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The gut microbiota of farmed and wild brook trout (*Salvelinus fontinalis*): evaluation of feed-related differences using 16S rRNA gene metabarcoding

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Abstract: The gut microbiota has become a topic of increasing importance in various fields, including aquaculture. Several fish species have been the subject of investigations concerning the intestinal microbiota, comparing different variables including the intestine portions, the environment and diet. In this study, the microbiota of farmed and wild brook trout (*Salvelinus fontinalis*) was analysed, considering separately wall and content of the medial portion of the intestine. A total of 66 fish (age class 2+) were sampled, of which 46 wild and 20 farmed brook trout, along two different years. Microbiota data were obtained using a 16S metabarcoding approach by analysing the V3-V4 hypervariable regions of the 16S rRNA. Data showed that the core microbiota of these species is represented by *Proteobacteria* (*Alpha*- and *Gammaproteobacteria*), *Actinobacteria*, *Firmicutes* (*Bacilli* and *Clostridia*) and, only for farmed animals, *Fusobacteria*. The latter taxon is likely related to the fishmeal-based diet administered to farmed brook trout. Indeed, alpha and beta diversity analysis showed differences between wild and farmed fish. Finally, statistically significant differences in the microbiota composition were observed between intestinal wall and content in wild fish, while no differences were detected in reared animals. Our work represents the first study on the intestinal microbiota of brook trout, both for farmed and wild specimens. Future works might focus on the comparison of our data with those of other fish species and on the study of other portions of the brook trout intestine.

Keywords: intestinal microbiota; salmonids; brook trout; next generation sequencing; 16S rRNA.

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1. Introduction

The term "microbiota" indicates the microbial population that colonizes a certain body district or environment, while "microbiome" refers to the genetic heritage of a specific microbiota [1]. One of the most studied microbiotas is the gut one [2,3]. The intestinal microbiota of different animal species has also been studied, especially regarding mammals. An example is the work conducted by de Jonge and collaborators [4], who analysed the microbiotas of 54 different mammals, being able to cluster the data based on the different dietary habits and intestine morphology of the investigated species. Other studies have focused on species of zootechnical interest, such as cattle

[5,6], pigs [7,8], horses [9], sheep [10] and goats [11].

Given the growing importance of aquaculture for the animal-origin proteins production, several researchers have also begun to study the gut microbiota of aquatic organisms. Data on intestinal microbiota of economically important fish species are therefore available, including Nile tilapia (*Oreochromis niloticus*) [12], turbot (*Scophthalmus maximus*) [13], rainbow trout (*Oncorhynchus mykiss*) [14], Chinook salmon (*O. tshawytscha*) [15], Atlantic salmon (*Salmo salar*) [16], and common carp (*Cyprinus carpio*) [17]. Compared to mammals, the study of the intestinal microbiota of fish is more complex, as there is a greater number of variables that can influence its composition. In fact, in addition to the factors that have been extensively studied in mammals (e.g., species, diet, age), the aquatic environment plays a preponderant role in the composition of the microbial community constituting the fish gut microbiota [18-20]. Despite these difficulties, the study of intestinal microbiota of new fish species is desirable to increase the available knowledge regarding the composition and the influence of its modifying factors.

In this perspective, our study aimed at characterizing the intestinal core microbiota of the brook trout (*Salvelinus fontinalis*), a fish that can be found in Italy both farmed and in the environment as an invasive species [21]. This salmonid is a species of economic and environmental interest for several countries, including Italy. Regarding human consumption farming, the Italian data for 2021 reported a production of 850 tons, equivalent to a value of 3.65 million euros [22]. Brook trout is farmed not exclusively for food, but also for recreational fishing. This latter purpose leads to the release of brook trout in the natural environment as invasive species, with adverse ecological impact on the local ecosystem and fauna. Several studies have been conducted on this fish species, concerning distribution [23], production performance [24] and sanitary conditions [25,26]. Although several aspects have been investigated on this species, there are currently no studies concerning the intestinal microbiota according to our knowledge. Therefore, our work aimed to study the microbiota of *S. fontinalis* through next-generation sequencing (NGS) 16S metabarcoding, considering several variables: different sampling sites (natural environment and farm) and matrices (content and intestinal wall). Moreover, we selected wild and farmed fish to investigate the role of environmental factors, primarily the diet, in the microbiota composition.

2. Materials and Methods

2.1. Fish sampling

A total of 66 brook trout (46 wild fish, 20 farmed specimens) belonged to the age class 2+ were sampled in Piedmont region (North-Western Italy).

The 46 wild fishes were sampled from the Balma Lakes during summer 2019 and 2020. In particular, n=21 and n=25 specimens were captured on 4 August 2019 and 29 July 2020, respectively. Balma Lakes (Upper and Lower Balma) are located in the Cottian Alps at 2.101 m a.s.l. (Lower Lake Balma; 45°02′13.799″ N; 07°10′52″ E) and 2.213 m a.s.l. (Upper Lake Balma; 45°02′15.055″N; 07°10′27.724″ E). The lakes fall within the SAC IT1110006 Orsiera Rocciavré (Municipality of Coazze, Province of Turin, northwest Italy). The Upper Lake is S-shaped, with two sub-basins separated by a shallow mid-section. The lake perimeter is 774 m, with a 1.82 ha surface area and 2.77 m maximum depth. The lake is placed in a catchment core composed of ophiolite metamorphic bedrock and the landscape is dominated by rocky outcrops, ridges, and mountain walls. The Lower Lake is circular shaped with a perimeter equal to 414 m; the surface area is 1.21 ha, and the maximum depth is 6.42 m. The main catchment core has same composition described for the Upper Lake, and the landscape is dominated by same elements observed above, except for the meadow, that is absent near the Lower Lake. The small inlet is located at the western shore dividing into three small branches

