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Heterotrophic microbiota of intestinal mucosa in *Parasalmo mykiss* (Walbaum, 1792): Age-related differences

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Abstract:
Intestinal microbiome of commercial aquatic species is an important fish farming factor that prevents or reduces economic losses. Heterotrophic bacteria that inhabit intestinal mucosa are involved in digestion, vitamin synthesis, immune modulation, and resistance to pathogens. Age-related changes in the composition of heterotrophic bacterial flora affect health status, nutrient absorption efficiency, and growth rate during ontogenesis. Most studies focus on the luminal microbiota, while the mucosal layer remains understudied despite its reliable impact on the immune system. The current lack of data on the age-related bacterial dynamics limits the development of age-specific diet strategies and disease prevention. This article presents data on the correlation between the age of rainbow trout (*Parasalmo mykiss* (Walbaum, 1792)) and the composition of cultured heterotrophic microflora.

The research featured 40 fish aged from one to two years and grown on fish farms in the Republic of Karelia, Russia. The bacteria isolated from their intestinal mucosa were identified using standard microbiological methods. The analysis involved the morphotype and biochemical activity, as well as the tinctorial and cultural characteristics of the isolates. The species identification relied on the MALDI-TOF technology.

The indices of dominance (Simpson, Berger–Parker), evenness (Pielou), diversity (Shannon), and richness (Margalef, Menhinick) made it possible to reveal that the heterotrophic component of the rainbow trout intestinal bacteria was a stable microbial community with a predominance of *Bacillaceae* and *Enterobacteriacae* enterobacteria, as well as Gram-negative non-fermentative bacteria. The index values corresponded to moderate α-diversity, which is typical of natural communities that tend to combine several abundant species with some rare taxa.

These results may help develop a scientific system for managing the gastrointestinal microflora of commercial fish to improve their health and productivity.

Keywords: Aquaculture, bacterial flora, biodiversity, heterotrophs, intestinal microflora, rainbow trout, *Salmonidae*, cage culture

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INTRODUCTION

Fish is very sensitive to abiotic and biotic environmental factors associated with modern high-productivity fish farming. The rainbow trout (*Parasalmo mykiss* (Walbaum, 1792)) possesses high commercial value and unique biological characteristics that make it one of the most popular industrial fish species. It grows rapidly even under intensive aquafarming conditions [1, 2]. Considering the increasing demand in fish production, the physiology of aquaculture species has become a relevant research issue [3].

Intestinal microflora is a marker of physiological and metabolic changes. The intestinal biotope microflora, or enteral microflora, is responsible for nonspecific stimulation of immunocompetent cells and tissues. As a result, it exercises both direct and indirect effects on symbiotic digestion [4], immune development, and resistance to infectious and somatic diseases [5].

The intestinal microflora of fish is known to depend on the aquatic environment and the host genotype. The epithelial surfaces of fish guts are densely colonized by

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microorganisms that demonstrate a wide variety of mutual relationships with the host [6]. Fish intestinal microflora includes two groups of microorganisms. The first group is represented by allochthonous, or transient microflora that enters the host organism with water and food, i. e., the microbiota of gut contents. The second group includes autochthonous, or indigenous microbiota that lines the surface of the intestinal mucosa and is to be found at all stages of ontogenesis [7, 8].

Fish intestinal microflora includes *Acinetobacter* spp. [9], *Aeromonas* spp. [10], *Bacillus* spp. [11], *Bacterium* spp. [12], *Clostridium* spp., *Enterobacter* spp., *Flavobacterium* [13], *Micrococcus* spp. [10, 14], *Lactobacillus* spp. [15], *Pseudomonas* spp. [10], *Proteus* spp. [11], etc. Opportunistic anaerobic and aerobic bacteria dominate the intestinal microflora of freshwater fish [16].

The nutrition of intestinal microflora depends on the diet of the particular fish. Proteolytic bacteria are the most frequent while amylolytic groups are present only if the fish has plant components in the diet [17]. Fish intestinal microflora is also affected by indigestible food ingredients. Prebiotics stimulate the activity of enteric microflora [18] whereas probiotics contain live cultures of microorganisms that colonize the digestive tract [19].

Minich *et al*. [20] studied the environmental and biological factors that shape the microbiome of the intestinal mucosa in fish. They established that the composition of this microbiome changes with age. In larvae, it was simple, unreliable, and dependent on the environment. In adults, it was functionally specialized. At the embryonic stage, *β*-proteobacteria (*Janthinobacterium*, *Rhodoferax*) were dominant. As the intestine develops, fish turns to exogenous nutrition, especially in artificial feeds. The microbiome composition becomes more complex and stable [21]. In larvae and juveniles, it almost totally depends on the environment, especially on the microflora of water [22] and feed [23]. Domineering opportunistic bacteria involve species of *Aeromonas*, *Shewanella*, *Pseudomonas*, *Vibrio*, and *Flavobacterium* dominate. The intestinal mucosa is thin, making it difficult to clearly distinguish between the mucosal and luminal flora [23–25].

Adult fish develops a species-specific, or core microbiome. The composition of the mucosal flora starts to depend more on the host's diet and physiology than on the environmental microflora [26]. The intestinal mucosa of adult fish contains *Proteobacteria* (*Aeromonadaceae*, *Vibrionaceae*), *Firmicutes* (*Lactobacillus*, *Bacillus*, *Clostridium*), and *Bacteroidetes* (*Bacteroides*, *Flavobacterium*) [14, 27].

