Communication

Skin Microbiota of Salmonids: A Procedure to Examine Active Bacterial Populations Using an RNA-Based Approach

Alda Pardo [1], Alejandro Villasante [1,2,3] and Jaime Romero [1,*]

1   Laboratorio de Biotecnología de Alimentos, Instituto de Nutrición y Tecnología de los Alimentos (INTA), Universidad de Chile, El Líbano, Santiago 5524, Chile; alda.pardo@ug.uchile.cl (A.P.); alejandro.villasante@gmail.com (A.V.)
2   Doctorado en Acuicultura, Programa Cooperativo Universidad de Chile, Universidad Católica del Norte, Pon-tificia Universidad Católica de Valparaíso, Santiago 783090, Chile
3   Facultad de Medicina Veterinaria y Agronomía, Universidad de Las Américas, Santiago 7500000, Chile
*   Correspondence: jromero@inta.uchile.cl; Tel.: +56-22-9781524

Abstract: Fish microbiota studies have mostly addressed intestinal bacterial communities because of their role in fish physiology; however, the skin microbiota has been studied less despite its role as the first pathogen barrier in the host. DNA-based molecular techniques have contributed to improving our understanding of the skin microbiota, but this approach faces challenges, such as the low count of bacteria in healthy fish skin. To overcome this limitation, an RNA-based approach was developed to study the skin microbiota in salmonids, including tissue sampling, RNA extraction, and downstream procedures to obtain PCR amplicons for next-generation sequencing. The protocol originated in this work overcomes the limitations of low bacterial counts and is useful for describing active microbiota in fish skin. The application of the protocol to salmonids reared in both an experimental setting and on a farm revealed that the trout skin microbiota was dominated by bacteria from the phylum Proteobacteria (>65%). At the genus level, *Piscirickettsia* (46%) was highlighted as the most abundant in the experimental unit samples; in contrast, *Pseudoalteromonas* (26%), followed by *Escherichia_Shigella* (~25%), was the most abundant in farmed trout.

Keywords: RNA; skin; microbiota; fish; trout; salmonids

1. Introduction

The skin of teleosts is a flexible, scaly structure covered by dermis-secreted mucus. This mucus is a slim, slippery layer covering the epithelial surfaces in fish [1]. The skin and mucus constitute physical and biochemical barriers that separate the organism from the environment [2]. These surfaces are inhabited by a diverse and complex bacterial community that may contribute to fish homeostasis [3,4]. At the phylum level, the composition of the bacterial communities present on the surface of the fish has been described as dominated by *Proteobacteria* and as having a different composition from the surrounding water [5,6]. It has been reported that the composition of the skin-associated microbiota of teleosts is affected by exogenous factors, such as the physicochemical parameters of the aquatic environment (temperature, pH, and salinity) and, in the case of farmed fish, by other factors, including diet, treatments, handling, and stress [4,7,8]. In addition, endogenous factors, such as host genetics, health, and development stage, have been demonstrated to modulate the skin microbiota of fish as well [4,9]. The role of the skin microbiota has been related to the immune response, gas interchange, osmoregulation and excretion, and contributes to the dynamic balance between host and the bacterial community in fish [6,10,11]. A disruption in the balance of the bacterial community of the skin mucus is commonly associated with a decrease in microbial diversity and a higher prevalence of pathogenic or opportunistic bacteria [9,12,13]. Therefore, comprehensive knowledge of composition of the skin microbiota is crucial to better understand its role in fish health.

With the development of molecular techniques based on DNA extraction and the analysis of 16S rRNA gene sequences, it has been possible to determine not only the composition and diversity of the skin bacterial community but also significant differences between bacterial communities and the relative abundances of specific taxa in fish under different experimental conditions. This has allowed us to better understand the dynamics of the microbial communities associated with the skin of teleosts [12,13].

The composition of fish skin microbiota has been mostly studied using DNA-based approaches; however, important challenges must be overcome when using this methodology. Several reports have shown that the number of bacteria associated with the skin of teleosts is low [14–16]. Recently, RNA extraction has been used to study active bacteria in different microbiota communities [17–19]. The purpose of these works was to obtain a clearer view of the roles of active bacteria in the structure of bacterial communities. RNA-based approaches can be very useful for studying the microbiota communities of samples with low bacterial counts, such as skin fish, and are of great value when describing active microbiota [20].