before entering the lake. Although a true outlet is not evident, the Balma Creek originates from water filtration through the sediments at the eastern side of the basin. The most relevant anthropogenic impacts in the Balma Lakes area over the last four decades of the 20th century are represented by the long-distance airborne transport of pollutants from the urban areas in the plain, grazing activities, and fishing. Although the Balma Lakes were originally fishless, *Salvelinus fontinalis* was introduced for recreational fishing in the 1970s [21]. Fish sampling was performed following the standardized method for fish sampling in European lakes (EN 14757:2005) which requires a single session using both benthic and mesopelagic nets in relation to the lake type, depth, and surface. The benthic nets (length: 30 m; height: 1,5 m; total area: 45 m^2) were composed of 12 panels (length: 2,5 m) with a mesh size ranging from 5 to 55 mm. The mesopelagic nets (length: 27,5 m; height: 6 m; total area: 165 m^2) had one less panel than benthic nets (11 panels in total). The nets were placed according to the bathymetric profile of the lake at approximately 6 p.m. and recovered 12 hours later. Only fish belonged to the age class 2+ were selected and retrieved for the analyses.

Regarding farmed samples, 20 brook trout (age 2+) were sampled in October 2020 in a farm housing both this species and brown trout (*Salmo trutta*). The farm is located in a mountainous area (Cottian Alps) at about 900 m a.s.l. The water supply is represented by creek water (12 °C). Salmonids are reared at low density (25 kg/m^3) and fed twice days with commercial feed pellet (Premium, Skretting). Brook trout were captured using a landing net and euthanized using an overdose (170 mg kg^{-1}) of tricaine methanesulfonate (MS-222).

Then, the middle intestinal tract was harvested directly on the sampling site, using sterile scalpels and forceps. Gut contents were also collected by applying slight pressure on the intestinal wall to allow the contents to eject. Samples of intestinal wall and content were transported to the laboratory under refrigerated conditions and were stored at -80°C before further analyses.

Water temperature (°C), pH (unit of pH), conductivity (µS cm^{-1}) and dissolved oxygen (mg L^{-1}) were measured during fish sampling using portable probes (HI 9033 conductivity meter, HI 9125 pH/ORP meter, HI 9147 dissolved oxygen meter, Hanna Instruments Inc. Woonsocket, RI, USA). Three replicates were measured for each parameter.

2.2. DNA extraction

For DNA extraction, the QIAamp® PowerFecal® Pro DNA Kit (Qiagen, Germany) was used, according to the manufacturer instructions provided. A portion of 50 mg of each sample was transferred to homogenization tubes containing ceramic beads with 800 µl of CD1 lysis buffer and were subjected to homogenization using the MP Biomedicals™ FastPrep-24™ Classic Bead Beating Grinder and Lysis System (Fisher Scientific Italia, Italy) with 1 cycle of 40 sec at speed 10. A positive extraction control consisting of a ZymoBIOMICS Microbial Community Standards (Zymo Research, California, USA) and a negative extraction control (ultrapure water) were set up. The extracted DNA was immediately quantified by VivaSpec spectrophotometer (Sartorius Stedim Biotech, Germany) and Qubit™ 3 Fluorometer (ThermoFisher, Massachusetts, USA) using the dsDNA HS Assay Kit (ThermoFisher, Massachusetts, USA), and then stored at -20°C.

2.3. 16S Ribosomal RNA (16S rRNA) gene Metabarcoding

The samples were amplified using the 16S Metagenomic Sequencing Library Preparation protocol (Illumina, California, USA). The primers 341FB (5′-CCTACGGGNGGCWGCAG-3′) and 806RB (5′-GACTACHVGGGTATCTAATCC-3′) targeting the hypervariable V3-V4 regions of the 16S rRNA gene were used following the manufacturer protocol. Amplicon PCR (final volume: 25 µl) was set up using 12,5 µl of NEBNext® Q5® Hot Start HiFi2X Master Mix (New England BioLabs, Massachusetts,

USA), 1,25 µl of each 10 µM primer and 10 ng of DNA. The thermal profile was the following: 98°C x 30s; 40 cycles at 98°C x 10s, 55°C x 30s, 72°C x 30s; final extension at 72°C x 2m. The PCR products were visualized on a 2% agarose gel to verify the successful amplification of the target. Samples with the amplicon of interest (430 bp) were purified using magnetic beads Agencourt AMPure XP (Beckman Coulter, California, USA).

Index PCR was performed using the Nextera XT Index Kit v2 Set A (Illumina, California, USA). Specifically, 25 µl of NEBNext® Q5® Hot Start HiFi2X Master Mix (New England BioLabs, Massachusetts, USA), 5 µl of Nextera XT Index Primers (Primer 1 and 2), 10 µl of H20 and 5 µl of purified DNA were added. The thermal profile used has thus been set: 98°C x 30s; 12 cycles at 98°C x 10s, 55°C x 30s, 72°C x 30s; final extension at 72°C x 2m. After purification using magnetic beads Agencourt AMPure XP (Beckman Coulter, California, USA), a quality control of the purified libraries was performed using the dsDNA HS Assay Kit (ThermoFisher, Massachusetts, USA) on a Qubit™ 3 Fluorometer (ThermoFisher, Massachusetts, USA) and the Agilent High Sensitivity DNA kit (Agilent Technologies, California, USA) on the BioAnalyzer 2100 Instrument (Agilent Technologies, California, USA). The libraries were then normalized and pooled before being quantified using the NEBNext® Library Quant Kit for Illumina (New England BioLabs, Massachusetts, USA). Finally, pooled libraries were normalized to 4 nM and subjected to 2x300 paired-end sequencing on a MiSeq™ System (Illumina, California, USA) with the MiSeq Reagent Kit v3 (Illumina, California, USA).