In adult fish, symbionts are important for carbohydrate hydrolysis. For example, Bacteroidetes produce carbohydrases that break down plant polysaccharides. They also stimulate the immune response. For example, *Lactobacilli* provide competitive exclusion of pathogens by synthesizing adhesins and antimicrobial peptides [14].

Piazzon *et al*. [28] analyzed the intestinal microflora of one-, two-, and 4 y. o. sea bream (*Sparus aurata*). Regardless of age, the fish microbiota was dominated by three phyla, namely *Actinomycetota, Firmicutes*, and *Proteobacteria*. However, *Actinomycetota* exceeded *Firmicutes* and *Proteobacteria* in 2 y. o. sea bream, and *Proteobacteria* predominated in 4 y. o. bream. All age groups were poor in *Firmicutes*.

Merrifield *et al*. [29] performed a pioneering study of the composition and diversity of microbiota associated with the intestinal mucosa of adult rainbow trout, not with the entire gut contents. *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, and *Fusobacteria* were the most common phyla.

Despite ample knowledge about the intestinal microflora of teleost fish, little is known about the heterotrophic microflora of intestinal mucosa and its correlation with age. Age-related changes in the heterotrophic bacterial flora can affect health, nutrient absorption, and growth rate, which is especially important at some stages of ontogenesis. Most studies focus on the luminal microbiota while the mucosal layer remains mostly understudied, in spite of its connection with the immune system. The lack of data on the age-related bacterial flora dynamics limits the development of age-specific diet strategies and disease prevention. This study describes the correlation between the age and the content of heterotrophic bacterial flora in *P. mykiss*.

STUDY OBJECTS AND METHODS

The research featured cultured heterotrophic bacteria from the parietal or mucosal layer of the intestine of the rainbow trout (*Parasalmo mykiss* (Walbaum, 1792), Fig. 1). *P. mykiss* belongs to the salmon family Salmonidae, genus *Salmo Linne*.

The samples were obtained from 40 fish at the age of one year (n = 10; 292.5 ± 20.2 g; 28.0 ± 1 cm), two years (n = 10; 740.8 ± 13.7 g; 37.7 ± 0.4 cm), three years (n = 10; 1,203.2 ± 33.7 g; 40.6 ± 0.5 cm), and four years (n = 10; 4,262.3 ± 95.3 g; 65.8 ± 1.3 cm).

The rainbow trout were kept in cages from an early stage, under the same conditions. They received Dibaq Solution trout feed (Spain; Table 1).

The fish were sampled from May to July 2023 at a commercial trout farm on the Lake Ladoga, Karelia. During the study period, the water temperature in the cages ranged from 16.3 to 17.1°C, the pH was between 6.1 and 6.7, and the oxygen concentration ranged from 8.9 to 10.2 mg/L. The studies followed the international ethical standards (European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes; Order No. 755 of the USSR Ministry of Health, August 12, 1977: Organizational Forms of Work with Experimental Animals). The ethical approval was obtained from the Animal Research Ethics Committee at Petrozavodsk State University (No. 274, May 7, 2020). After extracting the fish from the cages, we anesthetized the specimens with clove oil (*Caryophylli floris aetheroleum*) as recommended by Hamackova *et al*. [30].



Figure 1 Rainbow trout (*Parasalmo mykiss* (Walbaum, 1792))

Table 1 Dibaq Solution (Spain) trout feed

Granule diameter, mm	2.0	3.5	5.0	7.0	9.0
Crude protein, %	45.0	42.0	42.0	42.0	42.0
Crude fat, %	24.0	25.0	25.0	25.0	25.0
Fiber, %	1.5	1.8	1.8	1.8	1.8
Ash, %	8.0	8.0	7.5	7.5	7.5
Carbohydrates, %	11.8	14.0	14.0	14.0	14.0
Moisture content, %	8.5	8.5	8.5	8.5	8.5
Calcium, %	1.6	1.7	1.7	1.7	1.7
Phosphorus, %	1.2	1.2	1.2	1.2	1.2

The standard microbiological analysis methods presupposed isolating pure bacterial cultures from the trout intestinal microflora, followed by the study of their phenotypic properties. The sampling was performed 36 h after the last feeding to empty the intestine and minimize the effect of transient microbiota.

The isolation was performed under strict aseptic conditions. The left side of the fish was cleaned of mucus and descaled, the pelvic and pectoral fins were removed, and flamed with an alcohol swab. After that, we excised the abdominal wall with sterile scissors performing a crescent-shaped incision from the anus to the operculum. The intestine was removed through the incision near the pseudodiaphragm and the anus.

After collecting the parietal microflora from the isolated intestine, we placed it in sterile tubes with buffered peptone water for non-selective bacterial accumulation, including sublethally inhibited enterobacteria. We applied the differentiated cultivation approach to isolate the heterotrophic bacterial flora from the intestinal mucosa. Psychrotrophic bacteria are associated with aqueous environment. To identify them, we incubated the cultures at 4 and 15°C for 7–14 days in nutrient media, namely buffered peptone broth and R2A agar. Mesophilic forms are associated with food and intestinal epithelium. To identify them, we incubated the material at 25 and 30°C for 24–72 h in standard media (MPA, Endo, MacConkey, etc.).