Another issue to be considered is the collection of superficial samples. Previous researchers have separately used mucus and epithelial tissue to determine the composition of the microbiota. To increase mucus collection, those studies used swabs along the lateral line between the operculum and caudal peduncle or performed a gentle scraping of the dorsal lateral skin surface. Hence, most of those studies described mucus-associated bacteria [21,22]. Therefore, an adequate protocol for the extraction of high-quality RNA from skin samples is needed [5,12,21]. Hence, the main objective of this work was to develop a protocol to study the skin microbiota using tissue sampling, RNA extraction, and downstream procedures. The setting of the method was based on trout from an experimental unit. The obtained protocol was applied to describe the skin microbiota in trout collected from a commercial farm.

2. Materials and Methods

2.1. Samples

To set the protocol, rainbow trout skin samples were obtained from specimens reared in the experimental facilities of INTA, University of Chile. The protocol was applied to study the skin microbiota of specimens from a commercial trout farm located at Región de Los Lagos, Chile. The selected raceway to be sampled had no previous antibiotic treatment in the last 6 months and no handling procedures in the last 30 days. The skin samples were obtained using three specimens of rainbow trout reared in one raceway. The average weight of the sampled fish was 421 ± 110 g.

2.2. Skin Sampling

The sampling procedure was performed in a necropsy room under aseptic conditions. For fish euthanasia, a single net was used to place each trout in a bucket with an overdose (200 mg/L) of tricaine methanesulfonate (MS 222, Merck, Darmstadt, Germany), according to the recommendations established in the "Guide for the Care and Use Laboratory Animals of the National Institutes of Health" and the animal ethics guide of INTA, University of Chile [23]. Using gloves, each trout was handled by the gills, leaning one side on a disinfected surface to avoid touching the skin of the opposite side from which the sample was obtained. A single scalpel was used to make an incision up to the muscle in a vertical direction from the dorsal fin to the pectoral fin (using the lateral line as the midpoint), obtaining a rectangle of epithelial tissue of approximately 0.5 mm × 0.8 mm. Each skin–mucus sample was placed in a 1.5 mL Eppendorf tube with 0.7 mL of TRIzol and refrigerated at 4 °C for 12 h. Then, the samples were stored at −20 °C until processing.

2.3. Nucleic Acid Extraction

For DNA–RNA extraction, 100 mg, 200 mg, and 300 mg samples of skin were weighed and thawed at room temperature. The samples were placed in 2 mL Precellys tubes and

washed with 1 mL of PBS (phosphate-buffered saline) to remove the TRIzol. A lysozyme solution [20 mg/mL] (4 μL per 100 mg of sample) was added for 30 min at 37 °C in a thermoregulated bath, and then a proteinase K solution [20 mg/mL] was added (0.5 μL per 100 mg of sample) for one hour at 37 °C in a thermoregulated bath. Subsequently, the samples were homogenized using beads (0.1–0.5 mm) in Precellys (5000 rpm, 3 × 20 s, with 30 s pauses) to subsequently proceed to the extraction of nucleic acids using the AllPrep Powerfecal/DNA/RNA Kit (Qiagen, Hilden, Germany). Once the DNA and RNA elutions were obtained, the concentrations were quantified using a Qubit. In the case of RNA, cDNA was synthetized using random primers and a Reverse Transcription System Promega kit (Promega, Madison, WI, USA). PCR amplifications were performed as previously described [24]. PCR products were purified using a QIAquick® PCR Purification Kit (Qiagen, Hilden, Germany). Libraries were sequenced on the paired end Illumina platform Hiseq PE250 adapted for 300-bp paired-end reads at the CD Genomics (http://www.cd-genomics.com, accessed on 1 January 2023) as described [24].

2.4. Bioinformatic Analysis

The quality of the readings was determined using FastQC version 0.11.8 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/; accessed on 1 January 2023). Subsequently, duplicate sequences and chimeras were removed using the DADA 2 pipeline in RStudio version 4.1 (https://cran.r-project.org/ accessed on 1 January 2023). The taxonomic assignment was performed using the Silva database, and the sequence table (OTU) and the taxonomy table were built, using packages as described [24]. Then, the MicrobiomeAnalyst platform was used for the analysis of the reads, composition, and relative abundances [25,26].

3. Results

3.1. DNA and RNA Extractions and PCR Products

DNA and RNA were successfully extracted using the proposed protocol. The extractions yielded 60.3 ng/μL ± 13.4 in the case of DNA and 65.3 ng/μL ± 3.8 in the case of RNA. However, the DNA extracted from the skin samples rendered no amplification products. In contrast, all RNA samples rendered PCR products of the expected size.

3.2. Coverage and Sample Size

Considering the RNA extracted from the skin samples, 76,931 reads were obtained, with an average of 12,821 reads per sample. A larger number of reads and better coverage in species richness were obtained in the 300 mg samples compared to the samples of smaller sizes (100 mg and 200 mg). Therefore, the size of the sample for the protocol was defined as 300 mg of epithelial tissue (Figure 1).

