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REVIEW

REVIEWS IN Aquaculture

A meta-analysis revealing the technical, environmental, and host-associated factors that shape the gut microbiota of Atlantic salmon and rainbow trout

Shuowen Cao[1] | Johan Dicksved[1] | Torbjörn Lundh[1] | Aleksandar Vidakovic[1] | Parisa Norouzitallab[1] | David Huyben[2]

[1]Department of Animal Nutrition and Management, Swedish University of Agricultural Sciences, Uppsala, Sweden

[2]Department of Animal Biosciences, University of Guelph, Guelph, Canada

Correspondence
David Huyben, Department of Animal Biosciences, University of Guelph, Guelph, Canada.
Email: huybend@uoguelph.ca

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Abstract

Salmonids, specifically Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*), are commonly farmed and their gut microbiota plays important roles for optimal growth, health, and physiology. However, differences in experimental design, technical factors and bioinformatics make it challenging to compare the results from different studies and draw general conclusions about their influence on the fish gut microbiota. For a more comprehensive understanding of the gut microbiota, we collected all the publicly accessible 16S rRNA gene sequencing data with clearly stated sample metadata from freshwater Atlantic salmon and rainbow trout intestinal contents and mucosa sequenced on the Illumina MiSeq platform. A total of 783 samples from 19 published studies were included in this meta-analysis to test the impact of the technical, environmental, and host-accociated factors. This meta-analysis revealed that all the tested factors significantly influenced the alpha and beta diversities of the gut microbiota of salmon and trout. Technical factors, especially target region and DNA extraction kit, affected the beta diversity to a larger extent, while host-associated and environmental factors, especially diet and initial fish weight, had a higher impact on the alpha diversity. Salmon had a higher alpha diversity and higher abundance of *Enterococcus* and *Staphylococcus* than trout, which had higher abundance of *Weissella* and *Mycoplasma*. The results of this meta-analysis fill in a critical knowledge gap that demonstrate technical methodologies must be standardized and factors associated with host and environment need to be accounted for in the future design of salmonid gut microbiota experiments.

KEYWORDS
16S rRNA, fresh water, gut bacteria, microbiome, salmonid

1 | INTRODUCTION

The microbiota describes the collection of the microorganisms, such as bacteria, fungi, protozoa, and viruses, living in a certain environment. Microbes play key roles in the development and maintenance of different physiological functions of their eukaryotic host, in humans as well as other animals. The microbiota residing in the gut (gastrointestinal tract) within animals contributes to host nutrient

absorption,[1] metabolism,[2] aging,[3] immune system regulation,[4] and protection against pathogen invasion.[5] Meanwhile, the microbial communities are also constantly influenced by host factors, such as developmental stage,[6] health conditions,[7] and species,[8] as well as environmental factors, including temperature,[9] light,[10] and diet.[11]

In aquaculture, gut microbiota is important to aquatic animals due to their beneficial effects, especially the production of essential nutrients, for example, short chain fatty acids and vitamins. Studies have shown that intestinal microbiota are significantly affected by changes in environmental (abiotic) and host (biotic) factors, and therefore impair or promote their growth performance and health under different conditions.[12,13] Moreover, studies have shown impacts of environmental factors, including dietary composition, feed ration, temperature, rearing systems or habitat, as well as host-associated factors, including fish taxa, age, growth rates, and health status, on the gut microbiota of cultured fish in a laboratory setting as well as in the wild.[8,12–24] However, fish microbiota studies typically only investigate one or two factors per study, while controlling for several others, and it is difficult to compare studies with wide ranges in the factors mentioned above, in addition to differences in technical factors, for example, DNA extraction, PCR, and bioinformatic methods.

Salmonid fishes, particularly Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*), are two of the most commonly farmed fish species and are economically important to the global aquaculture industry.[25] However, there is a lack of research on the influence of technical, environmental, and host-associated factors on the salmonid gut microbiota, and the evaluated factors are often study specific. In this context, a meta-analysis, as a potent systematic method to re-analyse and summarize the results collected from multiple individual studies in a specific field,[26] can be applied to generalize the results of previous studies and give insights on future research within the area. To the best of our knowledge, there is no systematic meta-analysis reviewing studies focusing on the relations between salmonid gut microbiota and the potential influence of the three kinds of factors.[26]

The primary objective of this study was to perform a systematic meta-analysis on the freshwater salmonid studies to determine the effect size and rank of each technical, environmental and host-associated factors that influence the gut microbiota, specifically the alpha and beta diversities. The secondary aim was to correlate individual gut microbes with groups of these associated factors. We used the QIIME2 pipeline and SILVA 138 database to analyse 16S rRNA gene sequences from 19 studies composed of 783 samples from the gut of freshwater Atlantic salmon and rainbow trout.

2 | METHODS

2.1 | Systematic literature search

For the selection and collection of raw 16S rRNA sequence data for the meta-analysis in this study, all peer-reviewed published papers related to 'salmonid gut microbiota' were identified and then manually checked to ensure the suitability for the meta-analysis (Figure 1). The potential studies of interest were filtered by Title–Abstract–Keyword search on SCOPUS using 18 keyword combinations (['salmon', 'trout' or 'char'], ['gut' or 'intestine'], and ['microbiome', 'microbiota' or 'microbe']) and Title/Abstract search of PubMed database using the same combinations. The combined search from these two databases resulted in 229 full-text research articles published from 1 January 2011 to 31 December 2022.

Due to the low number of studies using salmonid species other than Atlantic salmon and rainbow trout, only these two species were selected for the meta-analysis (Figure 1). In addition, data collection was limited to in vivo studies sampling intestinal digesta or mucosa (rather than the whole intestinal tissue) from healthy (not obviously diseased or infected) non-triploid freshwater salmonids (Atlantic salmon before smoltification and freshwater-raised rainbow trout) without selection for any purposes in the meta-analysis to reduce the complexity and generalize the results for future research in this area. Moreover, only studies using Illumina MiSeq 16S rRNA gene sequencing were chosen because it was the most common sequencing platform. After that, 50 studies were checked for data accessibility due to the necessity of both clearly stated sample metadata and raw 16S rRNA gene sequencing data required to perform the meta-analysis. As a result, 19 studies meeting the selection criteria (Table 1) were identified for further processing and meta-analysis. All the raw 16S rRNA gene sequencing data and sample metadata were downloaded from NCBI Sequence Read Archive (SRA; https://www.ncbi.nlm.nih.gov/sra).

All the factors that potentially affected the gut microbiota were compiled and categorized for all the studies while evaluating them no matter if they were specifically addressed in the original study. After the final 19 studies (Table 1) were selected, only the 15 factors clearly stated in more than half of the studies (at least 10 studies) were analysed in this meta-analysis.

2.1.1 | 16S rRNA gene sequence data processing

All raw 16S rRNA sequence data generated by the Illumina MiSeq platform were processed by the next-generation microbiome bioinformatics platform QIIME2[42] (https://qiime2.org/) version 2022.2 following the tutorials provided by the QIIME2 team (https://docs.qiime2.org/2021.11/tutorials/). Raw sequences were imported into QIIME2, demultiplexed, end-joined, and denoised with chimera removal using QIIME2 built-in DADA2 method. To include most samples without compromising the quality of the data, the sequences were trimmed to maintain a minimum quality score of Q25. Samples with <2000 reads and taxa with fewer than 10 reads in that individual study were discarded to focus on higher abundant taxa in the meta-analysis. After that, the samples without enough replicates ($n < 5$) or proper control groups were also excluded. Then the sequences were taxonomically classified using the classifier pre-trained by RESCRIPT[43] on the full-length 16S rRNA gene SILVA v138 database[44] with a 99% confidence provided by QIIME2 (https://docs.qiime2.org/2023.5/

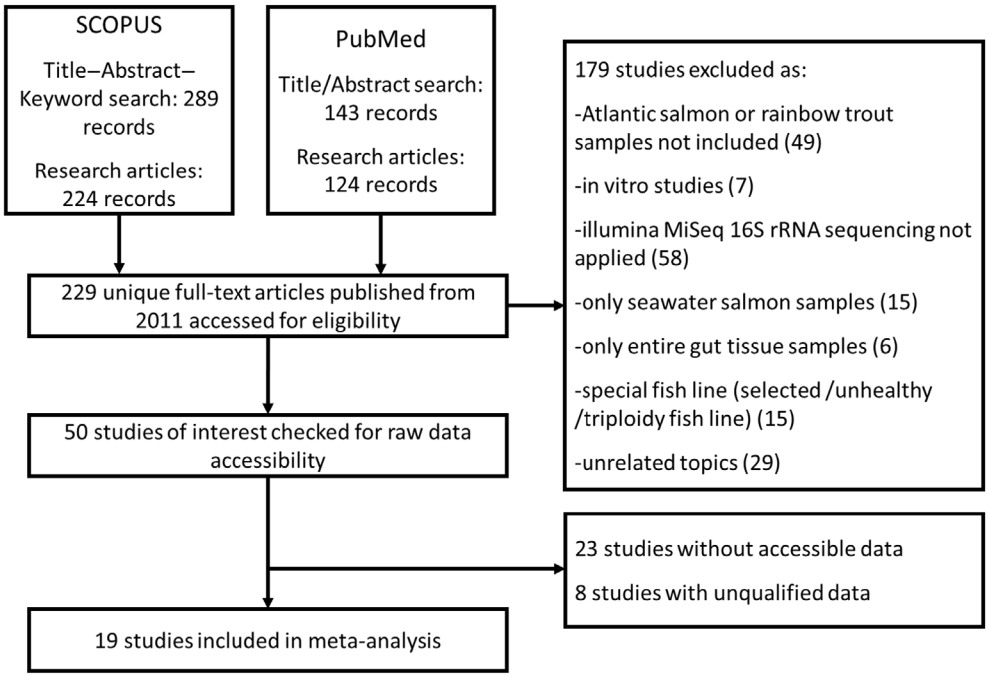


FIGURE 1 Flow chart illustrating the systematic literature search and data selection processes. The values in brackets indicate the total number of studies excluded next to each sub-criteria used.