The resulting enrichment cultures underwent identification based on the phenotypic characteristics in Bergey's Manual of Determinative Bacteriology [31]. The main phenotypic markers were as follows: morphotype; tinctorial and cultural characteristics; and

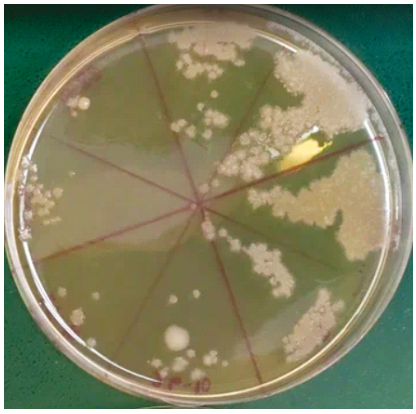

Figure 2 Bacterial cultures prepared for MALDI-TOF identification

biochemical activity (catalase, cytochrome oxidase, ammonification, and glucose metabolism). We used commercial reagent kits (Micro-Katalase, Micro-Cytochrome Oxidase, Research Center for Pharmacotherapy) to determine catalase and cytochrome oxidase. To assess protein ammonification and glucose metabolism, we used fish meal hydrolysate and standard nutrient media with carbohydrates and polyhydric alcohols.

To identify the dominant bacteria, we appealed to the method of Matrix-Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) on a VITEK MS mass spectrometer (Biomerieux, France). The MALDI-TOF technology compares ribosomal proteins obtained from intact microbial cells with a database of species-specific reference microbial proteins [32]. Before the analysis, the bacterial samples were cultured on agar nutrient medium for 18 h to obtain single colonies of ≥ 3 mm in diameter (Fig. 2).

Using a sterile bacteriological loop, we collected a small microbiological sample from each isolated colony using the direct smear/on-plate method and put it into a well of a plastic VITEK MS-DS slide. To obtain reliable results, each sample was distributed across at least three wells. To extract proteins (denaturation and lysis of cells), 2 µL of 70% formic acid was added to the sample, where it was allowed to dry at room temperature for 3–5 min. After drying the biomass of the isolate and formic acid, we added 2 µL of matrix.

It consisted of α-cyano-4-hydroxycinnamic acid in an aqueous solution of acetonitrile and trifluoroacetic acid, where a ratio of water, acetonitrile, and trifluoroacetic acid was 19:20:1. The resulting reaction mix was air-dried at room temperature for 3–5 min. The slide was then placed in the MALDI-TOF chamber of the mass spectrometer and exposed to several laser pulses. The resulting spectra were correlated with the data from the bioMerieux library using the proprietary bioMerieux algorithm, which generates microorganism identification. The instrument calibration involved the strain of *Escherichia coli* ATCC® 8739TM.

To study the species structure, we calculated the diversity-related indices of Simpson (1-D), Shannon (H′), Menhinick (D_{Mn}), and Margalef (D_{Mg}), as well as Pielou's evenness index (J′) [33]. To assess the dominance and biodiversity, we additionally calculated the Berger–Parker index [34]. The obtained data were analyzed using standard methods for bacterial count and statistical processing. The Microsoft Office Excel suite was used to work with spreadsheets.

RESULTS AND DISCUSSION

The heterotrophic microflora of the intestinal mucosa of *Parasalmo mykiss* (Walbaum, 1792) aged 1–4 y. o. belonged to seven phyla, i. e., *Actinomycetota, Bacillota, Bacteroidetes, Bacteroidota, Fusobacteriota, Proteobacteria,* and *Pseudomonadota*. We identified a total of 39 bacterial species belonging to 34 genera, i. e., *Arthrobacter, Micrococcus, Microbacterium, Propionibacterium, Bacillus, Carnobacterium, Lactobacillus, Listeria, Lysinibacillus, Eubacterium, Enterococcus, Lysinibacillus, Staphylococcus, Streptococcus, Bacteroidetes, Cytophaga, Flavobacterium, Sporocytophaga, Fusobacterium, Alcaligenes, Aeromonas, Acinetobacter, Hafnia, Citrobacter, Enterobacter, Escherichia, Klebsiella, Moraxella, Proteus, Pseudomonas, Pseudomonas, Pseudomonas, Serratia, Yersinia* (Table 2). Such low diversity is typical of fish microflora: it can be explained by the lack of dietary diversity and the limited habitat [35]. Table 3 summarizes the microflora biodiversity related to the Dibaq Solution trout feed.

Heterotrophic bacteria are present in the intestines of almost all fish species. During the period of active feeding, their count can reach 6×10^7 CFU/mL [35, 36]. In our study, the most representative enterobacteria belonged to the families *Bacillaceae* and *Enterobacteriacae*, as well as to the group of Gram-negative non-fermenting bacteria. Table 4 shows the proportion of isolated bacteria in the structure of the Bacillaceae family. A total of 578 strains were assigned to the *Bacillaceae family.* Three species belonged to the genus *Bacillus* (*Bacillus altitudinis, Bacillus firmus, Bacillus licheniformis*), and one species belonged to the genus *Lysinibacillus* (*Lysinibacillus fusiformis*). The intestinal mucous layer of the 2 y. o. trout demonstrated the highest abundance of bacilli, i. e., *B. firmus* (16.5%) and *B. licheniformis* (14.7%). *L. fusiformis* proved to be rare; it was isolated in pure culture only from the intestinal microflora of the 1 y. o. trout, and the proportion of this species stayed below 4.2% of the isolated bacteria within the family.