3.3. Taxonomic Analysis of Skin Microbiota from Experimental Trout

At the phylum level, *Proteobacteria* (65.3%) and *Bacteroidota* (16.1%) were the most abundant phyla. Minor components included *Firmicutes*, *Actinobacteriota*, and *Fusobacteriota* (Figure 2a). At the genus level, more than 30 genera were observed, highlighting *Piscirickettsia* (46%) as the most abundant. The *Ferruginbacter* genus showed a relative abundance near 20%, while for other genera, including *Vitellibacter*, *Stenotrophomonas*, *Streptococcus*, and *Staphylococcus*, the relative abundances were less than 5% (Figure 2b).

3.4. Sequencing and Taxonomic Results from Farmed Fish

A total of 159,456 reads with an average of 53,152 reads per sample were obtained. The rarefaction curves revealed good coverage (0.99) in all samples, as shown in Figure 3. At the phylum level, *Proteobacteria* (67%) was dominant, followed by *Firmicutes* (14.3%) and *Actinobacteriota* (13%) (Figure 4a). At the genus level, more than 100 genera were observed. For the most part, a low relative abundance (<0.5%) was observed. The dominant genus was *Pseudoalteromonas* (26%), followed by *Escherichia_Shigella* (~25%). Other genera,

including *Staphylococcus*, *Corynebacterium*, *Vibrio*, *Williamsia*, and *Ralstonia*, showed relative abundances of less than 5% (Figure 4b).
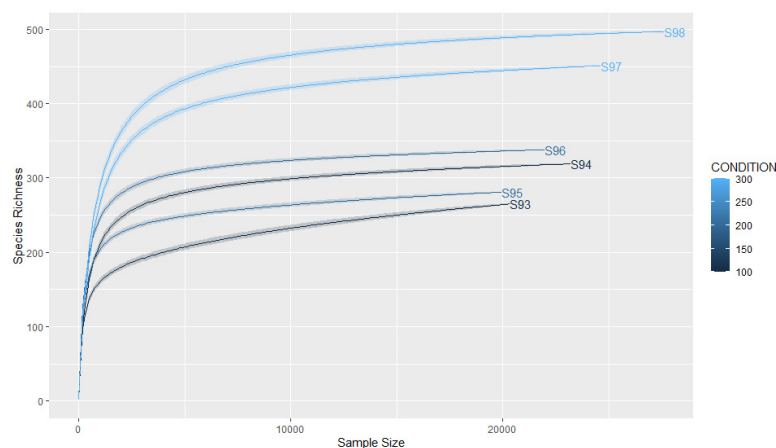

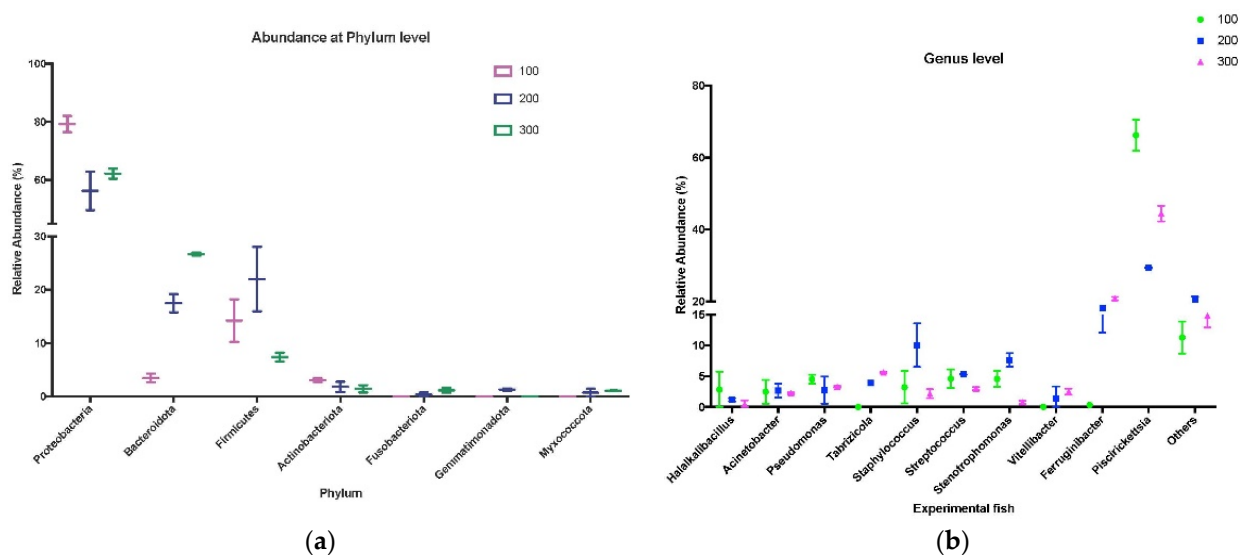**Figure 1.** Rarefaction curves obtained from the experimental samples.