data-resources/#public-microbiome-data). Classified sequences were taxonomically filtered to remove mitochondria, chloroplast, Archaea, and Eukaryotes. After that, all the pre-processed sequence and sample metadata were merged using QIIME2 merge commands for the following combined processing.

2.1.2 | Combined sequence data processing across studies

A phylogenetic tree was generated by QIIME2 built-in fast tree command using the merged data. The merged files were then piped to R[45] version 4.2.0 for further analysis. The data were cleaned and then filtered to discard the Amplicon Sequence Variants (ASVs) unclassified on phylum level. or with a prevalence lower than 3 throughout the whole dataset including all the samples using R tidyverse[46] ver1.3.1, stringr[47] ver1.4.0, and dplyr[48] ver1.0.9 packages. A phyloseq[49] (version 1.40.0) object was built and then all the samples were rarefied to 2838 sequences (the lowest number of sequences over 2000 sequences in one sample) to reduce the influence of sampling depths. The taxa were agglomerated on genus level for beta diversity analysis as not all the ASVs were classified on the lower level.

2.2 | Meta-analysis

Alpha diversity indexes and beta diversity distance matrixes were generated by R picante[50] ver1.8.2 and R vegan[51] ver2.6-2 and then visualized via ggplot2[52] ver3.3.6 and ggpubr[53] ver0.4.0 packages. Shapiro–Wilk normality tests[54] were used to evaluate the normality of the distribution of alpha diversity values, whereas non-parametric Kruskal–Wallis tests[55] were used to evaluate significance of the influencing factors on alpha diversity. Post hoc tests were done by Dunn tests[56] using Benjamini–Hochberg method[57] to determine pairwise differences between the groups. Faith phylogenetic diversity values were also regressed in generalized linear mixed-effects models by R lme4[58] and car[59] packages to analyse the contribution of the factors. Permutational Multivariate Analysis of Variance (PERMANOVA)[60] (9999 permutations were performed) with weighted Unifrac distance were used to evaluate significance of the influencing factors on beta diversity. The *p*-values <0.05 were considered as significant. Pairwise PERMANOVA was performed on all the factors with more than two subgroups to learn the differences between every two subgroups. Multivariate homogeneity of groups dispersions was tested by PERMDISP[61] in which both ANOVA (distances to centroids were calculated) and permutational analysis (999 permutations) were performed. Linear discriminant analysis Effect Size (LEfSe)[62] analysis results were generated by R microbiomeMarker[63] ver1.2.2 to determine the differentially abundant genera associated with a specific subgroup of a factor. Kruskal–Wallis cutoff of 0.05 and Linear Discriminate Analysis (LDA) cutoff of 2 were applied. The *p*-values were corrected by Benjamini–Hochberg method[57] to account for multiple comparisons, and q values lower than 0.05 were regarded as significant. All the results were visualized by ggplot2[52] and ggpubr[53] packages.

A total of 15 factors were analysed in this meta-analysis: paper/study, species, fish initial weight (large salmon/LS: ≥40 g; small salmon/SS: <40 g; large trout/LT: >80 g; small trout/ST: ≤80 g),

TABLE 1 Studies of interest with accessible data used for the meta-analysis on the freshwater salmonid gut microbiota.

Reference	Country	Number of samples[a]	Species	Target hypervariable region	Intestinal region	Rearing system	DNA extraction kit	Accession number
Leeper et al.[14]	Iceland	54/54	*Salmo salar*	V3–V4	Proximal and distal intestine	NA	QIAamp PowerFecal Pro	PRJNA732903
Bruni et al.[27]	Italy	10/11	*Oncorhynchus mykiss*	V3–V4	Proximal intestine	Flow-through	QIAamp Fast Stool Mini	PRJNA703401
Rimoldi et al.[28]	Italy	35/36	*Oncorhynchus mykiss*	V3–V4	Proximal and distal intestine	Flow-through	DNeasy PowerSoil	PRJEB28677
Weththasinghe et al.[29]	Norway	77/78	*Salmo salar*	V3–V4	Distal intestine	Recirculation	QIAamp Fast Stool Mini	PRJNA762510
Terova et al.[30]	Italy	12/12	*Oncorhynchus mykiss*	V3–V4	Proximal and distal intestine	Flow-through	DNeasy PowerSoil	PRJEB38845
Terova et al.[31]	Italy	24/24	*Oncorhynchus mykiss*	V3–V4	Proximal and distal intestine	Flow-through	DNeasy PowerSoil	PRJEB28677
Webster et al.[32]	UK	48/48	*Salmo salar*	V3–V4	Proximal and distal intestine	Flow-through and wild[b]	DNeasy PowerSoil	PRJEB30953
Bugten et al.[15]	Norway	38/60	*Salmo salar*	V4	Distal intestine	Recirculation	QIAamp DNA Mini	PRJEB48548
Huyben et al.[33]	Sweden	63/72	*Oncorhynchus mykiss*	V4	Distal intestine	Flow-through	QIAamp DNA Mini	PRJNA351922
Huyben et al.[34]	Canada	10/10	*Oncorhynchus mykiss*	V3–V4	Distal intestine	Flow-through	QIAamp Fast Stool Mini	PRJNA767341
Rudi et al.[35]	Norway	38/40	*Salmo salar*	V3–V4	Distal intestine	NA	LGC Mag Midi	PRJNA413667
Wang et al.[36]	Norway	16/16	*Salmo salar*	V1–V2	Distal intestine	NA	QIAamp Fast Stool Mini	PRJNA660116
Li et al.[37]	Norway	103/103	*Salmo salar*	V1–V2	Distal intestine	NA	QIAamp Fast Stool Mini	PRJNA730696
Krogdahl et al.[38]	Norway	27/27	*Salmo salar*	V1–V2	Distal intestine	Recirculation	QIAamp Fast Stool Mini	PRJNA539907
Huyben et al.[39]	Sweden	46/46	*Oncorhynchus mykiss*	V4	Distal intestine	Flow-through	QIAamp Fast Stool Mini	PRJNA454155
Huyben et al.[16]	Sweden	95/96	*Oncorhynchus mykiss*	V4	Distal intestine	Flow-through	QIAamp Fast Stool Mini	PRJNA408116
Biasato et al.[40]	Italy	12/12	*Oncorhynchus mykiss*	V4	Proximal and distal intestine	Flow-through	DNeasy PowerSoil	PRJEB51166
Hines et al.[41]	USA	15/40	*Oncorhynchus mykiss*	V4	Proximal and distal intestine	Recirculation	DNeasy PowerSoil	PRJNA750741
Baumgartner et al.[17]	UK	60/60	*Salmo salar*	V1–V2	Distal intestine	Recirculation	QIAamp DNA Mini	PRJNA800661

[a]The number before the slash indicated the number of samples that were included in the meta-analysis that passed quality filtering, while the number after the slash was the original number of gut samples collected.
[b]It is a trans-location study in which half of the wild and farmed fish were transferred to a farmed or wild environment while the other half were kept in the same environment as before. All the listed DNA extraction kits except LGC Mag Midi were manufactured by the global provider Qiagen.
Abbreviation: NA, not available.