A total of 906 strains belonged to the Enterobacteriacae family. The genera *Escherichia* (*Escherichia coli*) and *Klebsiella* (*Klebsiella oxytoca*) were identified to the species level while *Citrobacter* spp., *Enterobacter* spp., *Moraxella* spp., and *Proteus* spp. were specified to the genus level (Table 5).

The older trout contained less *Citrobacter* spp., *Enterobacter* spp., and *Klebsiella* spp. in the intestinal parietal microflora. The count of *Citrobacter* in the 4 y. o. trout dropped by 5.5 times compared to the 3 y. o. The count of *Enterobacter* in 1 and 2 y. o. trout varied from 5.2 to 6.8%; in 3 and 4 y. o., it was as low as 2.7–4.1%. The highest share of *Klebsiella* belonged to the 1 y. o. (7.3%) whereas the lowest one (1.4%) was found in the 4 y. o. fish.

Such environmental indicators as *E. coli* were found in minimal quantities. The share of *Escherichia* was ≤ 0.4 and ≤ 1.9% in the 1 and 4 y. o. trout, respectively. Our results were consistent with the data published in [37, 38] and may indicate a disturbance in the ecological state of the fishery reservoir, which usually leads to the predominance of opportunistic enterobacteria species over indicator species.

Gram-negative non-fermentative bacteria were dominated by four genera, namely *Alcaligenes, Acinetobacter, Flavobacterium*, and *Pseudomonas*. A total of 810 strains were classified as Gram-negative non-fermentative bacteria. *Pseudomonas* dominated for all ages of trout. However, *Pseudomonas aeruginosa*, a pathogen for animals and humans, was not recorded in the younger trout and was isolated only from the 3 and 4 y. o. specimens, where its proportion in the total Gram-negative non-fermentative bacteria reached 4.6 and 8.4%, respectively. *Alcaligenes* spp. contaminated the younger trout, where their proportion among other Gram-negative non-fermentative species was 2.4%. The genus *Acinetobacter* spp. was represented by *Acinetobacter* calcoaceticus. The highest count of *A. calcoaceticus* belonged to the 1 and 2 y. o. trout (2.5 and 4.3%, respectively). This fact may be associated with low non-specific resistance in young trout. Conversely, intestinal contamination with *Flavobacteria* increased with age from 0.3% (1 y. o.) to 1.8% (4 y. o.). Bacteria of this genus mainly belong to the transient microflora, but they can provoke epizootics under stressful conditions, thus increasing the susceptibility of aquatic organisms to infections and enhancing the adaptability of bacteria to environmental factors [39–41].

The prevalence of *Bacillaceae, Enterobacteriacae*, and Gram-negative non-fermenting bacteria is a sensitive indicator of fish growing conditions. The negative environmental effect may exceed the adaptive capabilities of fish organism, provoke infectious diseases caused

Table 2 Biodiversity of intestinal microflora in rainbow trout (Parasalmo mykiss (Walbaum, 1792))

