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Anatomical site and environmental exposure differentially shape the microbiota across mucosal tissues in rainbow trout (*Oncorhynchus mykiss*)

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This study investigates the diversity, structure, and composition of the fish microbiota across different mucosal organs by comparing the bacterial communities in the oropharyngeal cavity, posterior intestine, gills, and skin of farmed rainbow trout (*Oncorhynchus mykiss*), along with water and biofilm samples from the surrounding environment. Four distinct skin regions across the dorsal-ventral and anterior-posterior axes were also compared in fish weighing 390.5 ± 36.8 g. Sample analyses showed that lower values of richness (observed) and diversity (Shannon and Faith indices) were observed in the posterior intestine compared to the gills, skin, and environmental (water and biofilm) samples ($P < 0.05$). Similarly, the oropharynx showed higher Faith diversity values than the intestine ($P = 0.01$). Furthermore, bacterial community structures differed significantly across organs based on unweighted and weighted UniFrac distances ($P = 0.001$ for both), with the posterior intestine showing the greatest divergence from other mucosal sites. Indeed, Pseudomonadota was the most abundant phylum across all sample types, except for the posterior intestine, where Firmicutes, particularly the genus *Mycoplasma*, showed a clear predominance. The posterior intestine showed facultative anaerobic genera, while the other mucosae and environmental samples were mainly composed of strictly aerobic members, like *Flavobacterium* and *Crocinitomix*. The microbial communities across the different skin regions were highly heterogeneous: while the dorsal area showed a consistent microbiota, the ventral region exhibited differences between the anterior and posterior sections in bacterial structure and composition at the genus level ($P < 0.05$). For instance, *Candidatus* Piscichlamydia was very abundant in the gills and ventral-anterior skin, but was scarcely detected in the rest of skin areas. Overall, these findings suggest a high differentiation of bacterial communities across fish organs, tailored to the specific physiological and environmental characteristics of each mucosal tissue, with a stronger modulation by the surrounding environment in the external mucosae and a higher influence of host innate factors in inner organs, such as the intestine.

Animal mucosae represent their primary interface with the environment, as they are constantly exposed to antigenic substances that induce specific humoral and cell-mediated immune responses at both local and systemic levels[1]. Among vertebrates, fish species rely more on their mucosae than their terrestrial counterparts, since aquatic environments provide a more favorable medium for the growth of microorganisms compared to the air[2]. In addition to their defensive functions, mucosal organs have also crucial physiological roles. For instance, the skin plays an important role in fish communication (*i.e.*, via coloration), sensory perception through cutaneous mechanoreceptors and chemoreceptors, locomotion, cutaneous respiration, and osmotic/thermal

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regulation[3–5]. The gills are involved in gas exchange, regulation of osmotic balance, acid-base equilibrium, and excretion of nitrogenous wastes by passive and active transport across the epithelium[6]. The gut is another well-known multifunctional organ responsible for feed digestion, absorption, and nutrient catabolism, through coordinated peristalsis and enzymatic digestion, as well as water and electrolyte homeostasis[7]. The former tissues comprise the three most intensively studied and characterized mucosa-associated lymphoid tissues (MALTs) in fish[8,9], even though other mucosal immune systems are currently under study, such as the buccal cavity and the pharyngeal mucosa[10], including its sub-pharyngeal lymphoid organ, the Nemausean lymphoid organ (NELO)[11]. The oropharynx is primarily involved in respiration through buccal pumping, which is a mechanism that enables water to flow from the mouth over the gills via rhythmic compression/expansion of the pharyngeal region[12]. To a lesser extent, the oropharynx is also involved in digestion through mechanical processing by pharyngeal teeth in some species of Cyprinidae and Cobitidae, and through mucus secretion, which facilitates the lubrication and swallowing of food particles[13,14]. In addition, the mucus secreted in the oropharynx and the gut also serves to protect the mucosal surfaces from damage caused by the grinding of food and as a first barrier against pathogen colonization[13]. In essence, while mucosal tissues share certain functions, each one is specialized and performs unique roles that are critical to the overall performance and health of fish[8].

The bacterial communities inhabiting these body surfaces are partly responsible for the specific functions of each mucosa. In particular, the skin microbiota can avoid pathogen colonization by antagonistic activities and/or competition for space and resources[15], while the gut microbiota contributes to nutrient digestion and metabolism, energy homeostasis, mucosal integrity, intestinal barrier function, and immune system modulation[16]. The importance of the microbiota in maintaining the host health becomes evident during dysbiosis, defined as a significant disruption in the diversity, composition, and function of bacterial communities which often compromises the host health[17]. Inversely, a beneficial microbiota composition can enhance animal growth, feed utilization, development, immunity, and health[18]. These host-microbiota interactions are reciprocal. In this regard, the host in turn modulates microbial diversity and composition through multiple mechanisms (i.e., through the induction of an immune response by the recognition of pathogen-associated molecular patterns (PAMPs), or through the secretion of bile salts to the intestine[19,20]. Apart from the interactions with the host, the microbiota is also shaped by the environment, such as the water temperature and salinity[21], as well as the environmental bacteria[22].

For the above-mentioned reasons, microbial research is becoming increasingly important in aquaculture and ecological studies, particularly as a target for identifying biomarkers of fish health and condition[23–25] and for detecting environmental threats or unsuitable rearing conditions[26]. As an example, a relevant review by Ikeda-Ohtsubo et al.[27] sough to define what constitutes an optimal microbiota for plants, fish, and livestock animals in terms of bacterial composition. A key conclusion of the above-mentioned review was that the functions and impacts of the microbiota are highly dependent on the host species, genetics, and environmental conditions. In particular, external mucosal sites like the skin and gills are especially sensitive to environmental microbes, mirroring a major part of the composition of surrounding water[28], while host genetics and physiological factors also contribute, to a lesser degree, to shaping these communities[29,30]. In contrast, the fish gut microbiota is generally more conserved and host-specific, often exhibiting phylosymbiosis; a phenomenon whereby microbial communities align with host phylogeny, which is potentially driven by genetic and phenotypic differences across species, host-microbe coevolution, and/or vertical transmission, among other factors[31]. Nonetheless, external factors such as the diet and the habitat also influence the fish gut microbiota, albeit to a far lesser extent than host-inherent factors[32–34]. Altogether, host and environmental factors interact to shape the mucosal microbiota in fish, reflecting a complex ecological and evolutionary interplay similar to that observed in terrestrial vertebrates. However, apart from the environmental influences, the skin microbiota of vertebrates seems to be primarily shaped by individual and species-specific traits[35–37], whereas some studies in birds and mammals have shown that external factors (i.e., diet and rearing conditions) can outweigh the effect of host factors on their gut microbiome[38–40]. These patterns highlight the need to investigate how different mucosal surfaces are structured in fish, given the differential environmental exposure and host control among them with respect to the rest of vertebrates.

Despite numerous studies on the gut microbiota of various fish species[41–43], further research should focus on describing the bacterial communities specific to different mucosal organs in both cultured and natural environments, considering that comparative studies over the past decade have highlighted that sampled body regions significantly influence bacterial diversity, structure, and composition[23,28,31,44–46]. However, knowledge about microbial changes between and within different fish mucosal organs is still scarce, which limits the understanding of how spatial variation and specific conditions (host and environmental factors) within a specific mucosal surface may contribute to shaping the microbial communities. For instance, very few studies have examined microbial communities along the digestive tract beyond the gut regions[18] and, to our knowledge, only one work has addressed whether there are different bacterial populations across distinct skin regions[29]. Consequently, the objectives of the present study were: (i) to conduct a comparative analysis of the microbial communities across different fish mucosal organs and with respect to the surrounding environmental microbiota, and (ii) to compare the microbiota across different sampling areas of the skin mucosa. For this investigation, we selected the rainbow trout (*Oncorhynchus mykiss*) as a biological model, which is one of the most widely farmed salmonid freshwater species[47].