2.4. Bioinformatics

The fastq data were analyzed using the CLC Genomics Workbench (Qiagen, Germany) software, using specific tools for the analysis of Operational Taxonomic Units (OTU) clustering contained in the CLC Microbial Genomics Module. Briefly, reads were filtered by a quality score (Qscore < 0.05), ambiguity for up to 2 nucleotides, adapter sequence cut-off, and minimum length (minimum 100 nucleotides). Consensus sequences with the forward and reverse sequences were created and submitted to the SILVA database (version 138) for the OTU classification.

Alpha diversity was estimated using bias-corrected Chao1 (total species richness), Simpson index (probability that two randomly chosen individuals belong to different species) and Shannon entropy (uncertainty average degree relating to the classification of an unknown individual). Beta diversity was estimated using the Bray-Curtis method and principal coordinate analysis (PCoA). Since the null hypothesis for the homogeneity of variance and/or for normal distribution could not be rejected, difference in Alpha diversity among groups was analyzed using the nonparametric Kruskal–Wallis test (and the relative Mann-Whitney U post-hoc test), whereas difference in beta diversity was assessed using the PERMANOVA test. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Environmental variables

In 2019, the mean water temperature of Balma Lakes ranged from 13.60±0.52 °C (Lower Lake) to 14.10±0.35 °C (Upper Lake). pH values ranged from 6.98±0.12 (Lower Lake) to 7.15 ±0.21 (Upper Lake). The conductivity was very low and ranged from 17±0.87 µS cm^{-1} (Lower Lake) to 20±0.87 µS cm^{-1} (Upper Lake). Oxygenation ranged from 8.10±0.68 mg L^{-1} (Lower Lake) to 8.70±0.57 mg L^{-1} (Upper Lake).

In 2020, the mean water temperature of Balma Lakes ranged from 14.40±0.61 °C (Lower Lake) to 15.21±0.35 °C (Upper Lake). pH values ranged from 7.16±0.10 (Lower Lake) to 7.31 ±0.12 (Upper Lake). The conductivity ranged from 18±0.17 µS cm^{-1} (Lower Lake) to 19±0.97 µS cm^{-1} (Upper Lake). Oxygenation ranged from 7.98±0.15 mg L^{-1} (Lower Lake) to 8.41±0.68 mg L^{-1} (Upper Lake).

The main physicochemical water parameters of fish farm were the follow: water temperature: 14.21±0.89 °C; pH: 7.10±0.54; conductivity: 102±1.12 μS cm^{-1}; dissolved oxygen: 7.95±0.99 mg L^{-1}

3.2. Sequences analyses

Following the selection of the sequences according to the established parameters, a total of 24,799,141 reads were obtained for the intestinal content samples and 22,108,700 for the wall samples. The SILVA database sequences were compared to 1,672,557 unique non-chimeric sequences from the intestinal content samples and 1,203,619 from the wall samples, leading to the assignment of 2,503 and 1,220 OTUs, respectively. Data from positive control agreed with the manufacturer indications for 16S sequencing protocol (all the bacterial taxa of the standard were detected in the correct percentages, whilst *Saccharomyces cerevisiae* and *Cryptococcus neoformans* were absent as expected), while the negative extraction and PCR controls didn't show any contamination.

Regarding the phyla, the analysis of the obtained sequences showed *Firmicutes*, *Proteobacteria* and *Actinobacteria* as the most abundant, representing about 90% of the microorganisms. Analysing sequences at the class level, *Bacilli*, *Gammaproteobacteria*, *Actinobacteria*, *Alphaproteobacteria* and *Clostridia* were the most represented, constituting over 80% of the samples' microbiota (Figure 1). Given the presence in most of the samples analyzed, these bacterial taxa can be considered the core microbiota of brook trout midgut, both considering the content and the wall. Moreover, the phylum *Fusobacteria* must be considered exclusively for the farmed fishes as part of the core microbiota.