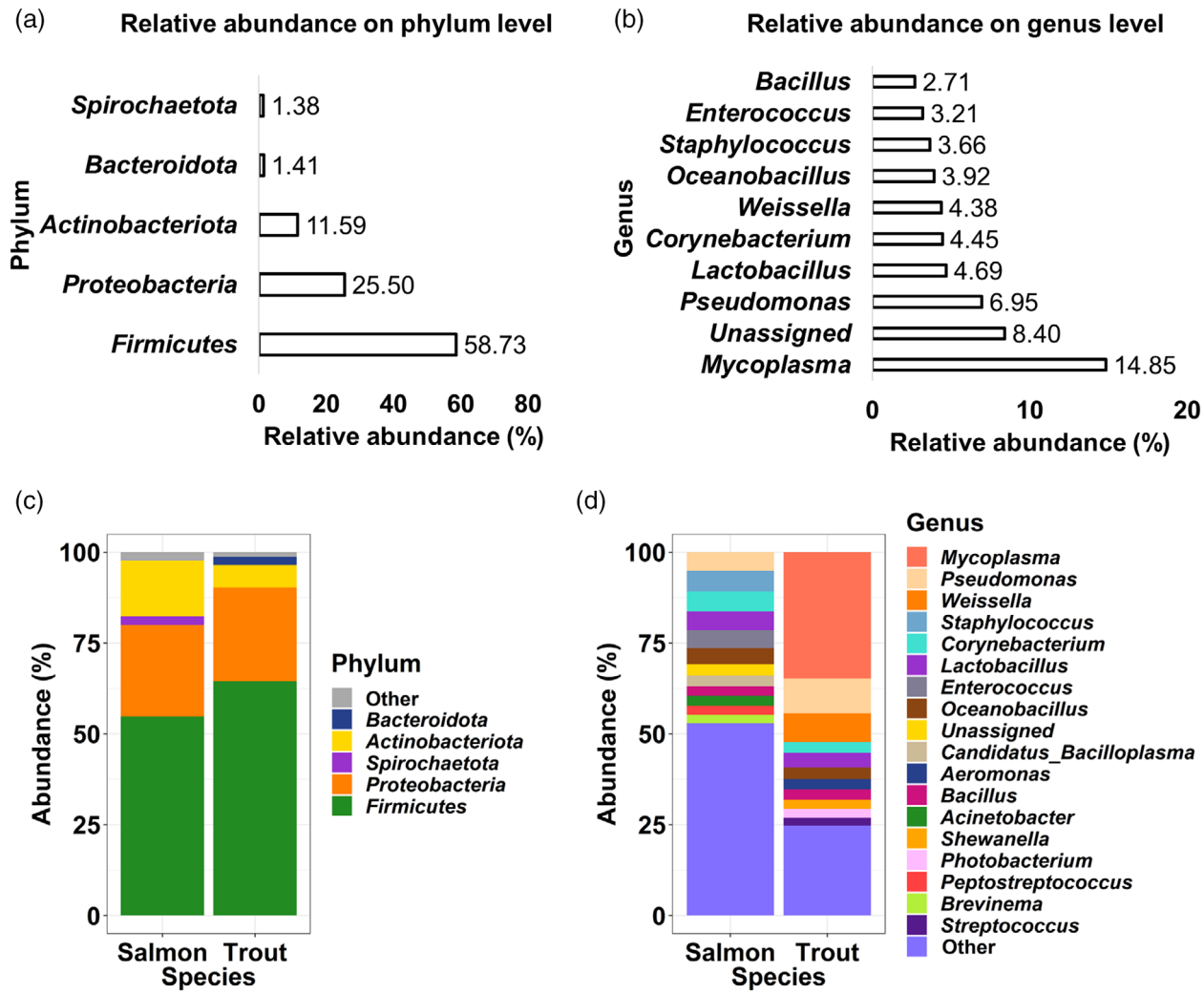


FIGURE 2 (A–D) Relative abundance of 783 gut microbiota samples from 19 freshwater salmonid studies. (A,C) phylum level. (B, D) genus level. Only the phyla more abundant than 1% and the genera with abundance values higher than 2% are shown in all four plots. The values beside the bars in plot A and B show the relative abundance of the corresponding phylum or genus. The *unassigned* is a collection of the genera unclassified on genus level from all the phyla.

specific growth rate (SGR; high: >1.2, otherwise considered as low), feed conversion ratio (FCR; high: >2, otherwise considered as low), weight gain (high: >140 g, otherwise considered as low), rearing system, daylight, temperature (high: >15°C, low: <13°C, other temperatures considered as mid), water flow rate (high: >8 L/min, otherwise considered as low), diet, target hypervariable region, intestinal segment, DNA extraction kit, and DNA polymerase.

3 | RESULTS

3.1 | Systematic literature search

Among all the 229 unique full-text studies after the combined search we excluded 49 studies using species other than Atlantic salmon and rainbow trout, 7 in vitro studies, 58 studies using sequencing platforms other than Illumina MiSeq, 15 seawater studies, 6 studies that only collected entire gut tissue samples, 15 studies using special fishlines, and 29 studies focusing on non-relevant topics. As a result, 50 studies were further checked for data accessibility. Among these, 23 studies were excluded for the absence of clearly labelled raw sequence data even after requesting assistance from the authors. Another eight studies including only sequence data of low quality (i.e., <Q25) or abundance (i.e., <2000 sequences) after processing by the uniform method described in the methods section. Only 19 studies met our requirements (Tables 1 and S1) and were included in the meta-analysis. The filtered studies included 783 samples that were represented by 7190 ASVs across 554 genera and 23 phyla.

Among all the samples, 96.8% were from Europe, while only 3.2% of the samples were collected in North America. Among the European countries, Northern European countries including Norway (38.2%), Sweden (26.1%), and Iceland (6.9%) provided 71.1% of all the samples. Exactly 58.9% of the samples were unsmoltified freshwater Atlantic salmon while 41.1% were rainbow trout. A total of 73.2% of all the

TABLE 2 The impact of the influencing factors on the alpha diversity of gut microbiota in freshwater salmonid fishes using Faith phylogenetic diversity.

Factor	Factor type	Sample size	*p*-value	Chi-squared
Paper/study	Mixed	783	<0.0001	609.08
Species	Host-associated	783	<0.0001	375.10
Initial weight	Host-associated	706	<0.0001	369.36
Rearing system	Environmental	572	<0.0001	357.53
Flow rate	Environmental	406	<0.0001	220.93
FCR	Host-associated	315	<0.0001	209.05
Target hypervariable region	Technical	783	<0.0001	208.17
Daylight	Environmental	549	<0.0001	205.48
SGR	Host-associated	356	<0.0001	192.67
Weight gain	Host-associated	193	<0.0001	135.44
Diet	Environmental	745	<0.0001	132.63
DNA extraction kit	Technical	783	<0.0001	127.98
Temperature	Environmental	680	<0.0001	126.48
Intestinal region	Host-associated	783	<0.0001	114.35
DNA polymerase	Technical	713	<0.0001	55.76

Note: The *p*-values and chi-squared values were generated from Kruskal–Wallis tests.
Abbreviations: FCR, feed conversion ratio; SGR, specific growth rate.

samples were distal intestine samples, whereas 25.5% were from both proximal and distal intestine and 1.3% were from the proximal intestine. As for the target hypervariable regions, 39.3% of the samples targeted V3–V4 region, followed by 34.4% of them targeting V4, while samples targeting region V1–V2 consisted of 26.3% of all the samples.

3.2 | General microbiota characteristics

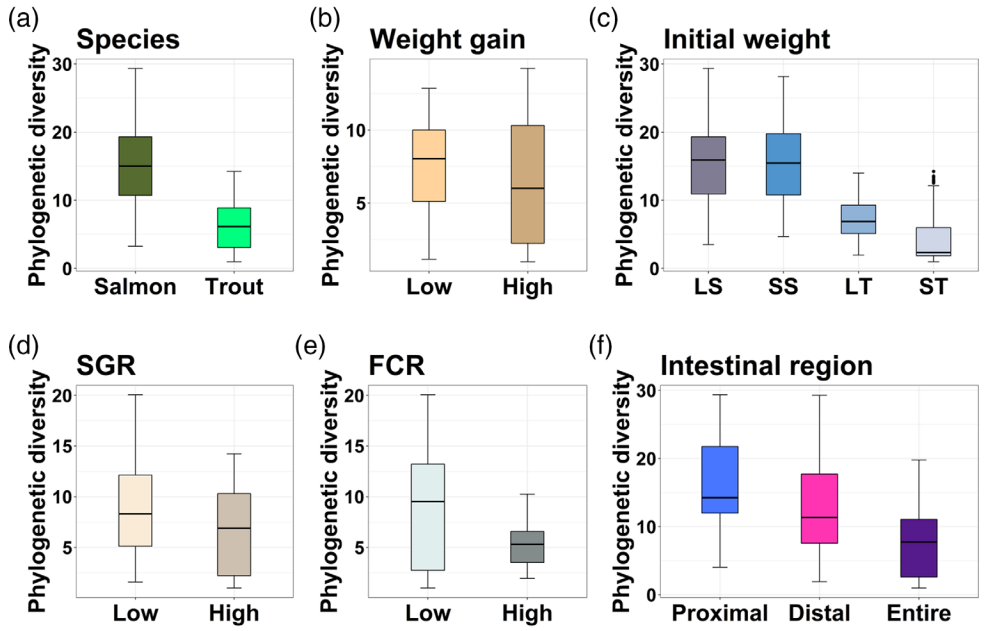
Among the 23 phyla present in the samples, three phyla dominated the salmonid gut microbiota with a total relative abundance of over 95.8% (Figure 2). Firmicutes contributed about 58.7% of the abundance as the most abundant phylum, followed by Proteobacteria that accounted for ~25.5% and Actinobacteria consisted of around 11.6% of the abundance. The alpha diversity indices were calculated after the ASVs were rarefied to 2838, which reduced the library size differences among the samples from different studies to facilitate alpha diversity comparisons.