Methods
Ethics statement
All procedures involving animal care, handling, and sampling were performed by trained and qualified personnel in accordance with Spanish legislation (Law 32/2007 and Royal Decree 1201/2015) and European

Directive 2010/63/EU. The study was approved by the Generalitat of Catalunya (CEEA 11264/2021) and the Ethics Committee of the Institute of Agrifood Research and Technology (Spain).

Experimental design and microbial sampling

Juveniles of rainbow trout were purchased from Truchas de Leiza SL (Navarra, Spain), and transported to IRTA, Centre de la Ràpita (Tarragon, Spain), where they were reared in captivity for 3 months in three 450 L-tanks connected to an IRTAmar™ water recirculation system. During this period, trained technical staff conducted daily inspections to check for external signs of disease or abnormal behavior, and no such signs nor mortalities were observed. Fish were maintained at a water temperature, pH, and dissolved oxygen of 15.9 ± 0.7 °C, 7.9 ± 0.2, and 8.8 ± 1.0 mg/L, respectively. Fish were fed an extruded diet (43% crude protein, 15% crude fat, 20.6 MJ/kg of energy), which was distributed twice a day with automatic feeders (Arvo-Tec T Drum 2000, Finland) at a feeding rate of 3.0% of the stocked biomass. Two hours after each feeding, uneaten pellets were collected, dried at 120 °C, weighed, and daily feed rations were adjusted to maintain 10–15% uneaten feed, ensuring ad libitum feeding. This experimental design was chosen to ensure standardized conditions for all fish (i.e., same age, diet, feeding regime, and environmental conditions) and to avoid the impact of ecological factors on the fish microbiota when samples were collected from wild fish populations[48].

At the time of sampling, following three months of rearing, rainbow trout had a body weight of 390.5 ± 36.8 g (mean ± standard deviation) and a standard length of 28.5 ± 0.8 cm. Fish were fasted two days prior to the sampling to clear the feces from the intestinal tract, given that gastric emptying occurs 8–12 h since the last feeding and gut evacuation takes 14 to 24 h at this temperature (16 °C), depending on fish size/age, and diet composition[49–52]. This approach ensures that only the mucosa-adhered microbiota was sampled, since previous studies have shown that this fasting period drastically reduces community diversity, and abundance of feed-associated bacteria to nearly undetectable levels, avoiding potential contamination from transient feed-associated microbes[53]. The samples collected for microbiota analysis are shown in Fig. 1. Sample collection was performed in the following order to standardize a similar sampling duration for all the fish. Each fish ($n = 5$ per tank; 15 in total) was individually netted and anesthetized with buffered tricaine methanesulfonate (MS-222, Sigma-Aldrich, Spain; 100 mg/L) to prevent cross-contamination of the skin mucus and ensure rapid sampling. Once anesthetized, each fish was carefully grasped by the head and the caudal fin using ethanol-sterilized gloves and placed in a support that secured the fish head and caudal fin to facilitate handling and prevent potential loss or contamination of mucus from manipulation (Supplementary Fig. 1). Skin mucus was collected according to the method described by Fernández-Alacid et al.[54]. Briefly, individual sterile glass slides were used to carefully collect the mucus from four distinct skin regions across the dorsal-ventral and anterior-posterior (both sides of the fish) in 2 mL-sterile tubes. The skin mucus from both sides of the fish was pooled (also for the gills' samples) to ensure sufficient content for downstream DNA extraction and to avoid the differential effect that the swimming direction may have on the microbiota of each body side. Mucus was first obtained along the ventral-anterior region (VA skin), from the pectoral fin to the beginning of the ventral fin below the lateral line. Then, skin mucus from the ventral-posterior area (VP skin) was sampled with a new sterile glass slide from the ventral fin to the base of the caudal fin, also below the lateral line. Similarly, the dorsal-anterior and posterior regions (DA and DP skin, respectively) were sampled, covering the same longitudinal areas but above the lateral line.

For the sampling of all inner mucosal tissues, we adopted swabbing as an animal-friendly method following the animal welfare guidelines to adhere to the 3Rs principle in animal research (replacement, reduction, and refinement)[55]. In addition, the omission of tissue excision prevented blood contamination of the samples. It has been demonstrated that this non-lethal approach ensures a highly accurate microbial representation in terms of bacterial diversity and composition in mucosal organs[56,57]. Regarding the gills' sampling, the second holobranch
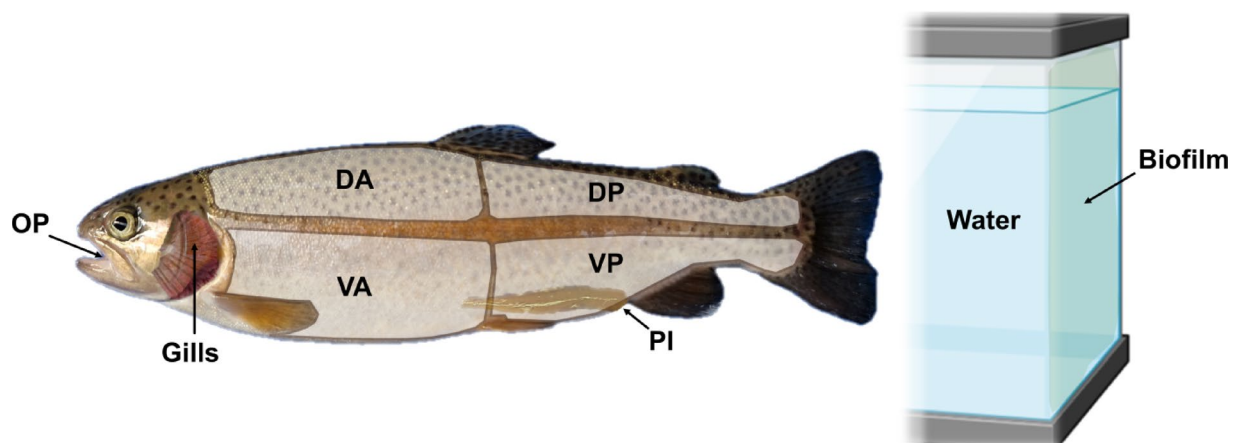
Fig. 1. Sample points selected for microbial comparison from the different mucosal organs of rainbow trout (*Oncorhynchus mykiss*) and surrounding environment (water and biofilms associated to rearing tanks). Abbreviations: OP: oropharynx; PI: posterior intestine; DA: dorsal-anterior skin; DP: dorsal-posterior skin; VA: ventral-anterior skin; VP: ventral-posterior skin.

of the right/left branchial arch was selected in order to avoid potential contamination with the microbiota of the skin mucus and to ensure a highly representative sample of bacterial diversity and abundance[58]. In brief, the operculum and the first holobranch were lifted with sterile tweezers, while the second holobranch was gently but insistently swabbed with a sterile flocked nylon swab (ref. MFS-96000BQ, Meidike Gene, China). The same swab was used for collecting mucus from the second holobranch of the right and left sides of the fish, and the swab tip was placed in a sterile tube. For sampling the oropharyngeal cavity (OP cavity), the use of the anesthetic MS-222 allowed to keep the fish's mouth open without applying any pressure that could damage the tissues. A sterile swab was wiped through the oral cavity in a clockwise and anticlockwise circular motion, which was repeated in the pharyngeal region between the oral cavity and the gill arches, and the tip was placed in a sterile tube. For the sampling of the posterior intestine (PI), the fish was laid on a sterile tray and its external surface was rinsed with absolute ethanol to avoid external contamination. Then, a flocked swab was inserted anally into the fish to a depth of ca. 5–6 cm until feeling the wall of the intestine folding up[57]. The swab was gently rotated in clockwise and anticlockwise directions, carefully removed, and the tip was placed in a sterile tube. All fish survived the sampling procedure, with no mortality observed during nor after the sampling. The sampling strategy was intentionally performed in a blinded and randomized order across individuals in order to reduce potential biases introduced by operators and temporal variability during the processing of numerous tissue samples per individual, while maintaining consistent handling across all specimens. Consequently, individual fish could not be traced back to their respective tanks, excluding the incorporation of tank as a covariate in subsequent statistical analyses.