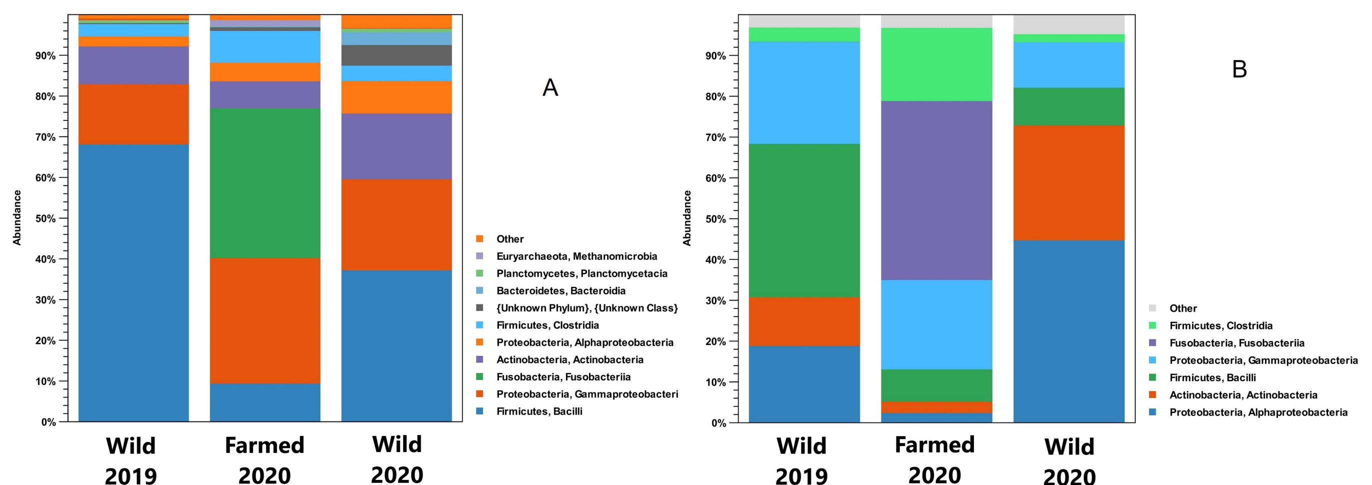

Figure 1. OTU abundance (%) grouped by class, with indication of phylum, of intestinal content (A) and intestinal wall (B) samples in wild and farmed brook trout.

3.3. Differences between wild and farmed samples: composition of the microbiota

Considering the data of the intestinal content microbiota, the presence of *Firmicutes* was about 71.0% (2019) and 41.0% (2020) in wild samples, while reached 18.0% in farmed animals. Focusing on samples taken from wild brook trout, *Proteobacteria* (*Alpha*- and *Gammaproteobacteria*) were the second most represented class (18.0% in 2019, 35.0% in 2020), followed by *Actinobacteria* (6.8% in 2019, 17.0% in 2020). Instead, the analysis of the intestinal contents of farmed fishes showed the presence of *Fusobacteria* (37.0%), not detected in the samples from natural environment. This high presence of *Fusobacteria* has

been almost entirely attributed to the genus *Cetobacterium*. Focusing on *Proteobacteria*, higher percentages of *Gammaproteobacteria* were observed compared to *Alphaproteobacteria* in all analysed samples, both farmed and wild. Substantial differences were observed about the *Bacilli* class, going from 9.5% for farmed fish to 68.0% for wild brook trout sampled in 2019. Less differences were observed for the other *Firmicutes* class analysed (*Clostridia*), with the highest percentages (7.8%) observed in farmed fish.

The analysis of intestinal wall allowed to see further differences between farmed and wild fishes. Farmed specimens showed the presence of *Proteobacteria* (24.0%), *Firmicutes* (41.0%) and *Actinobacteria* (2.9%) for the year 2019, while for the year 2020 the same situation was seen with different proportions (56.0% *Proteobacteria*, 11.0% *Firmicutes* and 29.0% *Actinobacteria*). In farmed samples a high percentage (44.0%) of *Fusobacteria* (genus *Cetobacterium*) was again observed, lacking in specimens collected in the natural environment. The class level analysis of gut wall microbiota showed differences compared to the intestinal content ones. Specifically, wild fish sampled in 2019 showed the same wall trend regarding *Alpha-*, *Gammaproteobacteria* and *Bacilli* distribution, albeit with different percentages. Instead, wild samples from 2020 showed a higher percentage of *Alphaproteobacteria* (45.0%) than *Gammaproteobacteria* (11.0%).

3.4. Differences between wild and farmed samples: alpha and beta diversity

Alpha diversity analyses showed a significant different richness and diversity in the intestinal content of analysed samples. Particularly, the Kruskal-Wallis test was significant for all indices except Simpson index, while the between-group Mann-Whitney test confirmed significance between farmed and wild brook trout of 2020 (Figure 2). 2).

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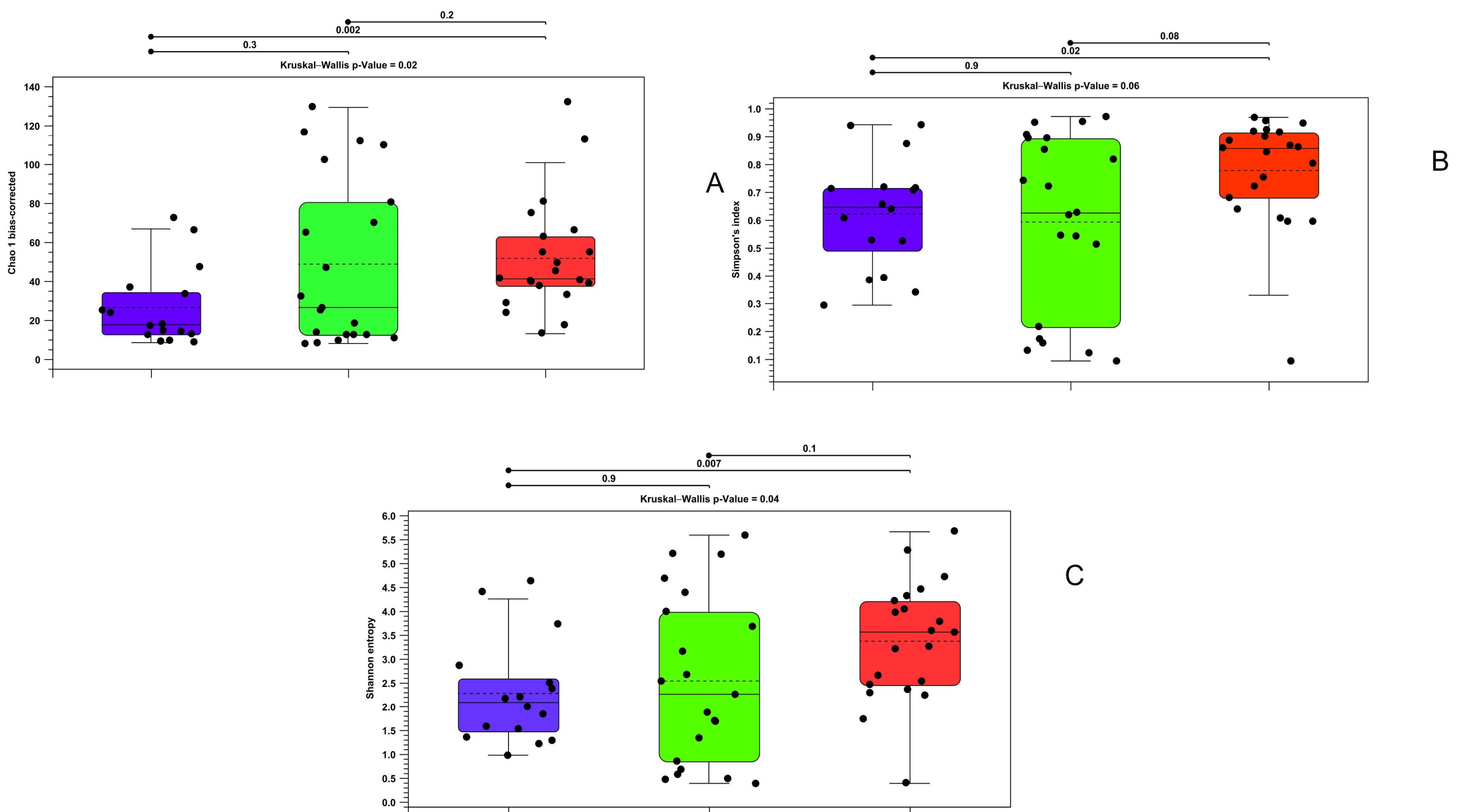