(**a**)   (**b**)

Figure 2. Relative abundances at the phylum: (**a**) and genus (**b**) levels in skin samples from experimental trout. Different samples are shown in colors (a) red, blue and green correspond to samples from 100, 200 and 300 mg of samples; in (b) green, blue and red correspond to samples from 100, 200 and 300 mg of samples.
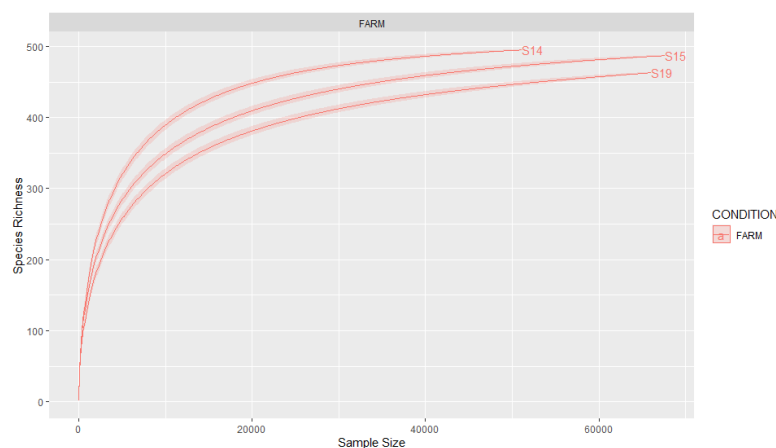

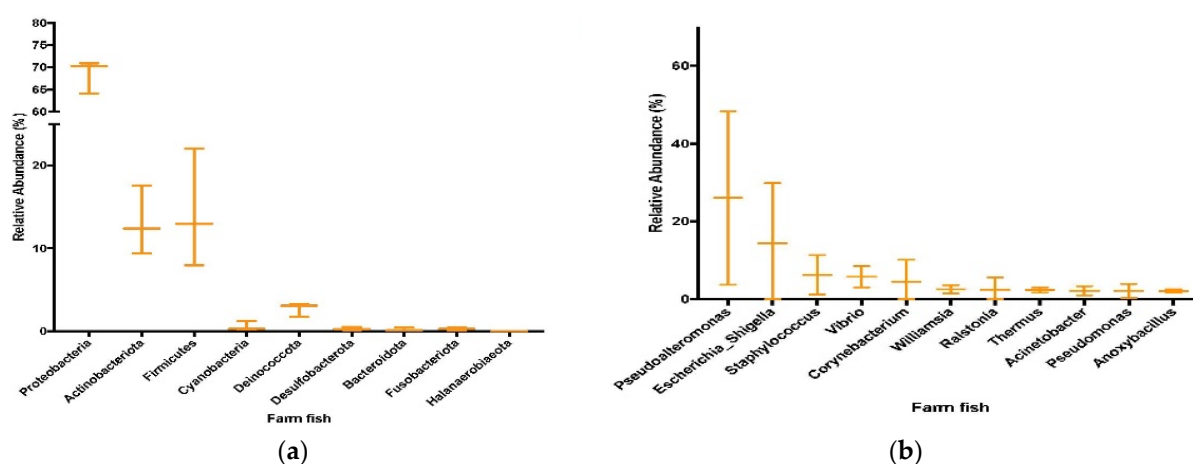**Figure 3.** Rarefaction curves obtained from the skin of farmed trout.

Figure 4. Relative abundances at the phylum: (**a**) and genus (**b**) levels from the skin of farmed trout.

