indigenous microbiota of the intestine of rainbow trout \textit{Parasalmo mykiss} (=Oncorhynchus)

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Abstract. This paper is devoted to finding the scientific support for the development of commercial aquaculture. The article presents the results of examining the microbiocenosis of the commodity trout (\textit{Parasalmo mykiss}) intestine. It was revealed that members of the genus \textit{Pseudomonas} prevail in the microbiocenosis of the aquaculture intestinal contents. A small number of lactic acid bacteria, representing autochthonous microbiota and rare types of bacteria, such as coagulase-negative \textit{Staphylococcus} spp. and \textit{Clostridium} spp., being the components of fish allochthonous microbiota were discovered. Lactic acid bacteria were not found on the mucous membrane of the intestinal tract, due to the weak attachment of microorganisms in fish exposed to stress. The enzyme product was obtained from the bacteria with proteolytic activity in order to introduce this supplement as a feed probiotic into the artificial diets of cultivated fish. The advantage of the developed product is that the raw material (source) for its production are the bacteria isolated from rainbow trout intestine from experimental cage farms. The maximum activity of selected proteases was observed at bacterial concentration \times 10^8 \text{ cells/ml}. Proteolytic activity in the enzyme product from bacteria of the genus \textit{Pseudomonas} spp. is higher than in the enzyme product obtained from the primary culture of \textit{E. coli}. Antibiotic resistance of the selected isolates from \textit{Pseudomonas} spp. Cefotaxime, which is recommended to use for prevention of bacterial infections when forming the broodstock was determined. \textit{Pseudomonas} showed high sensitivity to a number of broad-spectrum antibiotics, such as ciprofloxacin, gentamicin. The obtained results of microbiological research can be used to develop the strategy of antibacterial therapy in fish farming.

1. Introduction
Development of one of the most acute and economically perspective directions of obtaining high-quality fish products based on the use of natural bio-resource potential – aquaculture is of particular importance in the general trend to the depletion of commercial fish stocks of most seas and oceans of the planet.

Aquaculture is the one of the fastest growing sectors in food production, making up 50 per cent of the world's fish market [1]. It is assumed that the volume of seafood produced in 2020 will reach 150-160 million tons, the share of aquaculture is expected to be 75-80 million tons, while farmed fish will comprise 39 million tons. Nevertheless, negative interactions between fish and pathogenic microorganisms may cause unexpected deaths – low survival rate or a low (decreased) growth rate, which greatly complicates the hydrobiont culturing [2].

History of aquaculture development, in particular, trout farming on the Kola Peninsula was associated with the launch of the first stage of the Kola NPP in the early 70-ies, and by the early 90-ies
the Murmansk region was considered the leader in the commercial fish breeding. However, cases of mass disease (epizootics) of fish occurred on the fish farms which had a negative effect on the development of the industry. On the basis of long-term studies of disease rates the instructions on prevention and methods of controlling the main ichtipathogenic microorganisms have been developed, maximum allowable amount of farmed fish was set. The possibility and prospects of salmonids breeding in the Arctic region have been proven but the research is still ongoing [3].

In recent years, due to the noticeable decline in fish catches the interest to the aquaculture of the North has increased significantly. From the three sides the Murmansk region is bordered by the waters of two seas. From the North and North-East it is washed by the Barents sea and in the South by the White sea. The mainland is replete with lakes, rivers, streams water reservoirs. The most promising areas for cultivation of salmon family fish are considered natural reservoirs and the sea coasts of the Kola Peninsula. The unique climatic and natural conditions of the far North (long daylight hours, mild climate due to the warm Gulf stream, ice-free waters, etc.) allow successful commercial hydribiont cultivation [4].

Currently, in the Murmansk region there are 25 aquaculture farms located in marine areas and freshwater bodies which produce Atlantic salmon and rainbow trout, as well as cultivate Atlantic salmon juveniles [5]. Economic factors require the intensification of the systems of artificial breeding and the increase of aquaculture production [6]. In fish cultivation fish diseases often develop because of high population density. In such environments there is a disbalance of recovery and self-purification processes in the aquatic ecosystems, a high degree of eutrophication and an increase in saprobity index of fishery water bodies, deterioration of hydrochemical parameters of the environment due to the accumulation of products of fish metabolism and uneaten feed. It should be noted that the high content of organic substances in the aquatic environment creates conditions for intensified growth of the saprophytic, conditionally pathogenic and pathogenic microflora, causing fish diseases. Degradation of aquatic ecosystems and the strengthening of the destabilizing factors of natural and anthropogenic origin causes stresses in cultured fish [7], [8], [9].