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Figure 2. Alpha diversity calculated with bias-corrected Chao 1 index (A), Shannon entropy (B) and Simpson index (C) for gut content samples, in wild and farmed brook trout. Purple: farmed samples; green: wild samples of 2019; red: wild samples of 2020. 269 270 271

Similar results were obtained by analysis of the data obtained from the gut wall with the Kruskal-Wallis test, which showed significant differences for all indices. For the Mann-Whitney test, only the Simpson index and the Shannon entropy showed statistically significant differences between farmed and wild specimens for 2020. However, all the indices showed significant variations between wild animals taken in the two different years (Figure 3). 272 273 274 275 276 277

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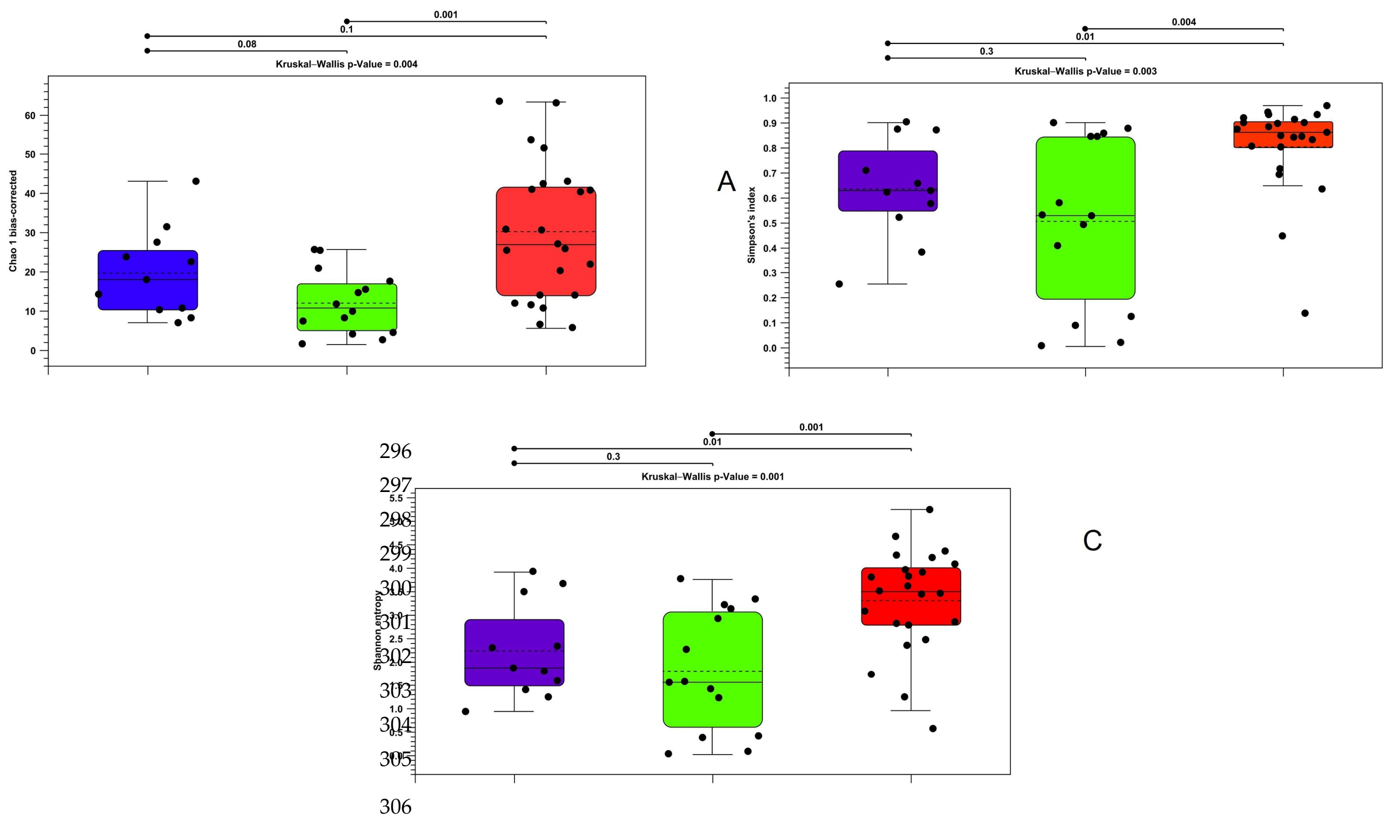

Figure 3. Alpha diversity calculated with bias-corrected Chao 1 index (A), Shannon entropy (B) and Simpson index (C) for gut wall samples, in wild and farmed brook trout. Purple: farmed samples; green: wild samples of 2019; red: wild samples of 2020.