Shapiro–Wilks test indicated Faith phylogenetic diversity (Faith PD) of the samples was not normally distributed ($W = 0.966$, $p < 0.001$), so non-parametric Kruskal–Wallis tests were performed to identify the significance of the factors. According to the Kruskal–Wallis tests, all 15 factors had significant effects on Faith PD (Table 2). Intestinal microbiota in Atlantic salmon had significantly higher Faith PD than the rainbow trout counterpart ($p < 0.001$, chi-squared = 375.1; Table 2 and Figure 3a). Furthermore, initial weight did not differentiate the Faith PD of either Atlantic salmon subgroups or rainbow trout subgroups significantly even though separated the species ($p < 0.001$, chi-squared = 369.4; Table 2 and Figure 3c). Regarding rearing systems, the recirculating system showed significantly higher Faith PD compared with both wild and flow-through counterparts, while the flow-through system had the lowest alpha diversity ($p < 0.001$, chi-squared = 357.5; Table 2 and Figure 4d). The samples collected from the mid-temperature ($p < 0.001$, chi-squared = 126.5; Table 2 and Figure 4b; Data S1) or high water flow rates ($p < 0.001$, chi-squared = 220.9; Table 2 and Figure 4c) had significantly higher Faith PD values than the others. As for the target hypervariable regions, primers targeting V1–V2 allowed for significantly higher phylogenetic diversity than V3–V4 and V4, whereas V4 alone gave the lowest phylogenetic alpha diversity ($p < 0.001$, chi-squared = 208.2; Table 2 and Figure 5b; Data S1).

In addition, Faith PD values were analysed by generalized linear mixed-effects models. In the best-fit model with the lowest Bayesian Information Criterion (BIC) value,[64] factor paper/study was considered as a random effect variable, while all other factors as fixed effect factors. The factors with low significance ($p > 0.05$) were removed one by one to find the factors making big impacts on Faith PD values. After the factors were selected, the interactions among the factors were also investigated. According to the ANOVA results (Table 4 and Data S1), species (chi-squared = 6.57, *p*-value = 0.01) and intestinal region (chi-squared = 8.19, *p*-value = 0.02) had significant influence on Faith PD, whereas all the other factors in the model were not significant.

Similar to alpha diversity, PERMANOVA also showed significant influences of all 15 factors on weighted UniFrac distances. Apart from that, group dispersions of all the factors except for factor species showed significant inhomogeneities in betadisper results (Figure S1).

The PERMANOVA of weighted UniFrac distances revealed a significant association between the microbiota of the gut samples and all



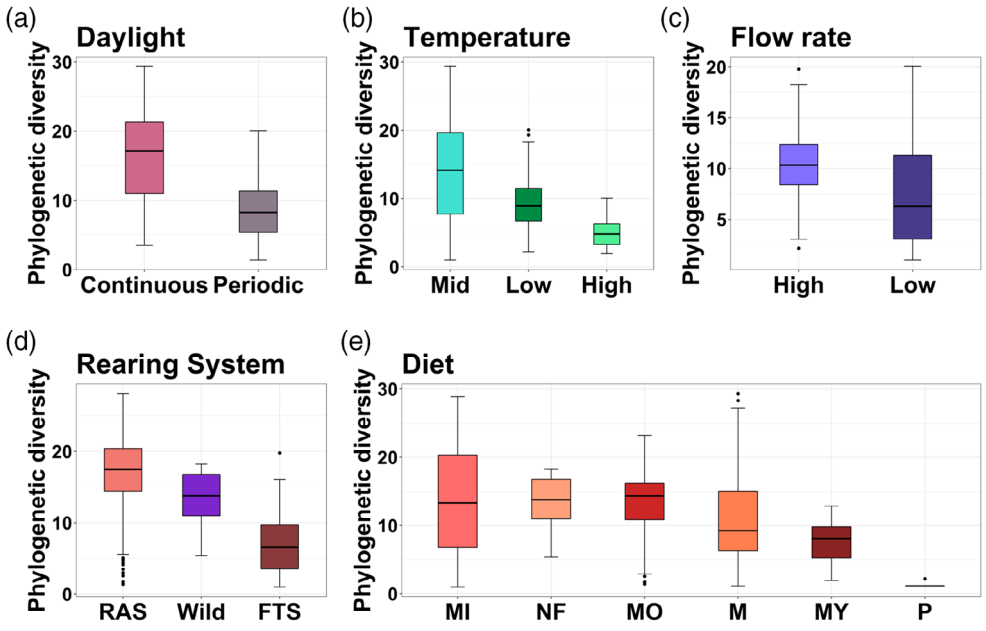
Tag	Factor	*p* value	Chi squared	Tag	Factor	*p* value	Chi squared
A	Species	<0.0001	375.1	D	SGR	<0.0001	192.7
B	Weight gain	<0.0001	135.4	E	FCR	<0.0001	209.0
C	Initial weight	<0.0001	369.4	F	Intestinal region	<0.0001	114.4

FIGURE 3 (A) Boxplots of Faith phylogenetic (alpha) diversity of 783 gut microbiota samples from 19 freshwater salmonid studies (host-associated factors). (B) Weight gains higher than 140 g are regarded as high, otherwise considered as low. (C) 'LS' stands for large salmon weighing at least 40 g while small salmon weighing lower than 40 g are labelled as 'SS'. 'LT' stands for large trout weighing more than 80 g while small trout weighing not more than 80 g are labelled as 'SS'. (D) specific growth rates higher than 1.2 are considered as high, otherwise regarded as low. (E,F) Feed conversion ratios higher than 2.0 are considered as high, otherwise considered as low. The table below the plots provides the *p*-values and chi-squared values from Kruskal–Wallis tests.

the technical factors including scientific paper/study, target hypervariable region, DNA extraction kit, and DNA polymerase. Technical factors explained most of the variation in data with significant inhomogeneities. Paper/study was the dominant factor explaining over 60% of the variance of the beta diversity (Table 3), which is more than twice the value for the primer target hypervariable region in the second place. In the weighted UniFrac beta diversity PCoA plot (Figure 6), PC1 explained 52.3% of the variation and the data points from each study were clustered together with half the studies overlapping while the other half clustered separately. Similarly, the target hypervariable region was also an important driver of the clustering of the intestinal microbiota (Figure 7f).However, in the best-fit generalized linear mixed-effects model, no technical factors was included (Table 4). In order to reveal which taxa that was most associated with the different technical factors, LEfSe at 0.05 significance level was applied. The LEfSe identified diverse microbial genera associated with different target hypervariable regions (Figure 8). High *Lactobacillus* abundance was significantly associated with region V1-V2 (Figure 8f and Table S2), while an enrichment of *Staphylococcus* instead was related to the V3–V4 region. In the samples targeting region V4, *Mycoplasma*, *Pseudomonas*, and *Weissella* were significantly more abundant.

3.3 | Environmental influence on salmonid gut microbiota

Among the other factors, the environmental factors explained more variance of the beta diversity than the host-associated factors. The most explanatory environmental factor was diet accounting for over 18.6% of the total beta diversity variance, while the rearing system, water flow rate, daylight, and the intestinal region explained around 11.6%–15.2% of the beta diversity variance (Table 3). Several abundant genera were identified using LEfSe that were associated with different dietary types. *Mycoplasma* was associated with plant-based diet, whereas the fish without extra feed supply related to high abundance of *Candidatus_Bacilloplasma* and *Brevinema* (Table S2). Marine-based feeds with different substitutes or supplements were also related to diverse bacterial groups. *Enterococcus*, *Weissella* and *Staphylococcus*, and *Pseudomonas* were enriched in the samples fed marine-based feeds with inclusion of insects, yeasts, and other ingredients not originally in the feeds, respectively (Table S2). The second most explanatory environmental factor was rearing system (Table 3 and Figure 7b) that showed recirculating aquaculture (RAS) and flow through systems (FTS) as separate clusters but with some overlap. Moreover, diet, temperature, and rearing system were also included in



Tag	Factor	*p* value	Chi squared	Tag	Factor	*p* value	Chi squared
A	Daylight	<0.0001	205.5	D	Rearing system	<0.0001	357.5
B	Temperature	<0.0001	126.5	E	Diet	<0.0001	132.6
C	Flow rate	<0.0001	220.9				