4. Discussion

Microbiota from fish external surfaces have been studied by different experimental approaches, including extracting bacterial nucleic acids from different samples, such as skin mucus samples obtained by using swabs along the lateral line between the operculum and caudal peduncle, obtaining skin surface samples by gently scraping the dorsal lateral surface [27], and sampling epithelial tissue [5,21]. Most of these studies used mucus without epithelial tissue. Other authors have separately analyzed epithelial tissue (without mucus); hence, it is difficult to obtain a comprehensive description of the skin-associated microbiota [5,28,29]. In this context, our protocol proposed the sampling of a rectangular area at the height of the pectoral fin, using the lateral line as the midpoint. We then described the procedures to obtain the sample and to process the nucleic acids (Figure 5).
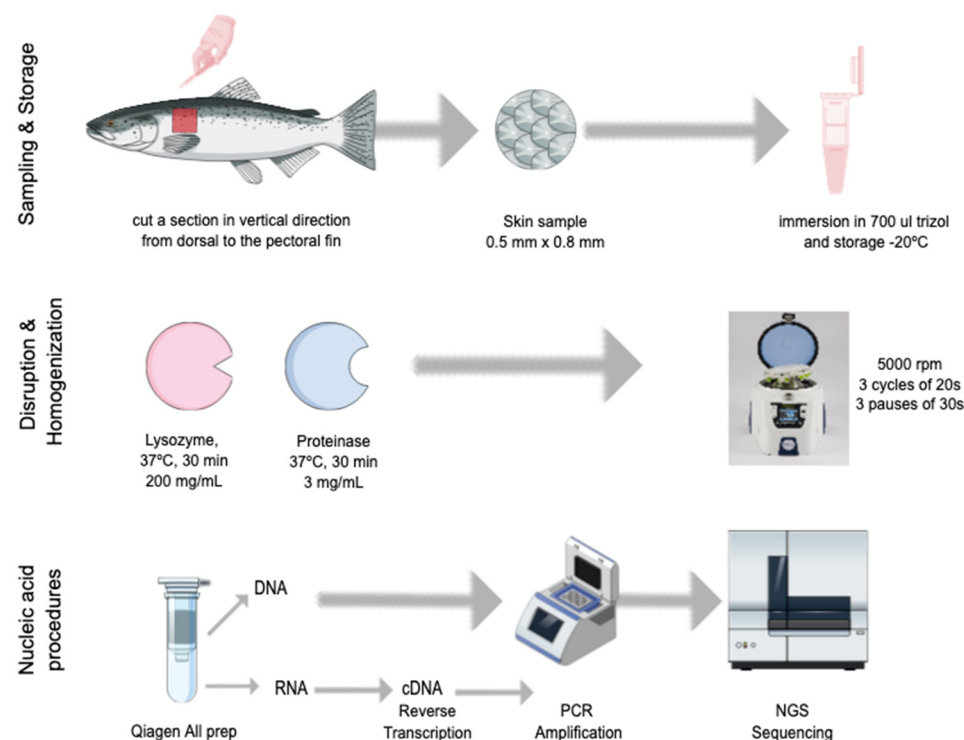

Figure 5. Protocol: Sampling and nucleic acid preparation from fish skin.

One challenge of DNA approaches is to obtain samples with sufficient bacterial biomass for PCR amplification. The minimum amount required for PCR is 1 ng/µL, or the equivalent of 10^2–10^4 DNA molecules [29,30], and the yield of DNA in our samples was

60 ng/uL; hence, it was enough to reach the minimum requirements. However, most of the DNA recovery could be derived from fish cells and not bacterial cells. Several reports have shown that the number of bacteria associated with the skin of teleosts is low; for example, in Atlantic salmon in the UK, the number is 10^2 to 10^3 per cm^2 in healthy skin, while in brown and rainbow trout, the counts are low at 10^2–10^7 per gram and 10^1–10^3 per cm^2 [14,15,18]. Hence, the range of bacterial numbers associated with healthy fish skin mucus is approximately 10^2–10^5 per cm^2, so it is difficult to collect enough genomic bacterial DNA for PCR amplification. This explains the failure in the PCR amplification when using DNA extracted from the sample as the template.

In contrast, the cDNA derived from RNA was an excellent template for PCR, with no failure, and this may be due to the copy number of the target molecules. According to the microbiology literature, the number of ribosomes in bacterial cells varies from 5000 to 35,000, depending on the growth phase [17,18]. Therefore, the RNA approach allows for an increase in the recovery rate of the template molecules by approximately 1000-fold when considering 16S rRNA molecules per bacterial cell; this is an advantage in overcoming the requirements for PCR amplification. Furthermore, the RNA extraction protocol provided good coverage in experimental and farmed samples, allowing for the description of the active bacterial community associated with rainbow trout skin.