Regarding the beta diversity, statistically significant differences were observed between farmed and wild microbiotas, especially in the case of intestinal content. The PERMANOVA analysis also demonstrated significant differences (p Bonferroni ≤ 0.05) between the three compared groups for both intestinal content and wall (Figure 4).

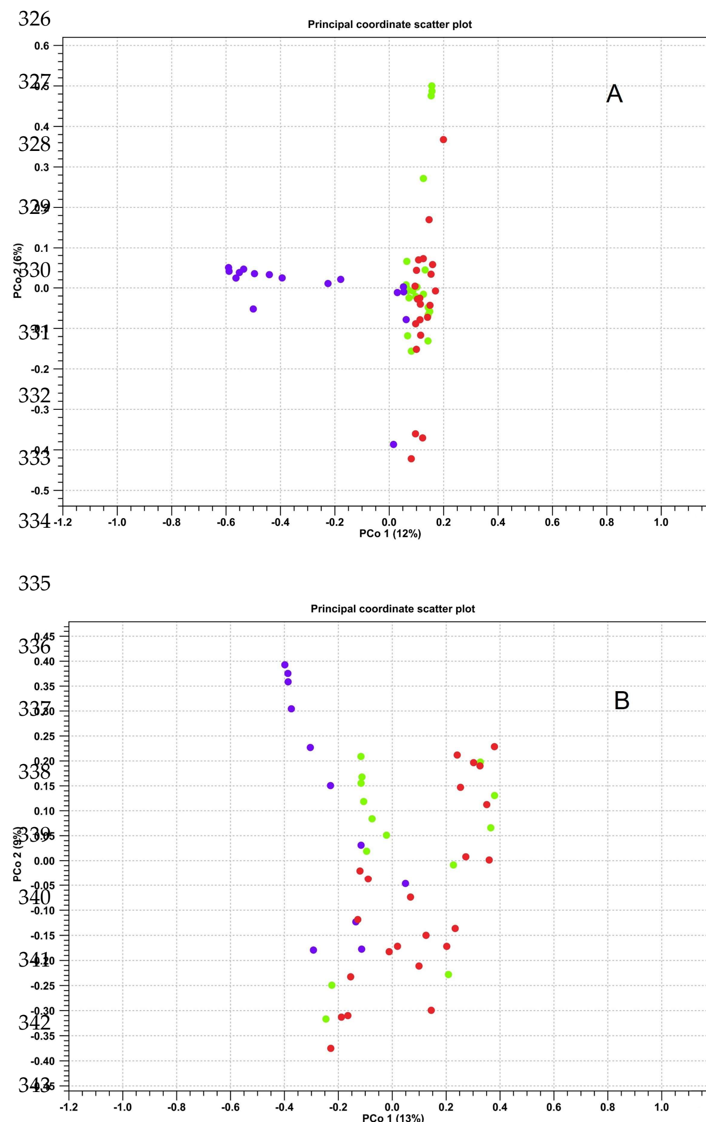

Figure 4. Scatter plot representing the beta diversity calculated with the Bray-Curtis index for the samples of intestinal contents (A) and intestinal wall (B). Violet: farmed samples; green: wild samples of 2019; red: wild samples of 2020.

3.5. Differences between intestinal wall and content: composition of the microbiota

Besides the comparison between wild and farmed fish, the microbiota of the two analysed matrices (content and intestinal wall) was also compared. In farmed brook trout the microbiota composition at phylum level was similar, with a very high percentage of *Fusobacteria* (37.0% in content and 44.0% in wall) and *Proteobacteria* (25.0% and 35.0%), followed by *Firmicutes* (18.0% and 25.0%).

Conversely, differences were noted on samples of wild fish, especially in the percentages of *Firmicutes* and *Proteobacteria*. *Firmicutes* was prevalent in the content (57.0% of the microbiota), while *Proteobacteria* in the gut wall (52.0%). *Bacilli* showed a higher percentage in content (54.0%) compared to the wall (18.0%). Finally, the greater prevalence of *Proteobacteria* in the wall was mainly determined by *Alphaproteobacteria* (37.0% against 5.1% of the content) (Figure 5).