Domain	Phylum	Class	Order	Family	Genus	Species
Bacteria	*Proteobacteria*	*γ Proteobacteria*	*Aeromonadales*	*Aeromonadaceae*	*Aeromonas*	*Aeromonas hydrophila*
	Proteobacteria	*β Proteobacteria*	*Burkholderiales*	**Alcaligenaceae*	*Alcaligenes*	*Alcaligenes* spp.
	Proteobacteria	*γ Proteobacteria*	*Pseudomonadales*	**Moraxellaceae*	*Acinetobacter*	*Acinetobacter calcoaceticus*
	Actinomycetota	*Actinomycetia*	*Micrococcales*	*Micrococcaceae*	*Arthrobacter*	*Arthrobacter* spp.
	Bacillota	*Bacilli*	*Bacillales*	**Bacillaceae*	*Bacillus*	*Bacillus spp.*
	Bacillota	*Bacilli*	*Bacillales*	**Bacillaceae*	*Bacillus*	*Bacillus altidudins*
	Bacillota	*Bacilli*	*Bacillales*	**Bacillaceae*	*Bacillus*	*Bacillus firmus*
	Bacillota	*Bacilli*	*Bacillales*	**Bacillaceae*	*Bacillus*	*Bacillus licheniformis*
	Bacillota	*Bacilli*	*Bacillales*	*Caryophanaceae*	*Lysinibacillus*	*Lysinibacillus fusiformis*
	Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Bacteroidaceae*	*Bacteroidetes*	*Bacteroidetes* spp.
	Bacillota	*Bacilli*	*Lactobacillales*	*Carnobacteriaceae*	*Carnobacterium*	*Carnobacterium* spp.
	Pseudomonadota	*γ Proteobacteria*	*Enterobacterales*	**Enterobacteriacae*	*Citrobacter*	*Citrobacter freundii*
	Bacteroidota	*Cytophagia*	*Cytophagales*	*Cytophagaceae*	*Cytophaga*	*Cytophaga psychrophila*
	Bacteroidota	*Flavobacteria*	*Flavobacterialesales*	*Flavobacteriaceae*	*Flavobacterium*	*Flavobacterium columnare*
	Bacteroidota	*Cytophagia*	*Cytophagales*	*Flexibacteraceae*	*Flexibacter*	*Flexibacter* spp.
	Fusobacteriota	*Fusobacteria*	*Fusobacteriales*	*Fusobacteriaceae*	*Fusobacterium*	*Fusobacterium* spp.
	Pseudomonadota	*γ Proteobacteria*	*Enterobacterales*	*Hafniacae*	*Hafnia*	*Hafnia* spp.
	Bacillota	*Bacilli*	*Lactobacillales*	*Lactobacillaceae*	*Lactobacillus*	*Lactobacillus* spp.
	Bacillota	*Bacilli*	*Lactobacillales*	*Streptococcaceae*	*Lactobacillus*	*Lactococcus plantarum*
	Bacillota	*Bacilli*	*Bacillales*	*Listeriaceae*	*Listeria*	*Listeria* spp.
	Bacillota	*Clostridia*	*Clostridiales*	*Eubacteriaceae*	*Eubacterium*	*Eubacterium* spp.
	Bacillota	*Bacilli*	*Lactobacillales*	*Enterococcaceae*	*Enterococcus*	*Enterococcus* spp.
	Pseudomonadota	*γ Proteobacteria*	*Enterobacterales*	**Enterobacteriacae*	*Enterobacter*	*Enterobacter* spp.
	Pseudomonadota	*γ Proteobacteria*	*Enterobacterales*	**Enterobacteriacae*	*Escherichia*	*Escherichia coli*
	Pseudomonadota	*γ Proteobacteria*	*Enterobacterales*	**Enterobacteriacae*	*Klebsiella*	*Klebsiella oxytoca*
	Bacillota	*Bacilli*	*Bacillales*	**Bacillaceae*	*Lysinibacillus*	*Lysinibacillus fusiformis*
	Actinomycetota	*Actinomycetia*	*Micrococcales*	*Micrococcaceae*	*Micrococcus*	*Micrococcus* spp.
	Actinomycetota	*Actinomycetia*	*Micrococcales*	*Microbacteriaceae*	*Microbacterium*	*Microbacterium* spp.
	Actinomycetota	*Actinomycetia*	*Micrococcales*	*Microbacteriaceae*	*Microbacterium*	*Microbacterium oxidans*
	Pseudomonadota	*γ Proteobacteria*	*Enterobacterales*	**Enterobacteriacae*	*Moraxella*	*Moraxella* spp.
	Actinomycetota	*Actinomycetia*	*Propionibacteriales*	*Propionibacteriaceae*	*Propionibacterium*	*Propionibacterium* spp.
	Pseudomonadota	*γ Proteobacteria*	*Enterobacterales*	**Enterobacteriacae*	*Proteus*	*Proteus mirabilis*
	Pseudomonadota	*γ Proteobacteria*	*Pseudomonadales*	**Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas aeruginosa*
	Pseudomonadota	*γ Proteobacteria*	*Pseudomonadales*	**Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas fluorescens*
	Pseudomonadota	*γ Proteobacteria*	*Enterobacterales*	*Yersiniaceae*	*Serratia*	*Serratia* spp.
	Bacteroidota	*Cytophagia*	*Cytophagales*	*Cytophagaceae*	*Sporocytophaga*	*Sporocytophaga* spp.
	Bacillota	*Bacilli*	*Bacillales*	*Staphylococcaceae*	*Staphylococcus*	*Staphylococcus* spp.
	Bacillota	*Bacilli*	*Lactobacillales*	*Streptococcaceae*	*Streptococcus*	*Streptococcus* spp.
	Pseudomonadota	*γ Proteobacteria*	*Enterobacterales*	*Yersiniaceae*	*Yersinia*	*Yersinia ruskeri*

* – dominant taxa

Table 3 Microflora biodiversity of Dibaq Solution trout feed (Spain)