Following the procedure of previous articles[59–61], a total of five water samples (50 mL) were taken from the fish tanks and each one was separately filtered through cellulose acetate membrane filters with a pore size of 0.22 µm (Ø = 25 mm; ref. 10404106; Millipore, MERK, Spain), which were transferred to 5 mL Eppendorf tubes for storage. Furthermore, five biofilm samples from the walls of the fish rearing tanks were collected using the flocked nylon swabs. In this sense, the swab model used in this study is specifically designed to collect large numbers of cells for clinical and research applications, and it was selected because of its thin tip (Ø = 2.5 mm), flexible polystyrene body, breakpoint that reduces its manipulation, and the proven superior performance of flocked nylon swabs in targeting microorganisms over different swab types and applicators[62]. All the samples were individually stored at − 80 °C until further DNA extraction.

## Extraction of DNA and sequencing library preparation

The DNA from the different samples was extracted using the DNeasy PowerSoil Pro Kit (ref. 47,016, QIAGEN, Germany) according to the manufacturer's instructions. Regarding the skin samples, ca. 100 mg of mucus per tube were used for each DNA extraction. For the swabbed and filtered samples, the flocked nylon tips and the cellulose acetate membrane filters were respectively maintained in the tubes up to the bead-beating step to ensure cell lysis and sample homogenization[57]. The concentration and purity of the extracted DNA were assessed using a Nanodrop-2000* spectrophotometer (Thermo Fisher Scientific, USA), with concentrations ranging from 80 to 200 ng/µL and the A260/A280 absorbance ratios being higher than 1.80.

The V3-V4 region of the 16 S rRNA gene was amplified using universal primers 341 F (5'-CCTACGGGNGGCWGCAG-3') and 805R (5'-GACTACHVGGGTATCTAATCC-3') with Q5* High-Fidelity DNA Polymerase (ref. M0491L, New England BioLabs, USA). The first PCR was performed using the following program: an initial step of 30 s at 98 °C for polymerase activation and DNA denaturation, followed by 25 cycles of 10 s at 98 °C, 30 s at 55 °C, 30 s at 72 °C, and a final extension step of 2 min at 72 °C. A second amplification of 8 cycles under similar conditions was then carried out to add specific barcodes to each template. The amplified regions were prepared for sequencing on an Illumina MiSeq Platform (2 × 300 bp paired-end) according to the 16 S Metagenomic Sequencing Library Preparation guide[63].

Data processing and statistics

Firstly, forward and reverse primers were removed from the FASTQ files using the Cutadapt tool in QIIME2 (v2022.2)[64]. Quality control and data analysis were performed following a workflow based on the package DADA2 (v1.24.0) in RStudio (v2023.06.1). The DADA2 package resolves differences at the single-nucleotide level, generating amplicon sequence variants (ASVs)[65]. Briefly, forward and reverse reads were filtered by quality, with a threshold set at a score of 26, which corresponded to truncation lengths of 280 nt for forward reads and 220 nt for reverse reads. Reads with an expected error rate higher than 2 were removed. Paired-end reads were merged and those with an overlap region of less than 12 nucleotides, any mismatch, or identified as chimeras were discarded. ASVs were then classified using the SILVA database (v138.1)[66]. ASVs with a classification confidence score below 80% were grouped as unassigned[67] and those identified as mitochondria or chloroplasts were excluded from the analysis. Based on the rarefaction curves generated with the *vegan* package in R (v2.6-4)[68], samples were rarefied to the lowest sample depth (52,494 reads per sample), which was a representative size for the ASVs present in the samples (Supplementary Fig. 2).

The phylogenetic tree of the different ASVs was obtained in QIIME2[64], and all statistical analyses were performed using the R package *microeco*[69]. The *trans_alpha* function was used to calculate observed richness (number of ASVs) and Shannon diversity index[70] and Faith's phylogenetic index, which estimates diversity based on the presence, abundance, and phylogeny of ASVs[71]. Significant differences in the relative abundances of the bacterial communities at different taxonomic levels and in alpha diversity were evaluated using the Kruskal-Wallis test followed by Dunn's test, adjusting the P-values with false discovery rate (FDR)[72]. Regarding inter-individual diversity, dissimilarities among samples were qualitatively and quantitatively estimated using unweighted and weighted UniFrac distances respectively[73] and visualized by principal coordinate analysis (PCoA) with the *trans_beta* function. After confirming the homogeneity of group dispersions using a permutational analysis of multivariate dispersions (PERMDISP; $P > 0.05$), significant differences in beta diversity were assessed using
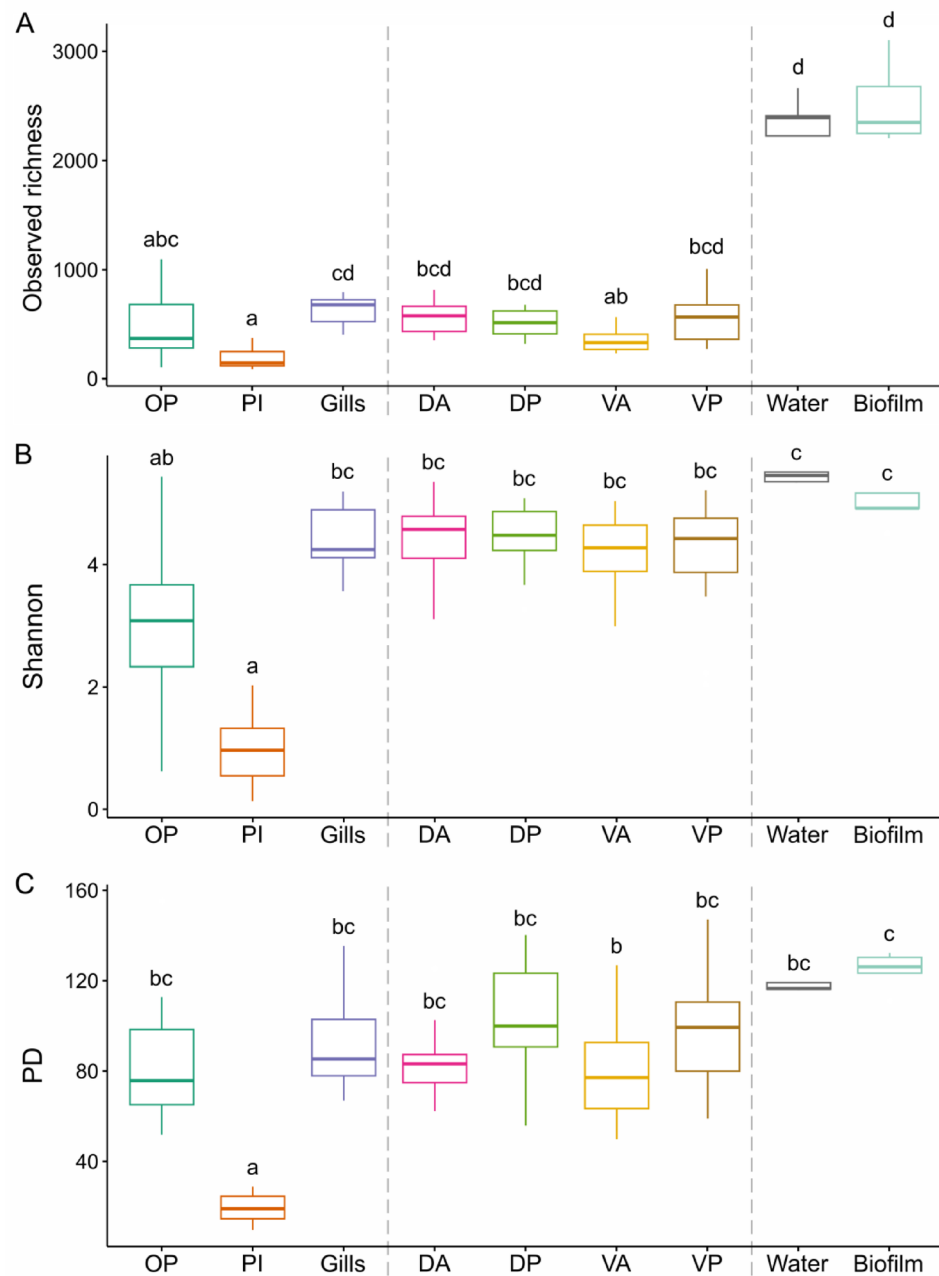
Fig. 2. Alpha diversity values of the bacterial communities in the different mucosal organs of rainbow trout (*Oncorhynchus mykiss*) and in the tank water and biofilm samples measured using **(A)** observed richness, **(B)** Shannon diversity index, and **(C)** Faith phylogenetic diversity index (PD). Different letters represent significant differences among groups ($P \leq 0.05$). Abbreviations: OP: oropharynx; PI: posterior intestine; DA: dorsal-anterior skin; DP: dorsal-posterior skin; VA: ventral-anterior skin; VP: ventral-posterior skin.