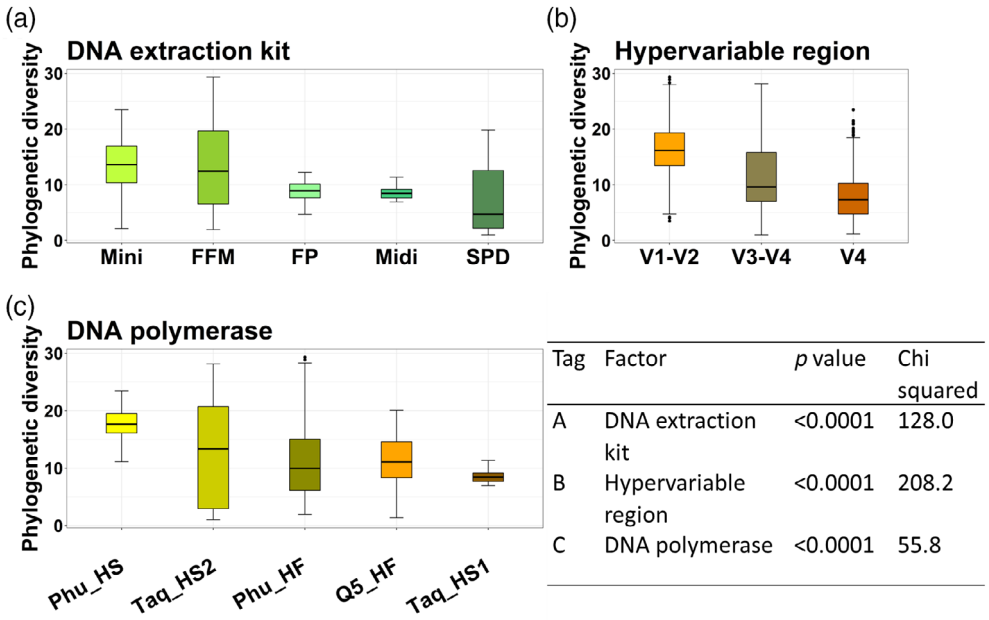
FIGURE 4 (A) Boxplots of Faith phylogenetic diversity of 783 gut microbiota samples from 19 freshwater salmonid studies (environmental factors). (B) Temperatures lower than 13°C are considered as low temperature, while 'High' indicates temperatures higher than 15°C. All other temperatures are considered as mid temperatures. (C) Water flow rates higher than 8 L/min were categorized as high, otherwise considered as low. (D) 'RAS' stands for recirculating aquacultural system, while 'wild' indicate a wild-like environment, and 'FTS' is the flow-through system. (E) 'M' stands for marine-based feeds. 'MI', 'MY', and 'MO' indicate marine-based feeds with inclusions of insects, yeasts, and other nutrient sources such as other prebiotics or oils. 'P' indicates plant-based feeds, whereas 'NF' indicates a wild-like environment without any feed provided. The table below the plots provides the *p*-values and chi-squared values from Kruskal–Wallis tests.

the best-fit model, though without any significance (Table 4). ANOVA (Data S1) showed that no feed diet (co-efficient = 2.06, *t*-value = 1.84) and mid-temperature (co-efficient = 2.78, *t*-value = 0.94) had higher impacts on Faith PD. Several abundant genera were identified using LEfSe that were associated with different rearing systems. *Mycoplasma*, *Weissella*, and *Pseudomonas* were enriched in the gut samples collected from the FTS, whereas *Bacillus* and *Enterococcus* were significantly more abundant in RAS (Figure 8d). There are also some genera related to the wild environment, such as *Candidatus_Bacilloplasma*, *Aeromonas*, *Brevinema*, and unassigned genera in *Enterobacterales* (Figure 8d).

Most of the abundant genera associated with diet and rearing system were also related to other environmental influencing factors including water flow rate (Figure 8e), daylight (Table S2), intestinal region (Table S2), and water temperature (Figure 8b). Unlike diet and rearing system, these factors only explained <14.2% of the beta diversity variance (Table 3). *Mycoplasma* and *Pseudomonas* were abundant in fish living in low flow rate water, while *Weissella*, *Candidatus_Bacilloplasma*, *Lactobacillus*, *Brevinema*, and *Streptococcus* were associated with high water flow rate (Figure 8e). *Staphylococcus*, *Enterococcus*, and *Oceanobacillus* were related to continuous daylight, whereas *Weissella* and *Pseudomonas* were abundant in the fish living in environments with periodic light (Table S2). *Pseudomonas* was found associated with the distal intestine, while *Mycoplasma* and *Candidatus_Bacilloplasma* were abundant in the entire gut samples (Table S2). Many genera were related to the proximal intestine, such as *Oceanobacillus*, *Lactobacillus*, *Phyllobacterium*, and *Enterococcus* (Table S2). High temperature was associated with *Mycoplasma and Pseudomonas*, whereas *Staphylococcus* were abundant in the samples collected from the fish living in environments of the low temperatures below 13°C (Figure 8b).

3.4 | Host-associated influence on salmonid gut microbiota

Host-associated factors, such as initial weight and species, only had minor influences on beta diversity. The most explanatory host-associated factor was initial weight and only accounted for 16.0% of the variation, whereas the other host factors (i.e., SGR, FCR, species, and weight gain) explained <11.0% of the beta diversity variation (Table 3). However, host-associated factors species (chi-squared = 6.57, *p*-value = 0.01; Table 4) and intestinal region (chi-squared = 8.19, *p*-value = 0.02; Table 4) had significant influence on



Tag	Factor	*p* value	Chi squared
A	DNA extraction kit	<0.0001	128.0
B	Hypervariable region	<0.0001	208.2
C	DNA polymerase	<0.0001	55.8

FIGURE 5 Boxplots of Faith phylogenetic diversity of 783 gut microbiota samples from 19 freshwater salmonid studies (technical factors). (A,B) 'Mini' and 'Midi' indicate the products designed for the extraction of microbial DNA from small amounts of samples. 'FFM' stands for the DNA fast extraction kit designed for small amounts of faecal samples, while 'FP' indicates the method designed for faecal samples with a built-in bead-beating step. 'SPD' stands for the DNA extraction kit designed for soil samples with a built-in bead-beating step and an addition of DNase. (C) 'Q5', 'Phu', and 'Taq' indicate Q5, Phusion, and Taq DNA polymerases followed by 'HS' or 'HF' which stands for hot start or high-fidelity characteristics. The numbers only show products from different companies. The table beside the plots provides the *p*-values and chi-squared values from Kruskal–Wallis tests.

TABLE 3 The impact of the influencing factors on the beta diversity of gut microbiota in freshwater salmonid fishes using weighted UniFrac and PERMANOVA.

Factor	Factor type	Sample size	*p* value	*R* squared	Variance explained (%)	Pseudo-*F*
Paper/study	Mixed	783	<0.001	0.618	61.8	68.57
Target hypervariable region	Technical	783	<0.001	0.244	24.4	125.90
DNA extraction kit	Technical	783	<0.001	0.191	19.1	46.00
Diet	Environmental	745	<0.001	0.187	18.7	29.75
DNA polymerase	Technical	713	<0.001	0.173	17.3	32.46
Initial weight	Host-associated	706	<0.001	0.160	16.0	37.02
Rearing system	Environmental	572	<0.001	0.152	15.2	46.41
Flow rate	Environmental	406	<0.001	0.141	14.1	64.26
Daylight	Environmental	549	<0.001	0.123	12.3	54.48
Intestinal region	Host-associated	783	<0.001	0.116	11.6	51.26
SGR	Host-associated	356	<0.001	0.110	11.0	48.10
FCR	Host-associated	315	<0.001	0.090	9.0	38.16
Species	Host-associated	783	<0.001	0.081	8.1	68.45
Temperature	Environmental	680	<0.001	0.075	7.5	20.98
Weight gain	Host-associated	193	<0.001	0.061	6.1	25.51

Note: 9999 permutations were performed in each of the PERMANOVA tests to obtain the *p* value, *R* square, and pseudo-*F* values.
Abbreviations: FCR, feed conversion ratio; SGR, specific growth rate.