Infectious diseases always pose a greater danger to the environment and the ecosystem as a whole. Use of open aquacultural systems of cage cultivation facilitates the spread of diseases both among farmed fish and indigenous populations. As a result, it can cause significant damage to the fishing industry and in future cause the reduction in fish products stock.

It is known that the damage to the cultured species may reach 25 % in case of epizootically dangerous diseases, mostly of infectious nature, while increasing the dynamics of the epidemic process will possibly cause 100 % death of cultured aquatic animals. Currently, on fish farms in order to reduce losses when reproducing the aquatic organisms systematic veterinary and sanitary supervision is arranged and therapeutic and prophylactic measures, using a wide range of medicines, including antibiotics, often added to fish forage, is adjusted.

According to the modern studies, the residual quantity of antibiotics is found both in food raw materials and finished products of aquaculture origin. The risk of eating food containing antibiotic components getting through the consumer body into the environment causes the formation and spread of antibiotic-resistant microorganisms and genes, and the increase in the cost of subsequent treatment and preventive measures and long-term bacteria carrying [10].

It is widely known that nutrition plays an important role in the functioning of innate immunity. Improving the survival rates, growth rates and normalizing the physiological state of fish after taking medication is possible by optimizing of forage and the ways of feeding.

A characteristic feature of most industrial feeds is the almost complete absence of natural organisms. Natural food contains the widest range of various biological substances that are involved in the regulation of many metabolic processes in the body of aquatic organisms [11]. Therefore, when developing artificial diets, it is necessary to consider the balance of fats, proteins and carbohydrates, and the content of biologically active substances, including probiotics as well.
In the water fish constantly interact with microorganisms of different taxonomic and ecophysiological groups which are found on mucous membranes [12]. Microbiota of the gastrointestinal tract is a single grouping, which is involved in several important physiological functions of marine organisms, including digestion, development of the innate immune system-mucous membranes, angiogenesis and protection from diseases [13]. The wall of the intestine and its associated lymphoid tissue is a direct link between the digestive system and innate immunity and the interaction between them is obvious to be of great importance in the study of nutrition and health. At the final stage of development in early fish ontogenesis gastrointestinal tract is heavily inhabited by a broad range of bacteria. Among them there are pathogenic bacteria passing into a dormant state, creating the resting forms of microorganisms and cells — "persisters" capable of causing pathology, under certain endogenous or exogenous stress factors [14]. However, microbiota changes after the introduction of probiotics into the fish diet [15].

Currently, fish intestinal microbiota can be examined in several aspects: the ability to antagonize bacterial pathogens; as a factor of internal potential benefit for an organism; as a means to identify potential probiotic bacterial candidates. The latter is one of the promising areas of research in aquaculture and biotechnology. It is important to note that the ability of probiotic bacteria to be cultivated in vitro is rather high, which allows to be detected using traditional microbiological methods of research.

In the manufacture of natural animal feed to breed 1 kg of marketable salmon (especially salmonids) requires 3-4 kg of wild fish, which is one of the factors causing a substantial reduction in reserves of valuable indigenous species of fish. Introduction of probiotics into the artificial diet will increase its efficiency by 80%. Therefore, it is relevant to obtain highly active fermentation product for introducing into the artificial diets of cultivated fish as a feed probiotic. The advantage of the product is that the raw material (source) for its production are bacteria isolated from the intestine of rainbow trout from experimental cage farm.

The purpose of this study is examining the intestinal microbiota of cage rainbow trout (Parasalmo mykiss), determining the antibiotic resistance of the selected isolates of Pseudomonas spp. and obtaining the enzyme product from the bacteria with proteolytic activity.

2. Materials and methods
Determining the microbial isolates from the intestine of cage rainbow trout, pure culturing of conditionally-pathogenic microorganisms, producing the protein desintegrates and measuring their optical density, were performed at the departments of "Microbiology and biochemistry", "Food Production Technology" and the center for the study of raw material and production of the Murmansk state technical university.

The object of the study was freshwater fish- cage rainbow trout Parasalmo mykiss of four generations, years-2015, 2016, 2017, 2018. The trout was imported from OOO "Artik-salmon".

In the period from February 2015 to March 2019 250 copies of rainbow trout were examined in fish-breeding farm "Nainas".

Trout cage farm OOO "Nainas" is situated in the town settlement Verkhnetulomskiy and consists of two cage lines placed at a distance of about 250 m from each other. A separate shift house is equipped for each line in the coastal area.

The dissection of fish was performed in the laboratory observing appropriate aseptic, only living fish was used. Studies of fish intestinal microbiota was carried out separately using the method of scraping the mucosa from a given surface area of the intestine, which allows to provide mathematical analysis of the numerical characteristics of microbiocenosis of the mucous surfaces and of the intestinal contents.