permutational multivariate analysis of variance (PERMANOVA) and pairwise PERMANOVAs for multiple comparisons, with P-value adjustment by FDR ($P \leq 0.05$)[74].

Results
Alpha diversity
No significant differences were found in observed richness (number of ASVs) and diversity estimated with Shannon index between the OP cavity and the PI ($P = 0.17$ and $P = 0.67$, respectively; Fig. 2A, B). On the other hand, the values of Faith's phylogenetic index were significantly lower in the PI when compared to the OP cavity of rainbow trout ($P = 0.01$; Fig. 2C). The bacterial communities from the gills exhibited values of observed richness, Shannon, and Faith's diversity higher than those observed in the PI ($P < 0.001$ for the thee indices) and similar to those in the OP cavity ($P = 0.24$, $P = 0.15$, and $P = 0.99$, respectively). Regarding the skin, there were

no significant differences in richness (observed) nor in diversity (Shannon and Faith's phylogenetic indices) across the dorsal-anterior (DA), dorsal-posterior (DP), ventral-anterior (VA), and ventral-posterior (VP) areas ($P > 0.05$). Furthermore, the values of observed richness, Shannon, and Faith's phylogenetic indices in the skin were similar to those observed in the gills and OP cavity ($P > 0.05$). However, these values were significantly higher in the skin than in the PI ($P < 0.05$), with the exception of the VA skin region, which was the only area showing similar richness to the PI ($P = 0.99$).

Regarding the bacterial communities in the water and biofilm from the fish rearing tanks, the richness was higher than in the PI and VA skin region ($P < 0.05$). Shannon values in the environmental samples (water and tanks' biofilm) were higher to both the OP cavity and the PI ($P < 0.05$). Faith's phylogenetic diversity in the environmental samples was also higher with respect to the PI ($P < 0.001$ in both cases). In contrast, the rest of the fish samples showed similar values to the environmental samples ($P > 0.05$).

Beta diversity

Regarding beta diversity results, when evaluating the inter-individual variation qualitatively based on unweighted UniFrac distances, which is computed considering the number of the common and exclusive ASVs among samples but excluding their abundance, there were significant differences among all sampled body organs and regions (PERMDISP, $P > 0.05$; PERMANOVA + FDR, $P < 0.05$; Supplementary Table 1), except among the DA, DP, and VP skin regions ($P > 0.05$).

When using the quantitative metric of weighted UniFrac distances, which also takes into account the abundance of each ASV, only the dorsal skin areas (DA and DP) exhibited a similar bacterial structure between them ($F = 0.64$, $R^2 = 0.02$, $P = 0.79$), while the bacterial communities from the rest of the mucosal organs were significantly different to these skin regions and among them ($P < 0.05$; Supplementary Table 1).

The water and biofilm microbial populations exhibited a clear separation for both unweighted UniFrac ($F = 3.68$, $R^2 = 0.32$, $P = 0.007$) and weighted UniFrac distances ($F = 43.90$, $R^2 = 0.85$, $P = 0.007$). The bacterial community structures of environmental samples were also significantly different to those of the fish mucosal organs ($P < 0.05$) and they clustered further apart from them, especially when considering the samples of the PI (Fig. 3A, B).

Taxonomical composition

As shown in Fig. 4A, the most dominant phylum in the OP cavity, gills, and skin regions (DA, DP, VA, and VP skin) was Pseudomonadota (Fig. 4A). This phylum did not exhibit significant differences among the sampled fish regions (Kruskal-Wallis test, $P = 0.02$). However, the composition and abundance of the rest of the predominant phyla varied among regions (Supplementary Table 2). Specifically, the OP cavity was mainly dominated by Pseudomonadota (50.1%), Bacteroidota (21.5%), and Firmicutes (13.0%). The most abundant phyla in the gills were Pseudomonadota (44.5%), Bacteroidota (24.4%), and Verrucomicrobiota (13.9%). Regarding the skin, the four sampled areas (DA, DP, VA, and VP skin) exhibited the same three most abundant phyla and at similar abundances among them (Dunn's test + FDR, $P > 0.05$): Pseudomonadota (40.5–56.2%), Bacteroidota (16.3–35.6%), and Actinobacteriota (8.6–13.7%). No differences between the anterior and posterior regions of the dorsal skin region were observed in the composition and abundance of any of the phyla with an abundance higher than 1% (Dunn's test + FDR, $P > 0.05$). In contrast, Verrucomicrobiota showed a significantly higher abundance in the VA skin (10.3%) than in the VP skin (1.4%; Dunn's test + FDR, $P = 0.002$), even though the abundances of the rest of phyla from these two ventral regions were also very similar between them and with respect to the dorsal areas (Dunn's test + FDR, $P > 0.05$). Contrary to the rest of organs, the most abundant phylum of the PI was Firmicutes (68.4%) followed by Pseudomonadota (31.2%), while the rest of intestinal phyla were almost non-detectable (< 0.5% in total). Concerning the environmental samples, the microbial communities from the biofilm of the tanks' walls were dominated by Pseudomonadota (42.4%), Bacteroidota (24.6%), and Actinobacteriota (14.1%), while the water was dominated by Pseudomonadota (42.7%), Bacteroidota (29.2%), and to a lesser extent by Myxococcota (5.0%).