Faith PD. Moreover, trout showed the highest impact among all the subgroups, with a co-efficient of −8.05 and *t*-value of −2.56 (Data S1), followed by both proximal and distal intestinal region (co-efficient = −5.97, *t*-value = −2.17; Data S1). Large salmon was associated with *Lactobacillus*, *Oceanobacillus*, and *Corynebacterium*, while *Staphylococcus* was abundant in small salmon (Table S2). In contrast to

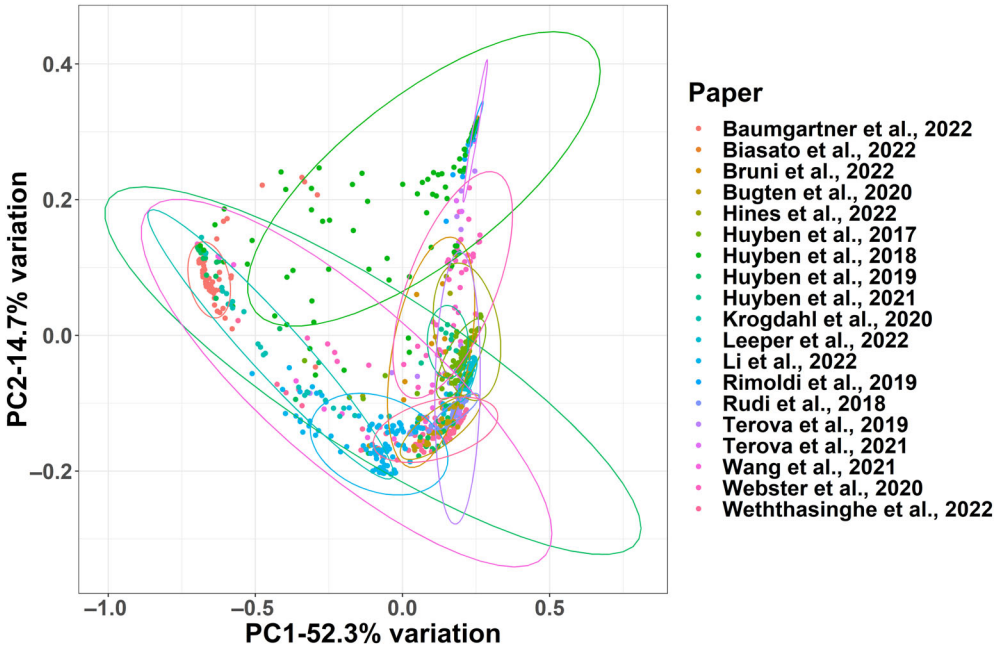


FIGURE 6 Principal coordinate analysis (PCoA) plot of weighted UniFrac distance of 783 gut microbiota samples from 19 freshwater salmonid studies (beta diversity). The studies are represented by different colours. Circles represent 95% confidence intervals.

salmon, different bacterial groups were found enriched in trout. Large trout were associated with *Pseudomonas* and *Weissella*, whereas *Mycoplasma* and *Shewanella* were abundant in small trout. For host-associated factors including specific growth rate (SGR), feed conversion ratio (FCR), and weight gain (WG), only a few genera were found to differ in abundance among the subgroups. *Shewanella* and *Mycoplasma* were associated with high SGR, while *Pseudomonas* was abundant in low SGR fish guts (Table S2). *Mycoplasma*, *Pseudomonas*, and *Aeromonas* were related to high FCR, while *Weissella* were identified as enriched groups associated with low FCR (Table S2). High WG was associated with *Shewanella* and *Mycoplasma*, while *Pseudomonas*, *Oceanobacillus*, and *Corynebacterium* were abundant in the fish with low WG (Table S2). Species only explained <8.1% of the variance but drove the separate clustering of gut microbiota between Atlantic salmon and rainbow trout in two directions in the PCoA plot (Table 3 and Figure 7a). As for the abundant microbial genera associated with these two species, *Mycoplasma* and *Weissella* were highly related to rainbow trout, while *Staphylococcus* and *Enterococcus* were associated with Atlantic salmon samples (Figure 8a).

4 | DISCUSSION

In this meta-analysis, we comprehensively collected all the available 16S rRNA gene sequence data from Atlantic salmon and rainbow trout from the literature, and then we filtered the data based on predefined criteria, e.g. high quality, well labelled and available data. The selected data were re-analysed using a standard set of parameters and the same bioinformatics tools were used for all the studies to minimize the bias arising from the experimental and analytical procedures. Our aim was to determine the contribution of technical, environmental, and host-associated factors that influenced the gut microbiota of salmonid fishes. In all the 783 samples from 19 studies, a dominant presence of Firmicutes, Proteobacteria and Actinobacteria on the phyla level indicated a core microbiota (Figure 2), which has been identified as dominant phyla in other salmonid (Salmonidae family) and ray-finned (Sparidae family) fish species, for example, Arctic charr[11] and gilthead sea bream, respectively.[65] In contrast, the gut microbiota of cyprinid (Cyprinidae family) fish species has been dominated more by Proteobacteria than Firmicutes, such as in Nile tilapia,[66,67] or dominated by Fusobacteria, for example, in common carp.[68,69] The differences in the core microbiota between genetic families of fish species are highly related to the host conditions (e.g. genetic and physiological divergences), the disparate environment they live in (e.g. water microbiota),[70] as well as the long-playing co-evolution between the host species/genus/family and their gut microbiota.[71]

Our meta-analysis indicated that paper/study is the overall most dominant factor that affects both the alpha and beta diversity of gut microbiota in freshwater salmonids (Table 3 and Figure 6). In addition, all the factors that were evaluated in this meta-analysis had a significant effect, which was similar to previously reported factors in the meta-analysis on the microbiota of shrimp.[72] A meta-analysis on the gut microbiota of 1046 healthy humans from around the world found that most factors influenced the beta diversity similar to the present study, specifically the environment (e.g. diet and housing) explained up to 20% of the variation whereas host effects (e.g. ancestry) had minor affects.[73] Aside from the paper/study, technical factors including target hypervariable region and DNA extraction kit were also dominant factors in shaping the beta diversity of

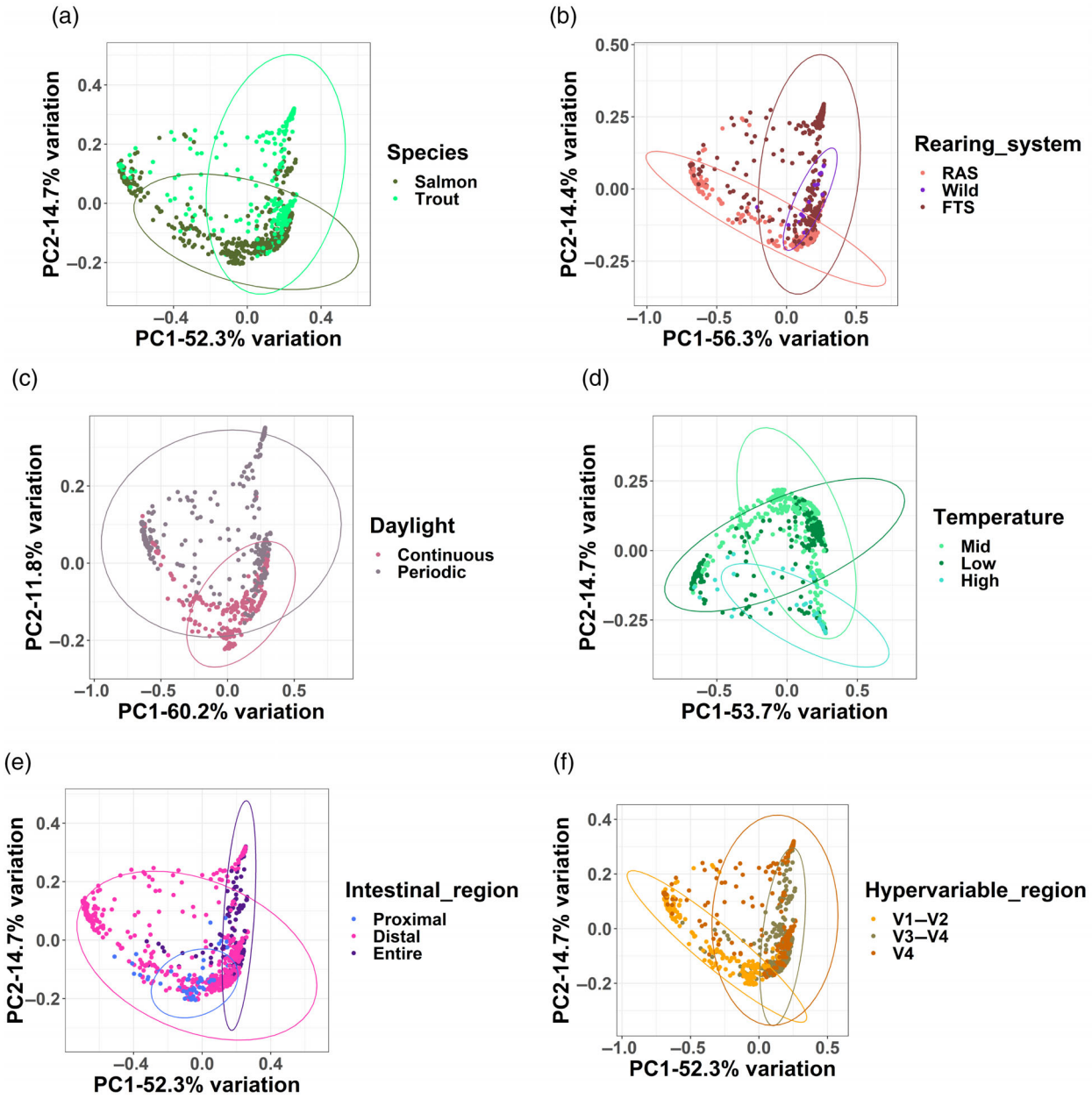


FIGURE 7 (A–F) Principal coordinate analysis (PCoA) plots of weighted UniFrac distances of 783 gut microbiota samples from 19 freshwater salmonid studies (beta diversity). Samples grouped by the host-associated factor (species), environmental factors (rearing system, daylight, water temperature, and sampled intestinal region), and technical factors (target hypervariable region). Circles represents 95% confidence intervals. 'RAS' stands for recirculating aquaculture system, while 'Wild' indicates a wild-like environment, and 'FTS' stands for flow-through system.