In our study, *Proteobacteria* was the most abundant phylum in both experimental and farmed skin samples. This is in line with other studies describing *Protobacteria* as the dominant phylum in skin mucosa. Using an RNA approach, Legrand et al. [22] found that the dominant phylum among the active microbiota of the outer mucosal surface in yellow tail kingfish was *Protobacteria*. Other studies using a DNA-based approach also found *Protobacteria* to be the dominant phylum. Lokesh and Kiron [13] observed that the *Proteobacteria* phylum was dominant in Atlantic salmon (*Salmo salar*) during the transition from freshwater to seawater. Similarly, Lowrey et al. [19] observed a relative abundance of *Proteobacteria* that was higher than 50% in rainbow trout skin. At the genus level, we observed that the composition of the microbiota associated with fish skin was different in experimental and farmed fish, which is consistent with other reports reviewed by Gomez and Primm [5].

Interestingly, the high abundance of *Piscirickettsia* found in the experimental samples is concomitant with a lower level of diversity at the genus level compared with farmed trout samples. This finding is in agreement with the dominance of one possible pathogen genus decreasing the diversity of the microbiota [4,15]. The presence of *Piscirickettsia* in the trout reared in freshwater has previously been reported. In 1992, an outbreak of Salmonid Rickettsial Septicemia (SRS) affected a batch of trout in Llanquihue Lake, with 10% related mortality and veterinary diagnoses [31]. *Piscirickettsia* is the most important bacterial pathogen in salmon aquaculture in Chile, demanding most of the antibiotic treatment to control the disease [32].

Similarly, Uren Wester et al. [33] found dominant taxa including Rickettsiaceae in the skin of Atlantic salmon. Although, these authors did not mention *Piscirickettsia*, they identified six taxa within the Rickettsiaceae family, accounting for >40% of the relative abundance in several samples. They also observed a marked reduction in these taxa when the fish were transferred to the natural environment, suggesting their abundance was environmentally determined. Sylvain et al. [34] studied Amazonian fish and reported that the skin mucus and bacterioplankton communities were significantly closer in composition; hence, the skin microbiome was influenced by environmental physico-chemistry and the bacterioplankton community structure. This may explain our findings when contrasting the skin microbiota of the fish raised in the experimental unit with those raised in the farm.

Recently, Berggren [7], reported that fish skin microbiomes are almost identical within individuals. They examined samples taken from the left and right side of the same fish individuals and showed highly similar microbiota compositions. This finding supports the idea applied in our protocol that the microbiomes present in fish skin can be accurately

evaluated and described, even when only a solitary sample from a particular body region is utilized.

Skin is relevant in fish health because it is the largest tissue in the body and, in addition to being a physical and immunological barrier, it represents an ecosystem composed of a great diversity of microorganisms, most of which are harmless or are in symbiotic relationships with the host. The bacterial community associated with fish skin affects susceptibility to infections. For example, according to Lowrey et al. [19], some of the bacteria described in trout skin showed antifungal properties. Therefore, a better understanding of the active microbiota composition is needed to improve farming culture practices. In this context, the protocol developed in this work will help to overcome the limitations of low bacterial counts and provide a more precise representation of active bacteria from the skin–mucus microbiota. Using this RNA-based approach, we reported differences in the skin microbiota of trout from different origins (experimental unit and farm) which were dominated by different taxa at the genus level. It is worth noting the dominance of *Piscirickettsia* (>40%) in the skin samples of the experimental units.

Author Contributions: Conceptualization, A.P. and J.R.; methodology, A.P. writing—original draft preparation, A.P. and J.R; writing—review and editing, A.V.; funding acquisition, A.V. and J.R. All authors have read and agreed to the published version of the manuscript.

Data Availability Statement: Data will be made available upon request.