At the level of genus, the microbiota of the OP cavity was characterized by high inter-individual variation, with a clear predominance of *Aeromonas* in two samples (14% of samples; 11.0% average abundance in the PI), *Flavobacterium* in other two samples (14% of samples; 9.6% average abundance), *Lelliottia* in one sample (7% of samples; 8.7% average abundance), and *Mycoplasma* in another sample (7% of samples; 8.2% average abundance) (Fig. 4B; Supplementary Table 3). On the other hand, the genus *Crocinitomix* showed a more even presence among the OP samples (6.5% average abundance). The PI was dominated by the genus *Mycoplasma* (66.7%) belonging to the phylum Firmicutes, and some individuals also exhibited relatively high abundances of the genera *Aeromonas* (12.9%) and *Lelliottia* (14.3%). Regarding the gills, *Candidatus* Piscichlamydia was the most abundant group in the branchial epithelium (12.0%), followed by *Crocinitomix* (9.0%), *Candidatus* Branchiomonas (6.8%), and *Flavobacterium* (5.3%). The skin of rainbow trout exhibited a highly uniform composition with low variability among individuals and across skin regions (DA, DP, VA, and VP skin). In this sense, the four areas were mainly composed of the genera *Pseudomonas* (15.1–21.4%), followed by *Crocinitomix* (3.8–6.2%) and *Rhodococcus* (3.3–4.6%). While *Candidatus* Piscichlamydia was almost absent in the majority of skin samples (DA, DP, and VP skin) (0.1–0.4%), it was very abundant in the VA skin (9.1%), which explained the high abundance of the phylum Verrucomicrobiota in this region. In regard to the environmental samples, the water microbiota was dominated by *Crocinitomix* (11.8%), followed by less abundant genera like *Sphaerotilus* (3.8%), *Emticicia* (3.0%), *Runella* (2.5%), and *Pseudorhodobacter* (2.1%). In contrast, the biofilms consisted of several low-abundance genera, such as *Nitrosomonas* (2.7%), *Crocinitomix* (2.6%), and *Flavobacterium* (2.1%) among many others, rather than being dominated by a single genus.
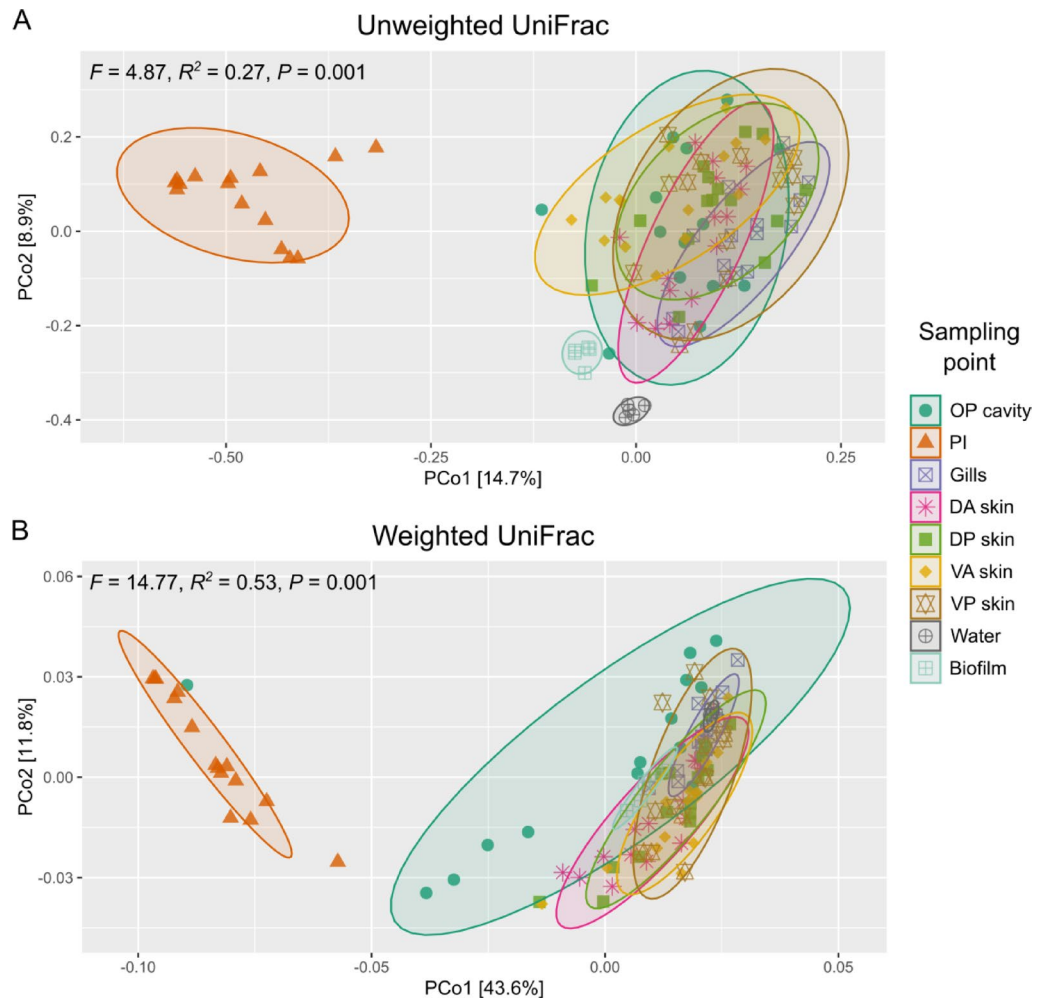
Fig. 3. **(A)** Principal coordinate analysis (PCoA) based on beta diversity metrics showing the spatial distribution of the bacterial communities among the different mucosal organs of rainbow trout (*Oncorhynchus mykiss*) and in the tank water and biofilm samples using **(A)** unweighted UniFrac distances and **(B)** weighted UniFrac distances. Abbreviations: OP cavity: oropharyngeal cavity; PI: posterior intestine; DA skin: dorsal-anterior skin; DP skin: dorsal-posterior skin; VA skin: ventral-anterior skin; VP skin: ventral-posterior skin.

Discussion

Considering the important role of the microbiota in fish health and condition[15,16], this study aimed to describe and compare the microbial diversity, structure, and composition, as well as the expected influence of environmental microbes on the bacterial communities within the different mucosal organs and body regions of rainbow trout. Since this research work adopted swabbing as a non-lethal sampling method in accordance with the 3Rs principle, internal sites of the digestive tract beyond the oropharynx and the PI, like the stomach and the anterior intestine, were not sampled, as it would have implied the sacrifice of animals.

Richness and diversity within the bacterial communities associated to fish mucosal organs and to the environment

Although the oropharyngeal mucosa is well-studied in mammals, few studies have compared the microbiota across the digestive tract beyond the different gut regions in fish[18]. Data from higher vertebrates have shown that the OP cavity is among the sections with the highest bacterial density[75–77]. In addition, earlier studies in rainbow trout using conventional microbiological approaches have reported a progressive decline in the number of culturable aerobic bacteria along the digestive tract from the esophagus to the lower intestine[78]. Similarly, Dong et al.[10] described a much higher observed (OTUs) and estimated richness (Chao1 and ACE indices) and diversity (Shannon index) in the buccal cavity and pharynx than in the hindgut of rainbow trout. These findings are in line with the numerically higher values for the Shannon index and significantly higher values for the Faith's index observed in the oropharynx compared to the PI of rainbow trout under current conditions.