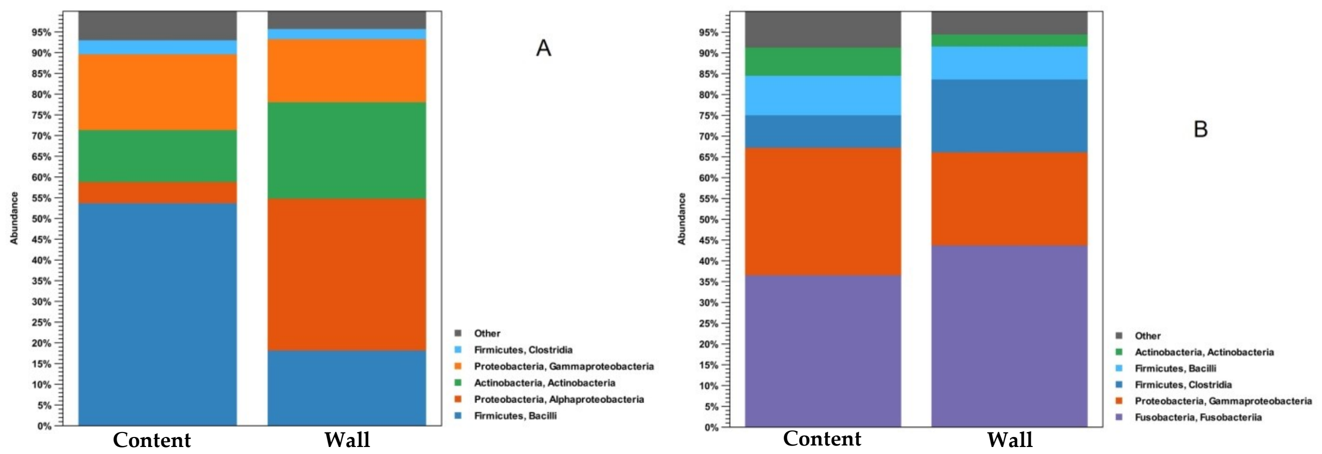

Figure 5. OTU abundance (%) grouped by class, with indication of phylum, of wild (A) and farmed (B) samples.

3.6. Differences between intestinal wall and content: alpha and beta diversity

The alpha diversity analysis did not reveal differences between the intestinal wall and content samples (Figure 6).

Kruskal–Wallis p-Value = 0.03
Chao 1 bias-corrected
A

Kruskal–Wallis p-Value = 0.3
Simpson's index
B

Kruskal–Wallis p-Value = 0.2
Shannon entropy
C

Figure 6. Alpha diversity calculated with bias-corrected Chao 1 index (A), Shannon entropy (B) and Simpson index (C) for gut wall samples, in wild and farmed brook trout. Blue: gut content of farmed samples; orange: gut wall of farmed samples; green: gut content of wild samples; green: gut wall of wild samples.

Instead, the comparison between groups with beta diversity showed differences between the two biological districts in wild fish, confirmed by significant values for the PERMANOVA analysis (P Bonferroni ≤ 0.05) (Figure 7).

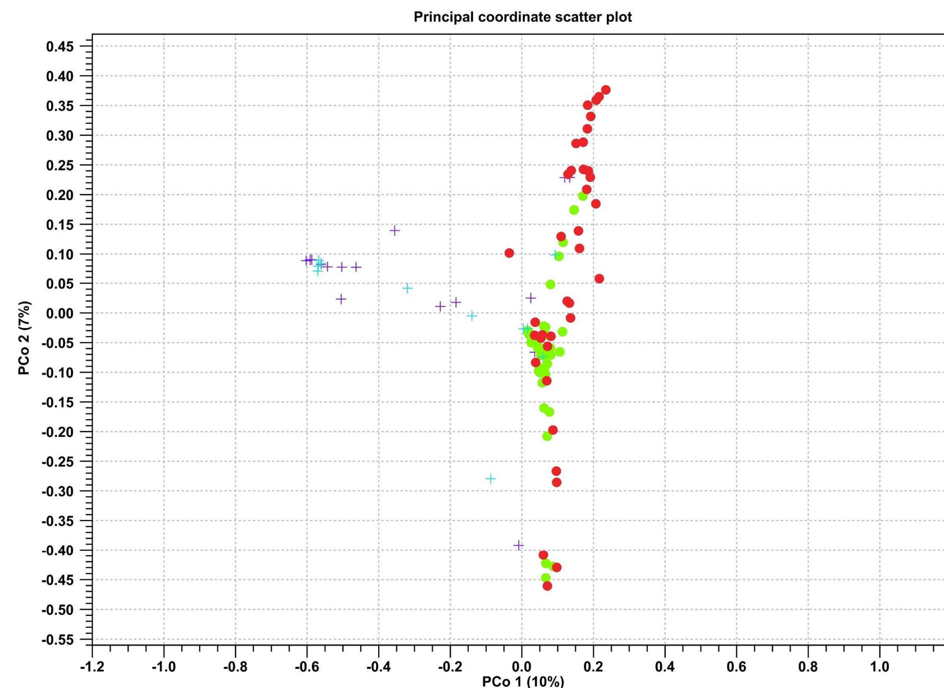

Figure 7. Scatter plot representing the beta diversity calculated with the Bray-Curtis index for the samples of intestinal contents (green dots) and intestinal wall (red dots) in wild samples and intestinal contents (purple crosses) and intestinal wall (blue crosses) in farmed samples.

4. Discussion and conclusions

The most represented microorganisms in the analysed microbiotas were *Proteobacteria* (*Alpha*- and *Gammaproteobacteria*), *Actinobacteria*, *Firmicutes* (*Bacilli* and *Clostridia*) and, only in reared specimens, *Fusobacteria*. These data are in agreement with previous studies. Ideed, Kim et al. [19] identified *Proteobacteria* and *Firmicutes* as the most abundant taxa in fish microbiota and reported high percentage of *Fusobacteria* in freshwater species (especially Perciformes, Tetraodontiformes, Siluriformes, Cypriniformes, and Lophiiformes); however, they did not consider salmonid species in their study. Specific studies have been conducted on the gut microbiota of salmonids. The intestinal microbial composition of reared Atlantic salmon (*Salmo salar*) fed with fishmeal-free feed showed *Firmicutes*, *Proteobacteria* and *Actinobacteria* as the most represented taxa [27]. The microbiota of juvenile rainbow trout (*O. mykiss*) considered by Michl et al. [28] consisted mainly of the phyla *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Fusobacteria* and *Actinobacteria*. They also studied the variations of the intestinal microbiota of this species in relation to the diet, reporting an increase in *Clostridiales* (*Firmicutes*), *Fusobacteriales* (*Fusobacteria*), *Vibrionales* and *Alteromonadales* (*Gammaproteobacteria*) in relation to an animal proteins-rich diet. Based on these data, the classes *Alpha*- and *Gammaproteobacteria*, *Actino*-

bacteria, *Bacilli* and *Clostridia* should be considered the "core" microbiota, as they are present in more than 80% of the samples.