Domain	Phylum	Class	Order	Family	Genus	Species
Bacteria	*Proteobacteria*	*γ Proteobacteria*	*Aeromonadales*	*Aeromonadaceae*	*Aeromonas*	*Aeromonas hydrophila*
	Proteobacteria	*β Proteobacteria*	*Burkholderiales*	**Alcaligenaceae*	*Alcaligenes*	*Alcaligenes* spp.
	Proteobacteria	*γ Proteobacteria*	*Pseudomonadales*	**Moraxellaceae*	*Acinetobacter*	*Acinetobacter calcoaceticus*
	Actinomycetota	*Actinomycetia*	*Micrococcales*	*Micrococcaceae*	*Arthrobacter*	*Arthrobacter* spp.
	Bacillota	*Bacilli*	*Bacillales*	**Bacillaceae*	*Bacillus*	*Bacillus* spp.
	Bacillota	*Bacilli*	*Bacillales*	**Bacillaceae*	*Bacillus*	*Bacillus amyloliquefaciens*
	Bacillota	*Bacilli*	*Bacillales*	**Bacillaceae*	*Bacillus*	*Bacillus subtilis*
	Bacillota	*Bacilli*	*Bacillales*	**Bacillaceae*	*Bacillus*	*Bacillus licheniformis*
	Bacillota	*Bacilli*	*Bacillales*	*Caryophanaceae*	*Lysinibacillus*	*Lysinibacillus fusiformis*
	Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Bacteroidaceae*	*Bacteroidetes*	*Bacteroidetes* spp.
	Bacillota	*Bacilli*	*Lactobacillales*	*Carnobacteriaceae*	*Carnobacterium*	*Carnobacterium* spp.
	Pseudomonadota	*γ Proteobacteria*	*Enterobacterales*	**Enterobacteriacae*	*Citrobacter*	*Citrobacter freundii*
	Bacteroidota	*Cytophagia*	*Cytophagales*	*Cytophagaceae*	*Cytophaga*	*Cytophaga psychrophila*
	Bacteroidota	*Flavobacteria*	*Flavobacterialesales*	*Flavobacteriaceae*	*Flavobacterium*	*Flavobacterium columnare*
	Bacteroidota	*Cytophagia*	*Cytophagales*	*Flexibacteraceae*	*Flexibacter*	*Flexibacter* spp.
	Fusobacteriota	*Fusobacteria*	*Fusobacteriales*	*Fusobacteriaceae*	*Fusobacterium*	*Fusobacterium* spp.
	Bacillota	*Bacilli*	*Lactobacillales*	*Lactobacillaceae*	*Lactobacillus*	*Lactobacillus rhamnosus*
	Bacillota	*Bacilli*	*Lactobacillales*	*Streptococcaceae*	*Lactobacillus*	*Lactococcus plantarum*
	Bacillota	*Bacilli*	*Lactobacillales*	*Streptococcaceae*	*Lactobacillus*	*Lactococcus plantarum*
	Bacillota	*Bacilli*	*Bacillales*	*Listeriaceae*	*Listeria*	*Listeria* spp.
	Bacillota	*Clostridia*	*Clostridiales*	*Eubacteriaceae*	*Eubacterium*	*Eubacterium* spp.
	Bacillota	*Bacilli*	*Lactobacillales*	*Enterococcaceae*	*Enterococcus*	*Enterococcus* spp.
	Pseudomonadota	*γ Proteobacteria*	*Enterobacterales*	**Enterobacteriacae*	*Enterobacter*	*Enterococcus faecium*
	Pseudomonadota	*γ Proteobacteria*	*Enterobacterales*	**Enterobacteriacae*	*Escherichia*	*Escherichia coli*
	Pseudomonadota	*γ Proteobacteria*	*Enterobacterales*	**Enterobacteriacae*	*Klebsiella*	*Klebsiella oxytoca*
	Bacillota	*Bacilli*	*Bacillales*	**Bacillaceae*	*Lysinibacillus*	*Lysinibacillus fusiformis*
	Actinomycetota	*Actinomycetia*	*Micrococcales*	*Micrococcaceae*	*Micrococcus*	*Micrococcus* spp.
	Actinomycetota	*Actinomycetia*	*Micrococcales*	*Microbacteriaceae*	*Microbacterium*	*Microbacterium* spp.
	Actinomycetota	*Actinomycetia*	*Micrococcales*	*Microbacteriaceae*	*Microbacterium*	*Microbacterium oxidans*
	Pseudomonadota	*γ Proteobacteria*	*Enterobacterales*	**Enterobacteriacae*	*Moraxella*	*Moraxella* spp.
	Actinomycetota	*Actinomycetia*	*Propionibacteriales*	*Propionibacteriaceae*	*Propionibacterium*	*Propionibacterium* spp.
	Pseudomonadota	*γ Proteobacteria*	*Enterobacterales*	**Enterobacteriacae*	*Proteus*	*Proteus mirabilis*
	Pseudomonadota	*γ Proteobacteria*	*Pseudomonadales*	**Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas aeruginosa*
	Pseudomonadota	*γ Proteobacteria*	*Pseudomonadales*	**Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas fluorescens*
	Pseudomonadota	*γ Proteobacteria*	*Enterobacterales*	*Yersiniaceae*	*Serratia*	*Serratia* spp.
	Bacteroidota	*Cytophagia*	*Cytophagales*	*Cytophagaceae*	*Sporocytophaga*	*Sporocytophaga* spp.
	Bacillota	*Bacilli*	*Bacillales*	*Staphylococcaceae*	*Staphylococcus*	*Staphylococcus* spp.
	Bacillota	*Bacilli*	*Lactobacillales*	*Streptococcaceae*	*Streptococcus*	*Streptococcus* spp.
	Pseudomonadota	*γ Proteobacteria*	*Enterobacterales*	*Yersiniaceae*	*Yersinia*	*Yersinia ruskeri*

* – dominant taxa

Table 4 Bacillaceae family, %

Isolated bacteria	Age of *Parasalmo mykiss* (Walbaum, 1792), y. o.			
	1	2	3	4
Bacillus spp.	12.4 ± 0.3	13.9 ± 0.3	6.2 ± 0.2	7.9 ± 0.2
Bacillus altitudinis	0	3.9 ± 0.1	0	0
Bacillus firmus	0	16.5 ± 0.4	8.6 ± 0.2	9.4 ± 0.3
Bacillus licheniformis	13.4 ± 0.3	14.7 ± 0.3	5.1 ± 0.2	12.7 ± 0.4