In addition, our study showed a high similarity in the richness and diversity of the microbiota from the gills and the oropharyngeal mucosa, in agreement with results in common carp (*Cyprinus carpio*) reported by Meng et al.[79]. This was likely due to their spatial proximity, physiological function, and environmental conditions for bacterial growth, as the branchial cavity, where gas exchange occurs, is bilaterally situated on both sides of the
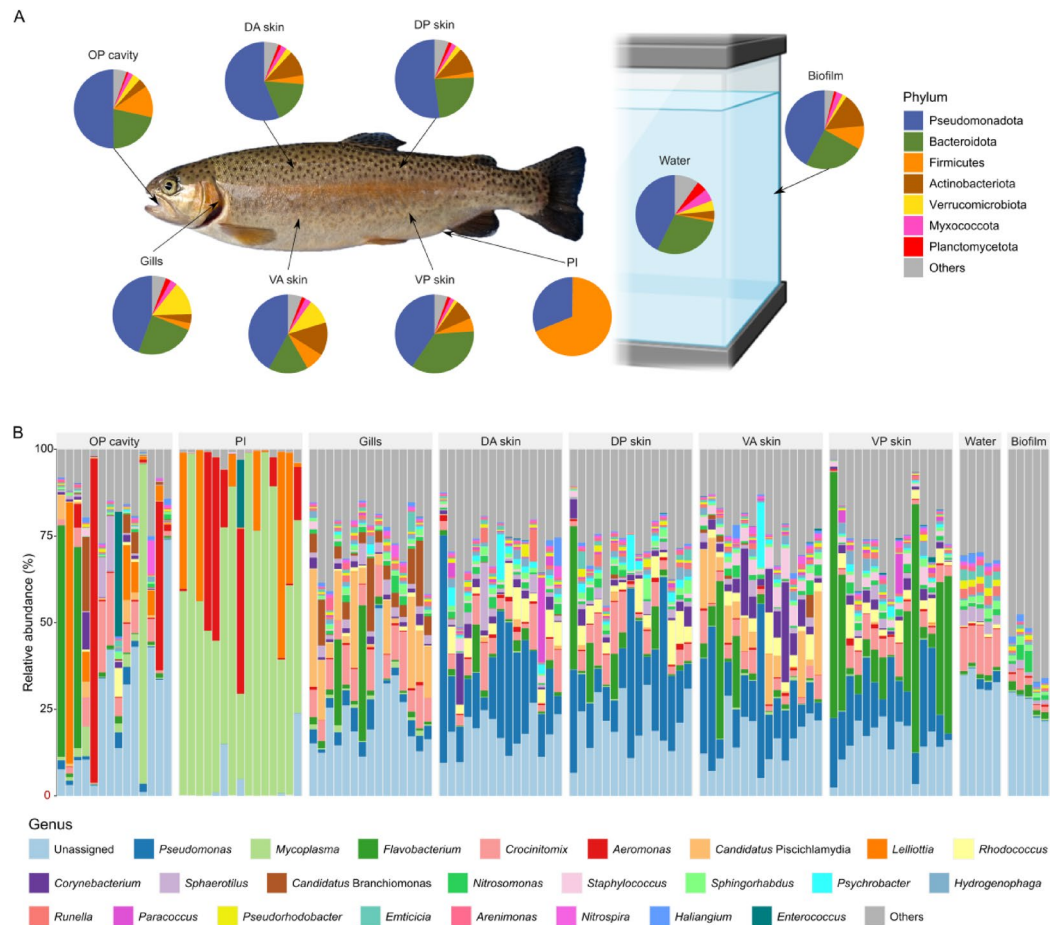
Fig. 4. Relative abundances at the level of **(A)** phyla and **(B)** genera of the bacterial communities among the different mucosal organs of rainbow trout (*Oncorhynchus mykiss*) and in the tank water and biofilm samples. Bacterial phyla with an abundance < 1% and genera with an abundance < 0.5% are classified as "others." Abbreviations: OP cavity: oropharyngeal cavity; PI: posterior intestine; DA skin: dorsal-anterior skin; DP skin: dorsal-posterior skin; VA skin: ventral-anterior skin; VP skin: ventral-posterior skin.

pharynx[80]. Indeed, the similarity in the values of richness, and Shannon and Faith's diversity indices among the OP cavity, gills, and skin may be attributed to a common modulation of their microbial communities by the exposure to the surrounding water[28], which aligns with the similar values observed for the environmental samples (water and biofilm). For instance, Sylvain et al.[31] observed that the fish skin mucus communities were associated to habitat-specific factors (environmental physiochemistry and bacterioplankton community), while the gut microbiota was mainly shaped by species-specific factors. These findings are also in line with previous studies showing a similar richness and diversity between the gills and skin in different fish species, such as rainbow trout[44], yellowtail kingfish (*Seriola lalandi*)[23], bullet tuna (*Auxis* sp.), common dolphinfish (*Coryphaena hippurus*), Atlantic little tunny (*Euthynnus alletteratus*), Atlantic bonito (*Sarda sarda*), Atlantic white marlin (*Kajikia albida*)[28], and gilthead seabream (*Sparus aurata*)[81]. However, different results have been observed in other studies on gilthead seabream, which showed higher values in the skin than in the gills[82], as well as in common carp and European seabass (*Dicentrarchus labrax*), which inversely exhibited richer and more diverse bacterial communities in the gills than in the skin[79,81]. The different results among studies may be caused not only by the different experimental conditions but also by the sampling methodology applied to the targeted organs. In this context, previous studies have demonstrated that the microbial composition of extracellular skin mucus differs from that within adjacent epidermal cells[44]. Thus, swabbing would predominantly target microbial communities from the mucus, whereas biopsy excision could recover deeper communities within the epidermis. In addition, it is important to note that the lack of individual fish tracking by tank prevented including tank as a covariate in statistical analyses due to blinded and randomized sampling order to reduce operator and time biases. However, tank effects are not expected to significantly influence the results of the study as it focuses on comparisons across tissues, sampled from all three tanks, and tanks were maintained under identical conditions with standardized diet, environment, and continuous water quality monitoring and regulation within a recirculating aquaculture system (RAS).

Regarding the PI, the lower microbial richness and diversity observed in comparison to the gills and skin are consistent with the higher richness and diversity observed by Meng et al.[79] in the buccal cavity and gills

than in the gut of the common carp. Similarly, previous studies on rainbow trout[83], tambaqui (*Colossoma macropomum*)[84], and gilthead seabream[82] have observed higher bacterial richness and diversity in the skin than in the gut. This pattern appears to persist across various fish species regardless of being farmed or collected from natural environments as Sadeghi et al.[85] described in 17 species collected from different lacustrine habitats. This trend may reflect a more restrictive habitat in the intestine that on the external mucosae because several factors intrinsic and extrinsic to the host constrain the gut bacterial colonization. These include, among others, the limited oxygen availability[86], pH changes induced by the secretion of acidic digestive products, bile salts, and pancreatic juices (containing bicarbonate) to the gut[87–89], the temperature[90,91], the antimicrobial effects of bile salts[20,92], and the competition for space and nutrients[93].

Inter-individual diversity of the bacterial communities associated to fish mucosal organs and with respect to the environmental microbes

Similar to our findings, previous studies have already reported a differential bacterial structure among distinct fish mucosal organs in terms of inter-individual variation in richness and diversity. For instance, a different distribution was observed for the bacterial communities in the caudal fin, gills, and gut in a pair of closely related species like the common nase (*Chondrostoma nasus*) and the Southwest European nase (*Parachondrostoma toxostoma*) when using qualitative (Jaccard, and unweighted UniFrac distances) and quantitative metrics (Bray-Curtis, and weighted UniFrac distances)[94]. Similarly, significant differences in the structure of the microbiota associated with the gills and the gut have been found in silver carp and bighead carp when using Bray-Curtis distances[45], and in common carp and zebrafish when using weighted UniFrac[46]. Interestingly, the three former studies agree that the mucosal tissues contribute more to the differentiation of the microbial communities than the fish species itself. Furthermore, Legrand et al.[23] showed a clear separation of the bacterial communities from the skin and the gills in yellowtail kingfish (*Seriola lalandi*) based on Bray-Curtis distances. A differential clustering of the bacterial communities from the skin and gut mucus samples when using Bray-Curtis distances was also reported in farmed rainbow trout[83]. Field studies conducted on diverse wild fish populations from lakes and freshwater streams have similarly revealed marked differences in the bacterial structure and composition between the skin and gut microbial communities[31,85]. The differential bacterial structure among mucosal organs may be related to the differential tissue architecture, physicochemical conditions, and host physiological pressures[24,44,95,96]. In this context, under present experimental conditions a pronounced separation of the PI from the rest of mucosal organs in the PCoAs for unweighted and weighted UniFrac distances was found; results that were consistent with previous studies where a clear divergence between the intestine and the rest of mucosae was observed[10,44,94]. This may reflect the challenging conditions that bacteria find in the gut for survival and proliferation[97], in line with the lower values of observed richness and diversity (Shannon index) of the PI with respect to the gills and skin, as well as of Faith's phylogenetic diversity, compared to the OP cavity, gills, and skin.