salmonid gut microbiota, but not in shaping the alpha diversity counterpart (Figures 5 and 7; Tables 2 and 3). In the studies testing the effects of target hypervariable regions and DNA extraction kits using the stool samples from humans and mice, significant shifts of microbiota composition related to experimental conditions were also found.[74] In addition, DNA extraction kits have been found to change the microbial compositions in faeces between zebrafish, horses, dogs, cats, and mice.[75] Hart et al.[75] suggested that the size of zebrafish and their intestine compared with the faecal biomass could change the yield of microbial DNA extracted using commercial kits since smaller intestines would have a higher proportion of host compared to bacterial DNA. These authors also mentioned that the fibre content in the diet, time post feeding and digestive enzymes could change the amount of faecal biomass and consequently the amount of DNA to be extracted. The variation in the amount of microbial DNA could bias the amplification efficiency of either target hypervariable region during PCR and sequencing of 16S rDNA. Therefore, the DNA extraction kit and target region would influence more of which microbes are identified rather than their diversity. In contrast, a study on human faeces found that DNA extraction method had little effect on microbial communities, while the target region had an immense impact.[76] The results shown in the present study revealed that both DNA

TABLE 4 The significance of the top influencing factors in the best-fit generalized linear mixed-effects model.

Factor	Chi-squared	*d.f.*	*p*-value
Species	6.57	1	0.01
Intestinal region	8.19	2	0.02
Diet	7.24	6	0.30
Temperature	2.17	3	0.54
Weight gain	0.22	2	0.90
Rearing system	0.19	2	0.91
SGR	0.01	2	1.00

Note: Type 3 ANOVA was performed to obtain the *p*-values and chi-squared values.
Abbreviations: d.f.: degree of freedom; SGR, specific growth rate.

extraction kit and target region had a high impact on beta diversity of salmonid gut microbiota, in line with the findings of the studies on other animals. Therefore, we suggest that these technical factors should be standardized across studies in order to improve comparisons of the microbiota between studies. If not, the influence of these technical factors should be considered when comparing the results from future studies.

The environmental and host-associated factors across the 19 studies had a large impact on the alpha diversity of salmonid gut microbiota (Figures 4 and 5). Among these factors, the environmental factor of diet ranked fourth place in explaining the variance of the beta diversity (Table 3) and this was visualized by the clustering of salmonid gut microbiota per diet (Figure S1). Aside from the large significant effects on beta diversity, diet only had a significant and moderate impact on alpha diversity (Figure 4e). The alpha diversity of the insect-fed salmonids was numerically higher than the fish fed with commercial marine-based feeds (Figure 4e), which aligns with a previous meta-analysis on fish fed with black soldier fly larvae (*Hermetica illucens*).[77] Previous studies have suggested that the presence of chitin in diets containing insects leads to an enrichment of chitinase-producing bacteria that would not normally be present, hence increasing microbiota richness.[39] Yeast based diets have been found to have beneficial effects on the gut health and microbiota of rainbow trout,[16] so it was unexpected to see the low level of alpha diversity (Figure 4e). Alpha diversity of the samples collected from the fish fed plant-based diets was significantly lower than in all the other groups, probably due to higher levels of antinutritional factors, such as phytate and saponins, that reduce microbial growth as previously reported.[78] Diet has also had similar effects on alpha and beta diversity in other non-salmonid fish species.[79,80]

Interestingly, environmental factors of rearing system, water flow rate and daylight were the next most impactful factors on beta diversity after diet (Table 3). In contrast to diet, these three factors showed large effects on alpha diversity rather than beta diversity (Figure 4). Daylight may be conflated with season and life-stage of the fish since young fry and fingerlings tend to receive continuous daylight while older broodstock may require shorter periods of daylight to stimulate breeding events. Higher alpha diversity in recirculation systems (Figure 4d) was expected since the bacterial load in the water entering the rearing tanks of recirculation systems is much higher than the counterpart in flow-through systems.[81] The higher hydraulic retention time without ozone or UV disinfection in the recirculation system results in a higher possibility that slow-growing microbes may stay longer and even grow after the initial disinfection.[21,82] In addition, the maturity of the biofilter can play a role in modulating the microbiota in recirculation systems.[83] Water temperature is better controlled in recirculation systems, resulting in different microbial communities compared to flow-through systems vulnerable to seasonal changes in temperature. However, the effect of temperature was one of the lowest factors influencing beta diversity in this meta-analysis (Table 3). This may be explained by the relatively low water temperatures salmonid fishes are typically reared compared with warm water fishes, for example, tilapias and carps.

Among the host-associated factors, initial weight, which was largely correlated by the life stage or the age of the fish, had a smaller impact on beta diversity compared with the top five factors that were environmental or technical (Table 3), although it was very similar to the effect of diet. Notably, limited to only freshwater samples, the salmon before smoltification were younger and therefore smaller than the trout counterpart, thus different weight ranges were applied while translating weight values into categories (large salmon: not lower than 40 g, large trout: higher than 80 g) based on the general condition of the fish weights. The effect of life-stage (weight or age) has been found previously to be a more influential factor than location or rearing system and water temperature for wild Atlantic salmon and Chinook salmon (*Oncorhynchus tshawytscha*).[22,84] Previous studies on Atlantic salmon have also found that alpha diversity decreases as the fish ages due to their reduced ability to filter microbial communities as they mature.[65,84,85]

In the best-fit generalized linear mixed model, all seven fixed effects were either host-associated or environmental factors, which is also in line with our finding that environmental factors have larger impacts on alpha diversity while technical factors impact beta diversity to a larger extent (Tables 2 and 3). Among the seven fixed effects, host-associated factors of species and intestinal region showed significant effects on shaping Faith PD of fish gut microbiota. It was expected that the factor of species has a significant influence on alpha diversity as previously reported,[86] and the statistics on Faith PD (Table 2) also supported this. However, according to the Kruskal–Wallis tests (Table 2), the intestinal region had the lowest chi-squared value among all the host-associated and environmental factors. The difference may be caused by the other factors in the model, such as random effect of paper/study or other fixed effects. Moreover, interactions among the factors were also investigated. All possible interaction effects between the seven fixed effects were tested, but no significant interactions were found. That did not mean these factors did not interact with each other, it is still possible that there are significant interactions between the factors not included in the best-fit model, or that the interactions were hidden by the simple linear regression. Regarding beta diversity analysis, significant