Cultivation of microorganisms in vitro and identification of the obtained isolates was done by standard methods of microbiology [16].

To establish the systematical classification of the isolated bacteria 2 volume Bergey's manual was used.
The strain of Escherichia coli K-12 F+StrR (KS-507), registration number B-3254, which is not genetically modified, non-pathogenic to humans and belongs to the 1st level of Biosafety served as a reference microorganism. The choice of the microorganism is explained by the fact that Escherichia coli is a natural inhabitant of the animal intestines and has significant proteolytic activity.

To obtain the disintegrant the method of alternate freezing and in the chamber of shock freezing and defrosting (thawing) in an ultrasonic bath was used.

General proteolytic activity was determined using Anson's method [17] with some modifications. The value of proteolytic activity ($A$) was calculated according to the formula (1):

$$A = \frac{1000 \times (D_{sample} - D_{control}) \times V_{total} \times 10^6}{t \times C_{EP} \times V_{EP} \times \varepsilon \times l \times 1000} = 1707.07 \times \Delta D, [\text{mkmoITYR/g}]$$

(1)

where 1000 is the conversion of mg to g (weight of enzyme preparation);

$D_{sample}$ – optical density of sample solution (280-320 nm) ODU;

$D_{control}$ – optical density of control solution (280-320 nm) ODU;

$V_{total}$ – total volume of the solution after adding trichloroacetic acid, ml (2+3.6+0.4+2 = 8 ml);

$10^6$ – conversion of mol of tyrosine to mkmo;

$t$ – incubation time, min (10 min);

$C_{EP}$ - concentration of enzyme preparation solution, mg/ml (1 mg/ml);

$V_{EP}$ – volume of enzyme solution, ml (0.4 ml);

$\varepsilon_{TYR}$ - molar extinction of tyrosine, l/(mol*cm) (1171.6 l/(mol*cm));

$l$ – length of optical path of pan, cm (1cm);

1000 (in denominator) – conversion of total volume from ml to l

Determination of proteolytic activity was carried out on spectrophotometer SPH 2000.

Antibiograms were examined using a standard flat disks produced by FBIS Research Institute of Epidemiology and Microbiology named after Pasteur (Russia), disk-diffuse method in accordance with MUK 4.2.1890-04. "Determining the sensitivity of microorganisms to antibiotics".

Sensitivity of the selected isolates Pseudomonas spp. to antibiotic substances, widely used in aquaculture: ofloxacin, ciprofloxacin, ampicillin, cefotaxime, doxycycline, gentamycin, tetracycline, chloramphenicol, clarithromycin was determined.

The experimental part of the work was carried out in accordance with the requirements of sanitary-epidemiological rules SP 1.3.2322-08. "Safety of work with microorganisms III-IV groups of pathogenicity (hazard) and agents of parasitic diseases”.

3. Results

Bacteria of the genus Lactobacillus (3 %) representing the autochthonous microbiota were found in microbiocenosis of the intestinal contents of trout.

The study also revealed the isolated bacteria of coagulase-negative Staphylococcus spp., its share is 35% and Clostridium spp. (15 %) related to the allochthonous intestine microbiota of fish.

Lactic acid bacteria were not detected in the intestinal mucosa of fish, probably due to the fact that representatives of the genus Lactobacillus are worse at attaching the intestinal mucosa of fish exposed to stress [18].

At the same time opportunistic members of the genus Pseudomonas (47 %) dominated in the intestinal microbiota on the fish studied.

The dominance of Pseudomonas in the composition of the normal microbiota of fish can cause mass disease of aquatic organisms [19]. According to microbiologists, in conditions of artificial cultivation there is often a combination of stress factors exceeding the adaptive capacity of aquatic organisms, which leads to their increased susceptibility to Pseudomonas [20].
Pathogenic microorganisms (Staphylococcus spp., Pseudomonas spp., Clostridium spp.) account for 97% of the intestine microbial makeup of the intestine of the studied fish (Figure 1).

Thus, we can assume that such a makeup could be viewed as a signal to an unfavourable situation in the ecosystem of intestinal microbionosis, but a few microorganisms visible under the light microscope are known to be capable of growing in laboratory conditions on usual environments, therefore, most likely, unpretentious representatives of microbial groups of the intestine, adapted to conditions in vitro were detected. Rather, traditional microbiological studies of the intestinal microbiota reflect only changes in the characteristics of the environment.