Strikingly, in the present study, the sample type that exhibited the highest inter-individual dispersion for both unweighted and weighted UniFrac metrics was the mucus from the OP cavity. This could be partly attributed to the combined sampling of microbiota from both the buccal and pharyngeal mucosa, which, despite their proximity, are functionally distinct and exhibit differences in their bacterial community composition[10]. In this sense, apart from the above-mentioned factors (oxygen, pH, temperature, nature of secretions, space, and nutrient competition), the differences in inter-individual microbial diversity found among organs may be promoted by their distinct cell types, functions, and mucosal activity specialization, which affect the housing conditions for microbial communities[98,99]. Indeed, a specialized secondary lymphoid organ associated to the pharynx recently described in certain teleost species, known as NELO, coordinates lymphocyte traffic and defense mechanisms within the fish respiratory mucosa[11]. Furthermore, the mucus cells in the gills and skin are specialized in respiration, protection against pathogens, and immune responses[100], while the mucus cells of the OP cavity and PI have adaptations for protecting the buccal epithelium and feeding[101], and for food digestion[102,103]. In addition, distinct mucin expression and glycosylation patterns have been reported between the fish skin, gills, esophagus, and intestine, reflecting tissue-specific functions and responses[104,105].

Concerning beta diversity across the different skin regions, the structures of the microbial communities throughout the entire dorsal areas (DA and DP regions) were similar, while the communities of the ventral were different between the anterior (VA) and posterior (VP) parts. In this sense, the VP region more closely resembled the dorsal parts than the VA region based on unweighted UniFrac distances. A plausible explanation for this may be that the hydrodynamic conditions of the pectoral fin during swimming, such as the generation of vortex rings during propulsion[106], may create unique conditions (i.e., water forces and friction) in the VA region because of their anatomical proximity. In this regard, previous studies have shown that abrupt behaviors such as fast swimming and skin sloughing may affect the microbial load[107], which may be related to the renewal of skin mucus considering its role in reducing frictional drag[108]. Moreover, the higher viscosity and increased content of metabolites and mucins, such as lactate, glucose, and mucopolysaccharides, in the ventral region than in the dorsal skin mucus might also influence microbial colonization by affecting nutrient availability, pathogen resistance, and locomotion[101,109,110]. In this sense, mucin sugars can be degraded and used as nutrient sources for the microbiota or act as microbial binding sites[111]. Additionally, structural differences like epidermal thickness, density of mucus cells, and microridge areas (apical epithelial projections involved in mucus retention) also vary between skin regions[112,113], potentially contributing to the discrepancies in beta diversity results between the dorsal and ventral areas.

Composition of the bacterial communities associated to fish mucosal organs and with respect to the environmental microbes

Several studies in rainbow trout have already demonstrated a consistent dominance of two bacterial phyla across mucosal organs, Pseudomonadota and Firmicutes. The majority of studies have shown very high abundances of

Pseudomonadota (synonym Proteobacteria) in the mouth, olfactory organ, pharynx, gills, skin, and stomach of rainbow trout, and a very consistent dominance of Tenericutes (reclassified as Firmicutes) in the gut[10,44,114–117]. Despite the lack of studies focused on the OP cavity in rainbow trout, Pseudomonadota, Bacteroidota, and Firmicutes have also been found among the most abundant ones in the buccal cavity of this species, together with Actinobacteriota[10], which in the present study was also observed at lower abundances. Regarding the gills, in consistency with our results, Bellec et al.[117] reported Verrucomicrobiota, Pseudomonadota, and Bacteroidota as the most dominant phyla in rainbow trout, while Verrucomicrobiota was not described in the gills by other authors[44,115], which may respond to differences in husbandry conditions and/or sample conservation among studies. In this sense, the presence of this phylum in fish gills has been reported to be highly sensitive to the water composition[118], and to sample collection (swabbing vs. excision) and storage[58]. Furthermore, the predominance of Pseudomonadota, Bacteroidota, and Actinobacteriota that we observed in the skin of rainbow trout was in agreement with many works studying the skin microbiota of rainbow trout, consistently demonstrating that Pseudomonadota represents the most abundant phylum in the skin of this species, followed by other phyla whose abundance ranking may vary, usually Bacteroidota, Firmicutes, and Actinobacteriota[44,116,119,120]. The composition and abundance of phyla in the skin are typically dependent on the water microbiota, as demonstrated in rainbow trout[115] and other fish species, such as Atlantic salmon[121] and European perch (*Perca fluviatilis*)[29]. However, in the current experiment, the composition at the level of phylum in the skin was closer to that of the biofilm from the tank walls than to the water. In this context, certain bacteria exhibit mechanisms that enable them to colonize both biotic and abiotic surfaces[122,123]. Although comparing these results at the phylum level with previous studies provides a global perspective on compositional changes, the genus-level analysis allowed us to hypothesize further about the origin and significance of the genera belonging to these phyla.

The dominance of Firmicutes in the PI was attributed to the high abundance of the genus *Mycoplasma*, in line with previous works on gut microbiota in this species[44,114,116,124]. This genus was also present in the OP cavity, but at lower average abundances, and was nearly absent in the rest of the mucosal organs and water and tank biofilm samples. These results indicate that *Mycoplasma* is highly specific to the gastrointestinal tract in fish. Accordingly, many studies have suggested a mutualistic relationship between *Mycoplasma* and the gut of salmonids, with this genus promoting fish growth, carotenoid synthesis, biosynthesis of B vitamins and essential amino acids, disease resistance, and host health[25,125]. Apart from *Mycoplasma*, some individuals also exhibited relatively high abundances of the genera *Aeromonas* and *Lelliottia* in the PI. The genus *Aeromonas* comprises some species considered to be pathogenic to fish, such as *Aeromonas salmonicida* and *Aeromonas hydrophila*. However, other species belonging to this genus are part of the gut commensal microbiota of freshwater fish and have even shown probiotic effects, like *Aeromonas media*[86,126]. Regarding *Lelliottia*, this genus has been frequently observed in the gills and skin of freshwater fish[127], but some studies have also reported it in the gut of salmonids[128,129]. Interestingly, *Mycoplasma*, *Aeromonas* and *Lelliottia* were also observed in the oropharyngeal mucosa, but their abundance widely varied among individuals, in line with the inter-individual dispersion observed based on unweighted and weighted UniFrac distances. In contrast, *Flavobacterium* and *Crocinitomix*, which were not present in any of the gut samples, showed high abundances in the oropharynx, as well as in the gills and skin regions (DA, DP, VA and VP). These differences in microbial composition were most likely determined by the distinct oxygen availability across these two regions of the digestive tract. In this regard, the absence of *Flavobacterium* and *Crocinitomix* in the PI of rainbow trout may be attributed to their strictly aerobic nature[130,131], considering that this microhabitat is more anaerobic than the rest of mucosal organs which are in direct contact with the environment. On the other hand, most of the species belonging to the genera *Mycoplasma*, *Aeromonas* and *Lelliottia* are facultative anaerobes[132–134], like the majority of bacterial members typically observed in the fish intestine[86]. This hypothesis is in consistency with the presence of *Flavobacterium* and *Crocinitomix* in the gills and skin, and the near absence of *Mycoplasma*, *Aeromonas*, and *Lelliottia* in these organs.