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

References

1. Benhamed, S.; Guardiola, F.A.; Mars, M.; Esteban, M.Á. Pathogen bacteria adhesion to skin mucus of fishes. *Vet. Microbiol.* **2014**, *171*, 1–12. [CrossRef] [PubMed]
2. Terova, G.; Gini, E.; Gasco, L.; Moroni, F.; Antonini, M.; Rimoldi, S. Effects of full replacement of dietary fishmeal with insect meal from *Tenebrio molitor* on rainbow trout gut and skin microbiota. *J. Anim. Sci. Biotechnol.* **2021**, *12*, 30. [CrossRef]
3. Easy, R.H.; Ross, N.W. Changes in Atlantic salmon (*Salmo salar*) epidermal mucus protein composition profiles following infection with sea lice (*Lepeophtheirus salmonis*). *Comp. Biochem. Physiol. D Genom. Proteom.* **2009**, *4*, 159–167. [CrossRef] [PubMed]
4. Reverter, M.; Tapissier-Bontemps, N.; Lecchini, D.; Banaigs, B.; Sasal, P. Biological and ecological roles of external fish mucus: A review. *Fishes* **2018**, *3*, 41. [CrossRef]
5. Gomez, J.A.; Primm, T.P. A slimy business: The future of fish skin microbiome studies. *Microb. Ecol.* **2021**, *82*, 275–287. [CrossRef]
6. Larsen, A.; Tao, Z.; Bullard, S.A.; Arias, C.R. Diversity of the skin microbiota of fishes: Evidence for host species specificity. *FEMS Microbiol. Ecol.* **2013**, *85*, 483–494. [CrossRef] [PubMed]
7. Berggren, H.; Tibblin, P.; Yıldırım, Y.; Broman, E.; Larsson, P.; Lundin, D.; Forsman, A. Fish skin microbiomes are highly variable among individuals and populations but not within individuals. *Front. Microbiol.* **2021**, *12*, 767770. [CrossRef]
8. Ingerslev, H.C.; Von Gersdorff Jørgensen, L.; Strube, M.L.; Larsen, N.; Dalsgaard, I.; Boye, M.; Madsen, L. The development of the gut microbiota in rainbow trout (*Oncorhynchus mykiss*) is affected by first feeding and diet type. *Aquaculture* **2014**, *424–425*, 24–34. [CrossRef]
9. Gomez, D.; Sunyer, J.O.; Salinas, I. The mucosal immune system of fish: The evolution of tolerating commensals while fighting pathogens. *Fish Shellfish Immunol.* **2013**, *35*, 1729–1739. [CrossRef]
10. Karlsen, C.; Ottem, K.F.; Brevik, Ø.J.; Davey, M.; Sørum, H.; Winther-Larsen, H.C. The environmental and host-associated bacterial microbiota of Arctic seawater-farmed Atlantic salmon with ulcerative disorders. *J. Fish Dis.* **2017**, *40*, 1645–1663. [CrossRef]
11. Larsen, A.M.; Bullard, S.A.; Womble, M.; Arias, C.R. Community structure of skin microbiome of gulf killifish, fundulus grandis, is driven by seasonality and not exposure to oiled sediments in a louisiana salt marsh. *Microb. Ecol.* **2015**, *70*, 534–544. [CrossRef] [PubMed]
12. Jurado, J.; Fuentes-Almagro, C.A.; Guardiola, F.A.; Cuesta, A.; Esteban, M.; Prieto-Álamo, M.J. Proteomic profile of the skin mucus of farmed gilthead seabream (*Sparus aurata*). *J. Proteom.* **2015**, *120*, 21–34. [CrossRef] [PubMed]
13. Lokesh, J.; Kiron, V. Transition from freshwater to seawater reshapes the skin-associated microbiota of Atlantic salmon. *Sci. Rep.* **2016**, *6*, 19707. [CrossRef] [PubMed]
14. Austin, B. The bacterial microflora of fish, revised. *Sci. World J.* **2006**, *6*, 931–945. [CrossRef] [PubMed]

15. Carbajal-González, M.T.; Fregeneda-Grandes, J.M.; Suárez-Ramos, S.; Cadenas, F.R.; Aller-Gancedo, J.M. Bacterial skin flora variation and in vitro inhibitory activity against *Saprolegnia parasitica* in brown and rainbow trout. *Dis. Aquat. Organ* **2011**, *96*, 125–135. [CrossRef]

16. Diler, Ö.; Altun, S.; Çalikuşu, F.; Diler, A. A study on qualitative and quantitative bacterial flora of the rainbow trout (*Oncorhynchus mykiss*) living in different fish farms. *Turk. J. Vet. Anim. Sci.* **2000**, *24*, 251–259.

17. Elekwachi, C.O.; Wang, Z.; Wu, X.; Rabee, A.; Forster, R.J. Total rRNA-seq analysis gives insight into bacterial, fungal, protozoal and archaeal communities in the rumen using an optimized RNA isolation method. *Front. Microbiol.* **2017**, *8*, 1814. [CrossRef]

18. Griffiths, R.I.; Whiteley, A.S.; O'Donnell, A.G.; Bailey, M.J. Rapid method for coextraction of DNA and RNA from natural environments for analysis of ribosomal DNA- and rRNA-based microbial community composition. *Appl. Environ. Microbiol.* **2000**, *66*, 5488–5491. [CrossRef]

19. Lowrey, L.; Woodhams, D.C.; Tacchi, L.; Salinas, I. Topographical mapping of the rainbow trout (*Oncorhynchus mykiss*) microbiome reveals a diverse bacterial community with antifungal properties in the skin. *Appl. Environ. Microbiol.* **2015**, *81*, 6915–6925. [CrossRef]