Table 5 Enterobacteriaceae family, %

Isolated bacteria	Age of *Parasalmo mykiss* (Walbaum, 1792), y. o.			
	1	2	3	4
Citrobacter spp.	2.4 ± 0.1	3.2 ± 0.1	7.1 ± 0.4	1.3 ± 0.1
Enterobacter spp.	5.2 ± 0.2	6.8 ± 0.5	4.1 ± 0.2	2.7 ± 0.1
Escherichia coli	0.40 ± 0.01	0.60 ± 0.01	1.70 ± 0.10	1.90 ± 0.01
Klebsiella oxytoca	7.3 ± 0.4	2.8 ± 0.1	3.2 ± 0.1	1.4 ± 0.1
Moraxella spp.	0	0	3.6 ± 0.1	4.4 ± 0.2
Proteus spp.	3.4 ± 0.1	8.5 ± 0.7	5.2 ± 0.2	5.6 ± 0.2

Table 6 Gram-negative non-fermenting bacteria, %

Isolated bacteria	Age of *Parasalmo mykiss* (Walbaum, 1792), y. o.			
	1	2	3	4
Alcaligenes spp.	2.4 ± 0.1	1.2 ± 0.1	1.7 ± 0.1	1.2 ± 0.1
Acinetobacter calcoaceticus	2.5 ± 0.1	4.3 ± 0.3	1.2 ± 0.1	1.4 ± 0.1
Flavobacterium columnare	0.30 ± 0.01	0.40 ± 0.01	1.40 ± 0.10	1.80 ± 0.10
Pseudomonas aeruginosa	0	0	4.6 ± 0.3	8.4 ± 0.5
Pseudomonas fluorescens	11.6 ± 0.6	7.1 ± 0.4	12.2 ± 0.6	11.6 ± 0.6

by opportunistic enterobacteria, and trigger reversible and irreversible pathologies of entire organ systems, which will eventually reduce the viability of the fish.

Figure 3 illustrates the inverse Simpson index, the Berger–Parker index, and the Pielou evenness index. The Simpson index varied from 0.06 to 0.07, indicating a low diversity of heterotrophic bacteria and a high dominance of one or several taxa. The alternative Berger–Parker dominance index of 0.90–0.97 confirmed the predominance of some species (Tables 4–6). These species included *B. lichenoformis* (1 and 4 y. o.), *B. firmus* (2 y. o.), and *Pseudomonas fluorescens* (3 y. o.). This change in closely related species may be associated with some minor changes in fish physiology, as well as with fluctuations in the feed composition. *P. fluorescens* became domineering in 3 y. o. trout, probably, as a result of water contamination with pseudomonads, some environmental factors (e. g., strain competition), or decreased immunity [42]. The biodiversity of species is not always obvious, so we calculated the Pielou index, which quantifies how equally species abundances are distributed in a community. It varied from 0.52 to 0.56, indicating a moderate degree of evenness.

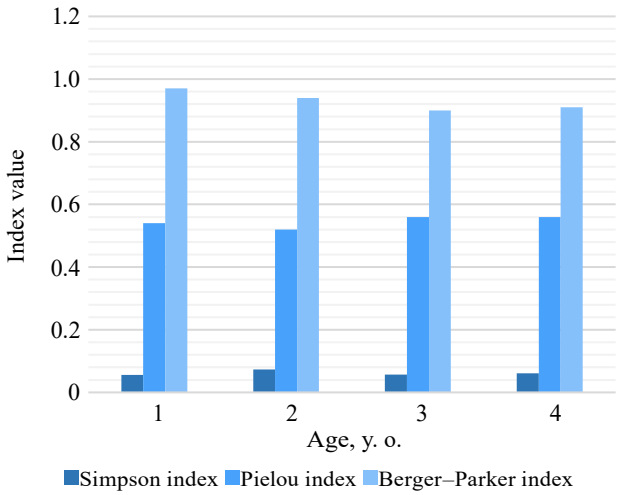

Figure 3 Dominance and evenness indices of heterotrophic bacteria

Figure 4 illustrates the species diversity and complexity. The Shannon index varied from 1.31 to 1.37. These results indicated a moderate α-diversity with very few domineering species. Few taxa were responsible for the colonization of potential niches. Moderate α-diversity is typical of many natural communities that combine several relatively abundant species with a number of rarer taxa [43, 44]. It denotes the presence of key functional groups of heterotrophic bacteria necessary for digestion and vitamin synthesis. It also indicates good resistance to moderate stresses, e. g., changes in diet or environment, due to the presence of reserve species. The lowest value was observed in the 2 y. o. trout (H' = 1.31) while the