In addition, considering the significant presence of *Crocinitomix* and *Flavobacterium* in the water and tank biofilm samples, it is plausible that the origin of these genera within the mucosal organs of rainbow trout, excluding the gastrointestinal tract, was influenced by environmental factors and rearing conditions. In this regard, previous studies have demonstrated an association between the presence in aquatic environments of bacterial members from the order Flavobacteriales, which includes *Crocinitomix* and *Flavobacterium*, and a healthy status in white shrimp (*Litopenaeus vannamei*)[17]. Furthermore, the genera *Flavobacterium* and *Crocinitomix* have also been reported as the two most abundant genera in the incoming water and in the tank water from a freshwater RAS containing healthy Chinook salmon (*Oncorhynchus tshawytscha*)[135]. In addition, *Flavobacterium* has also been observed in the tank water from both RAS and flow-through aquaculture systems growing healthy rainbow trout[136]. Consistent with the hypothesis that water may be the source of these two genera, the high abundance of *Crocinitomix* in the aquatic environment may account for the uniform inter-individual distribution of this genus in the oropharynx, gills, and four skin regions (DA, DP, VA and VP) that we observed. In contraposition, the lower abundance of *Flavobacterium* in the environment may explain the high variability of its abundance in the OP cavity, as well as in the gills and skin. In this regard, some individuals may be more susceptible to colonization by *Flavobacterium* based on their inherent differences in mucosal characteristics, including the content and level of glycosylation of mucins, which can be utilized by some species of this genus as nutrient sources[103,137], the variations in the mucus inhibitory properties on biofilm formation[138] and in the immune responses[139].

The remaining examined mucosal organs showed lower inter-individual variation. For instance, the high abundance of the phylum Verrucomicrobiota in the branchial epithelium was primarily due to the genus *Candidatus* Piscichlamydia. In agreement with our results, Bellec et al.[117] described *Candidatus* Piscichlamydia and *Candidatus* Branchiomonas as the most dominant genera in the gills of rainbow trout, while they were absent in the water community. Similarly, we did not find either of these genera in any of the environmental

samples, whereas they were present at lower abundances in the OP cavity, likely due to its proximity to the gills. In addition, the high abundance of *Candidatus* Piscichlamydia in the VA skin, which accounts for the high abundance of Verrucomicrobiota in this region, may be attributed to the water outflow from the gills to this region of the skin. Regarding this hypothesis, even though many common taxa are usually found between the gills and the skin[23,28,44,81,96,140], to our knowledge, no previous studies have compared the gills with different skin regions along the dorsal-ventral and anterior-posterior axes, which would be necessary to validate this hypothesis. Concerning *Candidatus* Branchiomonas, Brown et al.[114] reported it as the most abundant genus in the gills of rainbow trout. Upon observing that the fish were in a healthy state, both Brown et al.[114] and Bellec et al.[117] concluded that even though *Candidatus* Branchiomonas may be considered as a potential pathogen, it is part of the gill commensal microbiota of rainbow trout, in line with our results. Similarly, many studies have also noted the presence of *Flavobacterium* as a typical member of rainbow trout gills[44,114,117], even though some representatives of this genus have been described as the etiologic agent of bacterial cold water disease in rainbow trout[141]. These findings underscore the critical need for complementary omics-based and/or conventional approaches to resolve bacterial taxonomy at lower levels and to fully understand the host-microbe interactions.

Concerning the composition of the skin bacterial communities, other authors have observed high variability among individuals in different fish species, such as gilthead seabream, European seabass[142], and European perch[29]. Contrariwise, in the present study the skin of rainbow trout exhibited a highly uniform composition at the genus level within each skin region analysed (DA, DP, VA, and VP), with low inter-individual variation. These controversial results may be explained by innate host traits, such as the fish species, which strongly determine the skin microbial structure and composition[143]. For instance, although some *Pseudomonas* strains can be considered opportunistic fish pathogens under stressful conditions, this genus has been frequently reported as a commensal genus in the skin of rainbow trout[115,116,119,120]. In addition, *Pseudomonas* strains isolated from rainbow trout skin have shown inhibitory activities against *Flavobacterium psychrophilum*, which is the etiological agent of bacterial coldwater disease[144], and previous studies have reported a reduction in *Pseudomonas* after parasitic infection in rainbow trout[120]. Altogether, these findings suggest a positive association between the presence of this genus in the skin of rainbow trout and its health status. Similarly, some species belonging to the genera *Rhodococcus* and *Flavobacterium* isolated from the skin of rainbow trout have shown an antagonistic effect against *F. psychrophilum*[115], indicating that these species have a protective role in prevention of fish diseases. In this regard, in line with previous studies[116,120], the genus *Flavobacterium* was also very abundant in the skin of rainbow trout, especially in the VP area, even though it was very unequally distributed among individuals. As hypothesized above, the high inter-individual variability of *Flavobacterium* in the mucosae may be attributed to its potential environmental origin, where it is present in low abundances, but also to intrinsic host-bacteria specific interactions, which results in an uneven colonization of the fish mucosae at individual level. Furthermore, the increased abundance of *Candidatus* Piscichlamydia and *Corynebacterium* observed in the VA region was in line with the divergent structure of the bacterial communities from this region compared to the rest of the skin areas (DA, DP, and VP) based on weighted UniFrac distances. Although *Candidatus* Piscichlamydia may have its origin in the water incoming from the gills, the differences in *Corynebacterium* abundances give consistency to the hypothesis that there is a differential bacterial colonization across the skin in response to the specific hydrodynamic conditions created by the pectoral fin[106], as well as due to the described differences in the mucus characteristics between the dorsal and ventral areas (metabolites, mucins, viscosity, number and type of mucus cells)[109,112,113]. In agreement with these ideas, *Staphylococcus* was markedly more abundant in the ventral skin regions (VA and VP) compared to the dorsal skin (DA and DP). This genus has been detected at a wide range of abundances in both the skin mucus and the epithelial cells of the epidermis of rainbow trout[44,116]. In particular, Lowrey et al.[44], which sampled the fish left side-dorsal skin area, observed a clear predominance of the species *Staphylococcus epidermidis* in the epithelial cells, reflecting a preferential harboring of this species in the epithelium. In addition, *Staphylococcus warneri* has also been described as an ordinary resident of the skin mucus and epithelial cells of healthy rainbow trout, not pathogenic to this species, but exhibiting pathobiont characteristics under stressful conditions[145]. Further investigation into the role of these species on the skin of rainbow trout could provide valuable insights, particularly in explaining the factors driving changes in their abundance across the dorsal and ventral regions. On the other hand, Berggren et al.[29] showed a more similar composition when comparing the microbiota from the dorsal and ventral skin mucus of European perch. However, these authors did not distinguish between the anterior and posterior regions, which could have reduced the heterogeneity among samples. Overall, these results underscore the importance of accounting for location-specific anatomical factors when comparing the microbial composition among different body sites, and even within the same mucosal organ.

Conclusions

The results of the present study demonstrate a high level of bacterial differentiation across body regions of farmed rainbow trout, with the posterior intestine showing the greatest differentiation from other organs due to the predominance of *Mycoplasma*. In contrast, the oropharynx, gills, and skin harbored a more even bacterial population due to the influence of the environmental bacteria, as reflected by the presence of common strictly aerobic genera. Although bacterial composition was highly homogeneous across the skin, variations in *Candidatus* Piscichlamydia, *Corynebacterium* and *Flavobacterium* were observed between the ventral-anterior and -posterior regions, which underscores the relevance of considering anatomical sub-regions when analyzing mucosal microbiota. Overall, these findings reinforce the value of studying the intestinal microbiota as a robust indicator of host physiology and inherent characteristics, while outer mucosal organs may be further explored as potential indicators of husbandry conditions, particularly in relation to fish health and disease susceptibility. Expanding similar comparative studies to farmed and wild specimens of this and other fish species is essential to

clearly delineate what constitutes the fish commensal microbiota, as well as the expected variations within and among different mucosal tissues.

Data availability

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Author contributions

AR participated in the sampling, DNA extraction and amplification, microbiota analyses, and writing of the original draft. IS contributed to the conceptualization of the experiment, sampling, and writing of the original draft. ST participated in the sampling and in the writing through revision and edition of the manuscript. EG contributed to the conceptualization of the experiment, sampling, funding acquisition, and writing through revision and edition of the manuscript.

Declarations

Competing interests

The authors declare no competing interests.

ARRIVE guidelines

The study was conducted in compliance with the ARRIVE guidelines and all methods were conducted in accordance with relevant guidelines and regulations.

Additional information

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