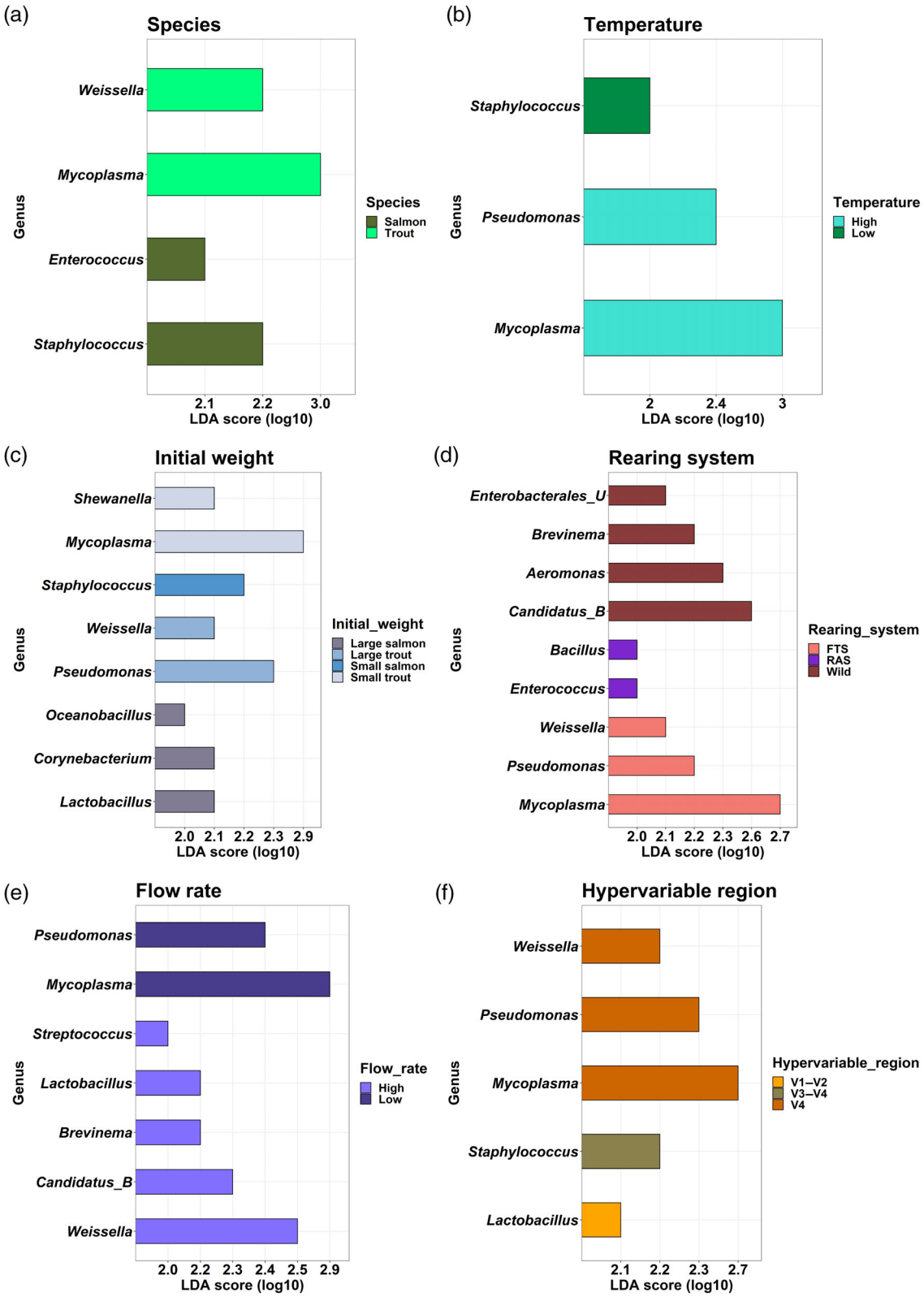


FIGURE 8 (A–F) Linear discriminant analysis Effect Size (LEfSe) results of abundant genera for 783 gut microbiota samples from 19 freshwater salmonid studies. The *y*-axis shows the enriched genera associated with different subgroups while the *x*-axis indicates the linear discriminant analysis (LDA) score (log10). High levels of classification were used for the genera not classified on lower levels (labelled with a *_U* suffix). Only the genera with an LDA score no lower than 2.0 are presented in the plots. The *q* values adjusted by Benjamini–Hochberg method lower than 0.05 are considered significant. '*Candidatus_B*' is short for '*Candidatus_Bacilloplasma*'.

heterogeneities of the beta diversity data were observed except for the factor of species, which may be a reason why all the factors showed significant influences on beta diversity. PERMANOVA was used due to its superior statistical power and insensitivity to heterogeneity in dispersions of comparisons with Analysis of similarities (ANOSIM) and the Mantel test,[87] although it is still possible that the PERMANOVA results we found were correlated between factors to some extent.

Regardless of the influencing factors, on the genus level, a core microbiota of *Mycoplasma*, *Pseudomonas*, *Lactobacillus*, *Corynebacterium*, *Weissella*, *Oceanobacillus*, *Staphylococcus*, and *Enterococcus* was found in the gut of Atlantic salmon and rainbow trout (Figure 2). Although, *Mycoplasma* and *Pseudomonas* were either found in very high abundance in half the studies and very low abundance in the other half, while the other genera were found more consistently (Figure 2). Previous studies have reported that these eight dominate genera in this meta-analysis are common in other freshwater fish species, such as grass carp (*Ctenopharyngodon idellus*)[88] and zebrafish (*Danio rerio*).[89] The dominant genus *Mycoplasma* accounted for over 14.8% abundance and was commonly found in both faeces and mucosa in the salmonid gut. A higher load of *Mycoplasma* in diseased Atlantic salmon[90] has made *Mycoplasma* a proxy for poor salmonid health, while its function is still not clear. A recent study has found that *Mycoplasma* produces the essential amino acid arginine[91] and may not only be associated with disease. *Lactobacillus* and *Weissella* were widely distributed across the gut microbiota of salmonids and these are usually considered as beneficial microorganisms due to their probiotic functions,[92,93] yet some of them have been reported as pathogens in salmonids.[94,95] Many species belonging to *Pseudomonas*, *Corynebacterium*, *Staphylococcus*, and *Enterococcus* are widely considered multidrug-resistant pathogens and opportunistic pathogens in fish as well as other animals,[96–99] and thus may be responsible for some differences in fish performance. *Oceanobacillus* is a relatively rare genus in freshwater fish species and little knowledge has been gained about them except their presence in fish. *Oceanobacillus* species are commonly distributed in seawater, but have also been found in a wastewater treatment system[100] and on rainbow trout skin.[101] A previous study performed on Beluga sturgeon (*Huso huso*)[102] indicated that fish feed with a higher inclusion of fishmeal resulted in gut microbiota more enriched in *Oceanobacillus*, therefore the fishmeal inclusion in marine-based diets may be responsible for the high abundance of this genus.

This meta-analysis for the first time presents a systematic re-analysis of all freshwater salmonid gut microbiota studies that provided 16S rRNA gene raw sequencing data generated by the Illumina MiSeq platform, but with some improvable limitations. At the very beginning of the meta-analysis, all possible influential factors were included, however, only the factors clearly indicated in at least half of the studies remained in the final analysis to generate meaningful results summarized across at least 10 out of the 19 studies. As a result, some factors of interest and importance, such as the pH and dissolved oxygen of the water, feeding frequency and fish density, were discarded. The lack of information on all these factors in the 19 studies resulted in their exclusion, while they may have significant impacts on the fish gut microbiota. Another limitation is the complexity of the influencing factors that overlap with each other, thus future improvement in the design of meta-analyses need to be performed. However, it is possible to control confounding effects by statistical analysis[103] or bioinformatics handling given adequate data[104] and the information about how each factor is generated. Apart from that, many studies of interest were also excluded before the final analysis due to inaccessibility of the authors and the raw data with clearly stated sample metadata. It would be very beneficial to promote publications with open access data and to include as much information as possible in future studies, which allows more secondary studies to compare and re-analyze the data to answer future questions. Only samples sequenced by the Illumina MiSeq platform were studied in this meta-analysis due to its high use in previous studies of interest and to avoid more complexity in analysing the results. Other more powerful Illumina sequencing platforms, such as HiSeq, and NovaSeq, provide higher resolutions and coverages, but also require higher computing capacity and longer time to process and analyse. Apart from Illumina, longer regions, such as full length 16S rRNA (all nine regions), can be sequenced on using Oxford Nanopore, Pacific Biosciences, Element Biosciences and other platforms to get more accurate representation of microbial compositions of the samples down to the species and strain level. Thus, a more in-depth systematic review should be performed in the future when there are sufficient sequence data and metadata on the gut microbiota of salmonid fishes, especially with future advancements in DNA extraction methodologies, sequencing technologies, microbial sequence databases, bioinformatic and meta-analysis tools.

5 | CONCLUSIONS

Overall, our findings indicate that all the factors mentioned in this study significantly influenced alpha and beta diversity indices of salmonid gut microbiota. PERMANOVA revealed that technical factors, such as paper/study, target hypervariable region and DNA extraction, heavily influenced the beta diversity and clustering of gut bacteria, whereas their impact on alpha diversity was not as strong. Paper/study was expected to be the most influential since the combination of different kinds of factors are combined for each individual study. Previous studies on humans and livestock animals agreed with our meta-analysis on salmonids that found target hypervariable region and DNA extraction kit highly impact gut microbiota results. Compared with the technical factors, host-associated and environmental factors influenced alpha diversity to a larger extent. Also, some of them, such as diet and initial weight, are much more explanatory than others in influencing beta diversity. The environmental factors led by diet impacted the beta diversity and clustering of gut bacteria among the host-associated and environmental factors. Aside from that, host-associated factors only contributed to the variance of beta diversity and clustering of gut bacteria to a minimal extent and fish initial weight was the most dominant host-associated factor, which was again supported by previous studies. These findings show three types

of factors influence the gut microbiota of salmonids, which further demonstrate that technical methodologies must be standardized and factors associated with host and environment need to be accounted for in the experimental design of future studies.

AUTHOR CONTRIBUTIONS

David Huyben: Conceptualization; supervision; writing – review and editing. **Shuowen Cao:** Writing – original draft; formal analysis. **Johan Dicksved:** Writing – review and editing; supervision. **Torbjörn Lundh:** Supervision; writing – review and editing; project administration. **Aleksandar Vidakovic:** Writing – review and editing; supervision. **Parisa Norouzitallab:** Supervision; writing – review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

All raw sequencing data analysed in this study are publically available on the NCBI SRA website under the accession numbers mentioned in Table 1. Data generated by this meta-analysis are available upon reasonable request.

ORCID

Shuowen Cao https://orcid.org/0009-0007-1167-2741
Torbjörn Lundh https://orcid.org/0000-0002-2780-3263
David Huyben https://orcid.org/0000-0001-7913-851X

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