Enzymatic activity could be the most reliable indicator of the intestinal microbiota functioning. The dominant group of microorganisms isolated from the intestinal contents of rainbow trout was pseudomonades with high proteolytic activity, therefore, tests aimed at determining their activity were conducted.

Living bacteria constantly secrete and absorb from a variety of macromolecules (proteins, polysaccharides) into the intestine. The majority of molecules cannot be transported through the cytoplasmic membrane of a microorganism and are subjected to disintegration due to the action of exofermentors. Proteolytic active bacteria are present in almost all species of fish. It should be noted that the microorganisms inhabiting fish intestines can synthesize a complex of proteases. For example, bacteria which belong to the genus Pseudomonas are characterized by a fairly high proteolytic activity of both extracellular and intracellular hydrolases.

Bacteria localized on the mucosa and in the contents of the intestine can produce a number of enzymes similar to hydrolases – lipases, phosphatases, glycosidases, proteases, and others [21].

As a result of a series of experiments, it was found that the enzymatic activity of the microorganisms (Table 1) is more reliably determined at cell concentration from $1 \times 10^8$ to $6 \times 10^8$ in 1 ml, in average $A = 1.49 \mu \text{mol Tyr}/(\text{g}^{-1} \times \text{min}^{-1})$.

| Suspensions $n \times 10^8$ | Bacterial cultures studied |
|---------------------------|---------------------------|
|                          | Escherichia col K-12      | Pseudomonas           | Pseudomonas           | Proteus         |
| Strain                   | Bacteria of the genus     | Bacteria of the genus | Bacteria of the genus |
|                          | *Escherichia coli* K-12   | Pseudomonas           | Pseudomonas           | Proteus         |

Table 1. Dependence of proteolytic activity of enzyme preparations on the number of bacterial cells in suspension.

Figure 1. The qualitative composition of intestinal microbiota of rainbow trout.
The maximum activity of the selected proteases is observed in bacterial cell concentration $1.5 \times 10^8$ cells/ml. Most likely, the higher the concentration of bacterial cells in suspension, the higher the percentage (%) of their survival in the process of disintegration.

At the same time, high turbidity of the bacterial suspension solution complicates determining the enzymatic activity in the spectrophotometer.

It is important to mention that the proteolytic activity in the enzyme preparation from bacteria of the genus *Pseudomonas* spp. (1.7 mkmolTYR/($g^{-1} \times min^{-1}$)), is higher than in the enzyme preparation of bacteria *E. coli* K-12 F+StrR (KS-507) (1.6 mkmolTYR/($g^{-1} \times min^{-1}$)).

The results obtained for proteolytic activity correlate to the literature data [22]. Proteolitic activity of the bacteria of the intestine content of different species of fish is in the range of 2-4 µmol($g^{-1} \times min^{-1}$).

The following results were obtained accounting for the formation of antibiotic-resistant bacteria in vitro. Cultures of Pseudomonads, appeared to be sensitive to ciprofloxacin, gentamycin, doxycycline, intermediate sensitivity was shown towards chloramphenicol, tetracycline, clarithromycin, ampicillin. The highest sensitivity to ciprofloxacin was revealed, so this antibiotic could be effective in curing the infectious diseases of aquatic organisms. The isolated strains of bacteria of the genus *Pseudomonas* spp. showing resistance to cefotaxime was determined. This antibiotic is used when working with manufacturers of aquaculture broodstock after obtaining gametes under the artificial conditions to prevent infectious diseases and death of aquatic organisms. The data obtained in the study of antibiogramme of the determined isolates of *Pseudomonas* spp. are presented in table 2.

**Table 2.** Interpretation of diameter values of growth inhibition zones of bacteria of the genus *Pseudomonas* spp. in determining the sensitivity of bacteria to antimicrobials

| Antimicrobials in disk | Diameter of growth inhibition zones (mm) |
|------------------------|----------------------------------------|
|                        | resistant | intermediate | sensitive |
| ofloxacin               |           |             |           |
| ciprofloxacin           |           |             |           |
| ampicillin              |           |             |           |
| cefotaxime              |           |             |           |
| doxycycline             |           |             |           |
| gentamycin              |           |             |           |
| tetracycline            |           |             |           |
| laevomycetin            |           |             |           |
| clarithromycin          |           |             |           |

Thus, knowledge of the resistance of the detected microbial association on a fish farm is important for developing a strategy for antibiotic therapy, which should be based on the results of a microbiological study.

The importance of examining the microbial community of fish intestines becomes most evident due to the studies of symbiotic digestion in mammals. Using a combination of methodological approaches,
including those that most accurately reflect the activity of bacterial digestive enzymes, allows to obtain reliable data concerning the diversity of micro-communities and their interaction with the host organism.

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