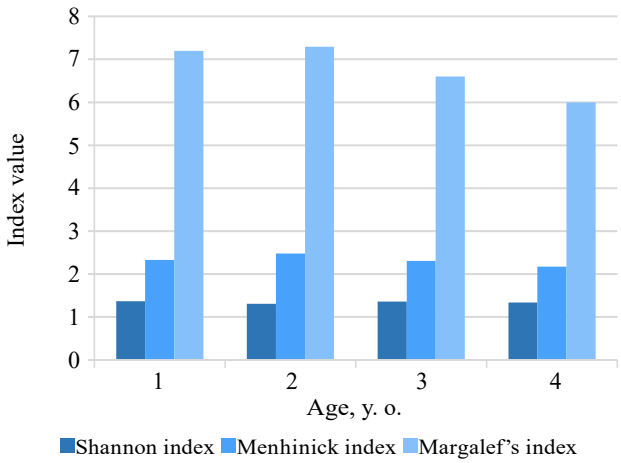

Figure 4 Values of the indices of species richness and complexity indices

highest was recorded in the 1 y. o. (H' = 1.37) and 4 y. o. fish (H' = 1.36). The amplitude of fluctuations between the maximum and the minimum was very small (0.06). The bacterial structure in terms of species diversity and evenness did not change radically after the first year of life and remained generally reliable over four years.

The Menhinick index (D_{Mn}) was necessary to describe the species richness. It was 2.33 (1 y. o.), 2.48 (2 y. o.), 2.31 (3 y. o.), and 2.17 (4 y. o.). The index revealed a clear trend: the species richness increased from year one to year two, when reached its maximum, and then consistently went down. Probably, the intestinal microbiome becomes more stable and specialized as the fish matures. As the Margalef richness index increases, species biodiversity also increases. The D_{Mg} was 7.2 (1 y. o.), 7.3 (2 y. o.), 6.6 (3 y. o.), and 6.0 (4 y. o.). Despite the initial slight increase (from year one to year two), the subsequent D_{Mg} values indicated a trend toward a lower species richness. The decrease may be related to dietary changes, immune system changes, or fish's adaptation to certain heterotrophic bacterial species in the intestinal mucosa.

CONCLUSION

The composition of heterotrophic bacteria in the intestinal mucosa of *Parasalmo mykiss* (Walbaum, 1792) aged 1–4 y. o. consisted of seven phyla, i. e., *Actinomycetota, Bacillota, Bacteroidetes, Bacteroidota, Fusobacteriota, Proteobacteria,* and *Pseudomonadota*. We identified 39 bacterial species belonging to 34 genera based on phenotypic characteristics and ribosomal proteins. The heterotrophic bacteria isolated and identified in pure culture represented a stable bacterial community. The observed changes affected the proportion of *Bacillota, Proteobacteria,* and *Pseudomonadota*, which dominated the intestinal microflora of rainbow trout across age groups. However, their proportion in the community varied depending on the age.

The rare species included *Lysinibacillus fusiformis* and *Bacillus altitudinis* (1 and 2 y. o. rainbow trout). *Pseudomonas aeruginosa* and some species of the genus *Moraxella* were found exclusively in the heterotrophic bacterial flora of older fish. The core of dominant phylotypes across all the age groups consisted of *Bacillaceae* and *Enterobacteriacae* enterobacteria, as well as Gram-negative nonfermenting bacteria (*Bacillus* spp., *Bacillus licheniformis*, *Citrobacter* spp., *Klebsiella oxytoca*, *Proteus* spp., *Alcaligenes* spp., *Acinetobacter calcoaceticus, Flavobacterium columnare, Pseudomonas fluorescens*).

The dominance (Simpson, Berger–Parker) and species richness (Shannon, Margalef, Menhinick) indices revealed the diversity of the heterotrophic bacteria. The structure of the bacterial community corresponded to moderate α-diversity and remained stable throughout four years of life. *B. licheniformis* gave its dominant position to *Bacillus firmus* between the first and second years of life, while *P. fluorescens* dominated in the third year.

These initial data on the correlation between the intestinal bacterial flora of *P. mykiss* and age are important for a better understanding of adaptive changes in trout, as well as the complex pathophysiological processes in the gastrointestinal tract caused by the adverse aquaculture conditions.

Specific results were as follows:

1. The intestinal mucosa of rainbow trout across four years of life was colonized by heterotrophic bacteria with moderate α-diversity, which did not change significantly with age (Shannon index H' = 1.31–1.37; Pielou evenness index J' = 0.52–0.56).

2. The microbial community demonstrated a high level of dominance (Berger–Parker index = 0.90–0.97), with the core microbiota across the age groups represented by *Bacillota, Pseudomonadota,* and *Bacteroidota.*

3. The age-related differences were manifested not in overall diversity changes, but in a shift in dominant species: *B. licheniformis* dominated in 1 and 4 y. o. fish; *Bacillus firmus* dominated in 2 y. o.; *P. fluorescens* dominated in 3 y. o. trout.

4. Such opportunistic pathogens as *Aeromonas hydrophila, Yersinia ruckeri, P. aeruginosa*, and *F. columnare* were present in the intestinal microbiota at all life stages, which indicated their constant supply from the external environment. Obviously, the sanitary conditions require stricter control.

5. The indicator species of *Escherichia coli* confirms that the intestinal microbiota was sustained by allochthonous (external) microbiota obtained from the feed and the aquatic environment rather than by a stable autochthonous resident community, which requires further study.

CONTRIBUTION

The authors were equally involved in the research and bear equal responsibility for any potential plagiarism.

CONFLICT OF INTEREST

The authors declared no conflict of interest regarding the publication of this article.

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