Fusobacteria were also identified in farmed samples. In the study conducted by Lyons et al. [29] on intestinal contents of rainbow trout a high presence of *Fusobacteria* was detected, although in this case the most represented class was *Mollicutes*, followed by *Bacilli*, *Clostridia*, *Gammaproteobacteria* and *Spirochaetia*; however, it should be considered that this work was focused on the medial portion of the intestine. Therefore, these data suggest that the high presence of *Fusobacteria* may be connected to an animal protein-based diet, as underlined by other studies carried out on reared teleost fed with fishmeal feed [19,30]. *Fusobacteria* found in our study are all attributable to the *Cetobacterium* genus. *Fusobacteriales*, especially *Cetobacterium* spp., were negatively correlated with the dietary availability of vitamin B12 (cyano-cobalamin) [31]. This vitamin is highly present in fish [32], so a diet rich in fishmeal-based feed can increase the presence of vitamin B12-synthesizing bacteria, such as *Fusobacteria*. The comparison between the intestinal microbiotas of wild and farmed brook trout seems to support this hypothesis, as demonstrated by the high percentages of *Fusobacteria* found in specimens fed with a commercial feed.

Analyzing the two biological matrices separately, data from our study do not show differences between the intestinal wall and content. Our results are in contrast with the observations derived from other previous studies. Nyholm et al. [33] and Gajardo et al. [34] compared the microbiota of intestinal wall and contents in three species of *Cyprinodontiformes* and in *S. salar*, respectively: both showed that the bacterial community in the wall was significantly less different than the content microbiota, indicating that only few bacteria taxa of the intestinal tract have the ability for colonize the host's mucosa. Other studies may be needed to confirm our data, considering a greater number of samples and other districts of the intestine (proximal and distal portions). The alpha diversity analysis of microbiotas of intestinal wall and contents did not show significant differences in the farmed brook trout. However, the analysis of beta diversity shows differences in wild fish, with a greater presence of *Firmicutes* in the content and *Proteobacteria* in the wall. Several studies have also found a high percentage of *Proteobacteria* associated with intestinal wall, usually corresponding to 30 – 40% of the total microbiota [34]. The lack of this difference in farmed fish could be linked to the environment standardized condition. It is known that even differences in the wild environment can cause variations at the level of the microbiota. Nyholm et al. [33] showed significant differences in the intestinal microbial community of three fish species (*Aphanius iberus*, *Gambusia holbrooki* and *Valencia hispanica*) in relation to the sample collection sites and demonstrated that localization can explain a large part of the variance found.

Our work therefore represents the first study on the characterization of the intestinal microbiota of brook trout. The core microbiota was determined both for farmed and wild specimens. The decision to analyze the gut microbiota of brook trout in natural conditions and in artificial housing derives from what was previously done by other authors for other fish species [35]. Differences were found in the composition of the microbiota of the groups taken into consideration: it remains to be clarified in future studies what these differences are related to. The presence of *Fusobacteria* in farmed specimens can be related to the commercial diet as previously discussed, but it remains to clarify whether the other differences may be related to diet, since environmental parameters here considered (water temperature, dissolved oxygen, conductivity, and pH) were quite similar between Balma Lakes and the fish farm. Both the intestinal wall and the contents were taken into consideration, however without significant differences between the two matrices as indicated in other studies. The differences were mainly found between wild and farmed fish, in agreement with other studies carried out on this topic. Future investigations might focus on comparing the microbiota of species that are phylogenetically similar (e.g., salmonids) or farmed in the same farms (e.g., rainbow trout). Furthermore, the

other gut districts can be studied to detect differences with the medial portion of the intestine.

Moreover, about wild brook trout, new studies should be focused on fish living in alpine lakes at different altitudes to understand if this factor can influence the gut microbiota. Indeed, the altitude could influence the diet of these fish (i.e., the presence or the absence of a particular prey due to its altitudinal range), introducing another variable to take into consideration. We say that three main groups (Diptera Chironomidae, Imenoptera, and Coleoptera) represent the preferred diet of brook trout in Balma Lakes [36]. However, we think that difference in gut microbiota between wild fish captured in 2019 and 2020 should be sought in other environmental variables.

All the main water physicochemical parameters except temperature were quite similar in both wild and farmed fish. On this path, the differences in microbiota richness and diversity observed in wild fish (2019 vs. 2020) could be related to the slightly increase in water temperature occurred in 2020. However, further studies are needed to better understand the influence of this key variable on fish gut microbiota.

Finally, the information derived from this study can represent a starting point for the evaluation of the effect of candidate probiotics for the prevention of infectious diseases, the modulation of the immune system and the implementation of production performance [37]. Although our study does not provide information on probiotics, the knowledge of the gut core microbiota of brook trout in healthy conditions could be the starting point for the application of probiotics in case of dysbiosis caused by infectious processes. Thus, experimental studies on the evaluation of microbiota changes due to infectious processes are needed in the near future. The analysis of the microbiota relating to infectious diseases is of crucial importance for the development of intensive aquaculture, as certified by the growing number of studies on the topic [38].

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