20. Blazewicz, S.J.; Barnard, R.L.; Daly, R.A.; Firestone, M.K. Evaluating rRNA as an indicator of microbial activity in environmental communities: Limitations and uses. *ISME J.* **2013**, *7*, 2061–2068. [CrossRef]

21. Caballero, S.; Galeano, A.M.; Lozano, J.D.; Vives, M. Description of the microbiota in epidermal mucus and skin of sharks (*Ginglymostoma cirratum* and *Negaprion brevirostris*) and one stingray (*Hypanus americanus*). *PeerJ* **2020**, *8*, e10240. [CrossRef]

22. Legrand, T.; Catalano, S.R.; Wos-Oxley, M.L.; Stephens, F.; Landos, M.; Bansemer, M.S.; Stone, D.A.J.; Qin, J.G.; Oxley, A.P.A. The inner workings of the outer surface: Skin and gill microbiota as indicators of changing gut health in yellowtail kingfish. *Front. Microbiol.* **2017**, *8*, 2664. [CrossRef]

23. Committee for the Update of the Guide for the Care and Use of Laboratory Animals. In *Guide for the Care and Use of Laboratory Animals*; National Academies Press: Washington, DC, USA, 2011.

24. Villasante, A.; Ramírez, C.; Rodríguez, H.; Dantagnan, P.; Hernández, A.; Figueroa, E.; Romero, J. Dietary carbohydrate-to-protein ratio influences growth performance, hepatic health and dynamic of gut microbiota in atlantic salmon (*Salmo salar*). *Anim. Nutr.* **2022**, *10*, 261–279. [CrossRef] [PubMed]

25. Chong, J.; Liu, P.; Zhou, G.; Xia, J. Using microbiomeanalyst for comprehensive statistical, functional, and meta-analysis of microbiome data. *Nat. Protoc.* **2020**, *15*, 799–821. [CrossRef]

26. Dhariwal, A.; Chong, J.; Habib, S.; King, I.L.; Agellon, L.B.; Xia, J. MicrobiomeAnalyst: A web-based tool for comprehensive statistical, visual and meta-analysis of microbiome data. *Nucleic Acids Res.* **2017**, *45*, W180–W188. [CrossRef]

27. Jung, T.S.; Del Castillo, C.S.; Javaregowda, P.K.; Dalvi, R.S.; Nho, S.W.; Park, S.B.; Jang, H.B.; Cha, I.S.; Sung, H.W.; Hikima, J.; et al. Seasonal variation and comparative analysis of non-specific humoral immune substances in the skin mucus of olive flounder (*Paralichthys olivaceus*). *Dev. Comp. Immunol.* **2012**, *38*, 295–301. [CrossRef]

28. Esteban, M.Á. An overview of the immunological defenses in fish skin. *ISRN Immunol.* **2012**, *2012*, 853470. [CrossRef]

29. Kramer, M.F.; Coen, D.M. Enzymatic amplification of DNA by PCR: Standard procedures and optimization. In *Current Protocols in Molecular Biology*; Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., Struh, K., Eds.; John Wiley & Sons, Ltd.: New York, NY, USA, 2001; Volume 56, pp. 230–257.

30. Lorenz, T.C. Polymerase chain reaction: Basic protocol plus troubleshooting and optimization strategies. *J. Vis. Exp.* **2012**, *63*, e3998. [CrossRef]

31. Gaggero, A.; Castro, H.; Sandino, A.M. First isolation of *Piscirickettsia salmonis* from coho salmon, *Oncorhynchus kisutch* (Walbaum), and rainbow trout, *Oncorhynchus mykiss* (Walbaum), during the freshwater stage of their life cycle. *J. Fish Dis.* **1995**, *18*, 277–280. [CrossRef]

32. Miranda, C.D.; Godoy, F.A.; Lee, M.R. Current status of the use of antibiotics and the antimicrobial resistance in the chilean salmon farms. *Front. Microbiol.* **2018**, *9*, 1284. [CrossRef]

33. Uren Webster, T.M.; Rodriguez-Barreto, D.; Castaldo, G.; Gough, P.; Consuegra, S.; Garcia de Leaniz, C. Environmental plasticity and colonisation history in the Atlantic salmon microbiome: A translocation experiment. *Mol. Ecol.* **2020**, *29*, 886–898. [CrossRef] [PubMed]

34. Sylvain, F.É.; Holland, A.; Bouslama, S.; Audet-Gilbert, É.; Lavoie, C.; Val, A.L.; Derome, N. Fish Skin and Gut Microbiomes Show Contrasting Signatures of Host Species and Habitat. *Appl. Environ. Microbiol.* **2020**, *86*, e00789-20. [CrossRef] [PubMed]